

Circadian rhythms in the development of obesity: potential role for the circadian clock within the adipocyte

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Summary

Obesity is one of the most profound public health problems today, and simplistic explanations based on excessive nutritional consumption or lack of physical activity are inadequate to account for this dramatic and literal growth in our world population. Recent reports have suggested that disruptions in sleep patterns, often linked to our '24-h' lifestyle, are associated with increased body fat and altered metabolism, although the cause–effect relationship for these associations has yet to be elucidated. Abnormal sleep/wake patterns likely alter intracellular circadian clocks, which are molecular mechanisms that enable the cell/tissue/organism to anticipate diurnal variations in its environment. The environment may include circulating levels of nutrients (e.g. glucose, fatty acids and triglycerides) and various hormones (e.g. insulin, glucocorticoids). As such, alterations in this molecular mechanism, in particular within the adipocyte, likely induce metabolic changes that may potentiate disrupted metabolism, adipose accumulation and/or obesity. Although diurnal variations in adipokines and adipose tissue metabolism have been observed, little is known regarding the molecular mechanisms that influence these events.

Keywords: Circadian, clock, obesity, sleep.

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Introduction

Obesity is one of the most profound public health problems today, and although much has been learned regarding the regulation of body weight and adiposity, the prevalence of obesity continues to rise. More than two-thirds of adults in the United States and more than 9 million children aged 6–16 years are currently considered overweight (1), and simplistic explanations based on nutritional overconsumption, poor diet and/or lack of physical activity are inadequate to explain this dramatic rise in obesity prevalence. Current treatments for obesity have been largely unsuccessful in maintaining long-term weight loss, demonstrating the urgent need for new insight into mechanisms that may lead to obesity and altered metabolism. Recently, a number of studies have provided support for a link

between the altered sleep/wake patterns associated with our '24-h' lifestyle and obesity (2,3). At the heart of the association between sleep and obesity may be a molecular mechanism intrinsic to all eukaryotic cells and organisms, namely the circadian clock.

Biological rhythms, such as sleep/wake cycles, are an integral component of virtually all aspects of life. These rhythms are controlled in large part by circadian clocks, intrinsically maintained molecular mechanisms that serve to condition the organism to changes in its environment (4,5). Mammals possess both central and peripheral clocks (6,7). The central clock is located within the suprachiasmatic nucleus (SCN) of the brain and is influenced primarily by light. Peripheral clocks are those clocks located within other cells of the organism besides the neurones of the SCN (including those clocks found in other cells of the

central nervous system) and are largely influenced by neurohumoral factors. Thus, altered sleep/wake patterns have the potential to disrupt both the central clock (via abnormal entrainment by light) and peripheral clocks (via perturbations of the central signals as well as altered release of neurohumoral factors stimulated by behaviours such as feeding, for example). This review will summarize our current knowledge of the molecular mechanisms linking altered sleep/wake patterns, circadian rhythms and adipocyte-specific clocks to obesity in humans.

Potential role for the circadian clock in obesity

The existence of highly conserved circadian clocks has long been recognized in eukaryotic cells, and recent studies have shown that even certain prokaryotic cells possess *bona fide* circadian rhythms (8). These observations suggest that the circadian clock confers a selective advantage. By possessing an internal clock mechanism, cells/organisms are able to anticipate temporal changes in their environment, optimizing biological processes so that they occur at an advantageous time in the day. This clock mechanism allows adipose and other tissues to anticipate diurnal variations in its environment, such as circulating levels of glucose, fatty acids and triglycerides, as well as various hormones, including insulin and adrenaline. In doing so, the circadian clock prepares these tissues for the anticipated stimulus, allowing an appropriately rapid response.

Natural fluctuations in body weight associated with seasonal changes in day length have been observed for many mammalian species, suggesting a central role for the circadian clock mechanism in body-weight control and adiposity. In rodents, decreasing the length of the light phase (and consequently increasing the wake/active time for these nocturnal animals) results in significant weight gain, apart from steroid hormone influences (9). In studies performed on sheep, increasing the length of the photoperiod resulted in increased activity of the lipogenesis-promoting proteins malic enzyme and lipoprotein lipase, independent of nutritional status (10,11). In Siberian hamsters, tightly controlled systems of energy balance have been identified that are coordinated by the length of the photoperiod acting via the temporal pattern of melatonin secretion from the pineal gland (12). These animal studies serve to illustrate that fluctuations in body weight associated with manipulations of the light/dark cycle occur naturally, independent of the societal and behavioural constraints present in humans, suggesting a global role for the circadian clock in regulating body weight.

Alterations in circadian patterns of metabolism have also been associated with disease states in humans. In both healthy and type-1 diabetes mellitus (T1DM) subjects, adipose lipolysis decreased in the afternoon, and increased again at night; however, in the T1DM group, lipolysis

increased earlier in the evening than in the healthy controls, and remained elevated throughout the night, indicating that lipolysis shows a distinct circadian rhythm that is altered in T1DM patients (13). Additional research in humans suggests a link between the lack of adequate sleep and metabolic disorders, such as obesity and type-2 diabetes mellitus. In a study of over 1000 outpatients at a family practice clinic, obese patients (body mass index [BMI] = 30–39 kg m⁻²) averaged the lowest total sleep time (TST), 16 min per day lower compared with normal subjects, even after adjusting for established sleep disorders such as insomnia and sleep apnea (2). A difference of 1-h per week in TST was equivalent to a 5.4-kg m⁻² increase in BMI. Interestingly, the extremely obese subjects in this study (BMI = 40–85 kg m⁻²) averaged greater TST than the obese subjects (2), indicating that increases in BMI associated with TST were not simply due to greater wake time spent on eating.

Previous studies have reported that obese patients were sleepier during the day and more likely to experience disturbed sleep at night compared with normal weight controls (14). Daytime sleepiness could not wholly be accounted for by disturbed night-time sleep, however, suggesting that a circadian abnormality likely underlies the daytime sleepiness observed in these obese patients (14). Morning levels of cytokines associated with obesity (e.g. tumour necrosis factor alpha [TNF- α] and interleukin-6 [IL-6]), were significantly elevated in patients with sleep apnea compared with controls and were also significantly correlated with a categorical measure of excessive daytime sleepiness (15).

In sleep-restricted subjects (4-h sleep per night for six nights), glucose clearance rate was almost 40% lower compared with controls (8-h sleep) (16). In addition, insulin-independent glucose disposal was 30% lower, as was the acute insulin response to glucose (an early marker of diabetes). Thyrotropin levels were decreased at night and throughout the day in the sleep-deprived subjects, and cortisol concentrations during sleep deprivation were higher in the afternoon and early evening than the sleep recovery period (16).

Shift work represents another type of sleep alteration in which the normal synchrony between light–dark phase, sleeping and eating is significantly disturbed. Shift work has been associated with cardiovascular disease, obesity, diabetes and other metabolic disturbances. Karlsson *et al.* reported that night-shift workers had higher triglycerides, lower high-density lipoprotein cholesterol, and were more likely to be obese compared with day workers (17). In another study, duration of shift work was positively associated with both BMI and waist/hip ratio in both men and women, independent of age, sex, smoking status, physical activity and educational level (18). Among offshore personnel, age and years of shift work were the best predictors of

BMI, with the highest BMI reported among the night-shift workers (19). Additional studies have supported these observations of altered plasma lipid metabolism and adiposity among night-shift workers (20,21). In a study of subjects working both day and night shifts, levels of antioxidants were significantly lower following the night shift compared with the day shift, suggesting that oxidative stress may be one mechanism linking night-shift work to obesity and cardiovascular disease (22). Shea *et al.* recently reported that significant endogenous circadian rhythms are present in leptin, glucose and insulin, even in subjects who experienced 38 h of continued wakefulness, with peaks for all measures around the usual time of waking (23). Feeding during the period of wakefulness was associated with systematic increases in leptin levels, while fasting during recovery sleep was associated with systematic decreases in leptin levels, glucose and insulin (23). Disturbances in lipid and glucose metabolism, leptin levels and adiposity point to the adipocyte as an important factor in the development of metabolic disease associated with shift work.

Although the association between sleep deprivation, shift work and weight gain has been well documented in the last several years, the mechanisms for this association are not well understood. Altered sleep-wake cycles undoubtedly affect the central circadian clock mechanism, which, in turn, may affect the timing of peripheral circadian clocks through neurohumoral influences. Altered feeding patterns dramatically influence peripheral circadian clock mechanisms, again through neurohumoral entrainment (24). Taken together, proper communication between the central circadian clock, peripheral circadian clocks and diurnal variations in the environment appears to be essential in the regulation of adiposity and body weight. One mechanism linking increased adiposity to disrupted sleep/wake patterns is likely through impairment of the circadian clock intrinsic to the adipocyte. Such impairment may occur via asynchrony between sleeping and eating or other types of behaviours and/or via alterations in the DNA sequence of genes comprising or regulated by the circadian clock mechanism. Although these factors could potentially induce both global and peripheral changes in circadian rhythms, disruption of the adipocyte-specific circadian clock may specifically potentiate the development of obesity, independent of global circadian clock disruptions, via increased proliferation, altered metabolism and/or other factors.

The molecular circadian clock mechanism

Circadian clocks are defined as a set of proteins that generate self-sustained transcriptional positive and negative feedback loops with a free-running period of 24 h (5). These circadian clocks are therefore intrinsic to the cell, and exist even when cells are isolated and cultured *in vitro* (25,26). There are three major components to the circadian

clock (i) input signals (zeitgebers or timekeepers) which reset the circadian clock; (ii) the circadian clock mechanism itself and (iii) the output from the clock (which manifests at the level of altered gene and protein expression, metabolism and/or function, depending upon the cell/organ).

The core mammalian clock machinery consists of at least eight distinct proteins: BMAL1 (brain and muscle ARNT-like protein 1), CLOCK (circadian locomotor output cycles kaput), PER1 (period 1), PER2, PER3, CRY1 (cryptochrome 1), CRY2 and REV-ERB α (Fig. 1) (27,28). BMAL1 (also known as MOP3) and CLOCK are basic helix-loop-helix/PER-ARNT-SIM (bHLH/PAS) transcription factors that form a transcriptionally active complex upon heterodimerization (through the PAS domain). This heterodimer binds to *cis*-acting elements (E-boxes) within the promoter of various target genes, including *per1*, *per2*, *per3*, *cry1*, *cry2* and multiple clock-controlled output genes (Fig. 1) (27,28). The BMAL1/CLOCK heterodimer also stimulates the transcription of *bmal1* itself, generating a positive loop of the clock, and the nadir-to-zenith of gene expression is greatest for *bmal1*. Conversely, upon heterodimerization, the CRY-PER complex forms a negative feedback loop of the clock by translocating into the nucleus and repressing the transcriptional activity of the CLOCK/BMAL1 heterodimer. A second negative loop of the circadian clock is governed by REV-ERB α , which again represses CLOCK/BMAL1 transcriptional activity following accumulation in the nucleus. *Per1* and *per2*, in particular, have an exceptionally high amplitude of circadian gene expression and are extremely sensitive to changes in response to various stimuli capable of resetting the behavioural rhythm, principally light (29,30). Mutations in *per2* are associated with advanced sleep phase syndrome with a short-period phenotype in humans (31).

Although the backbone of the mammalian clock machinery has been described, the full characterization of this

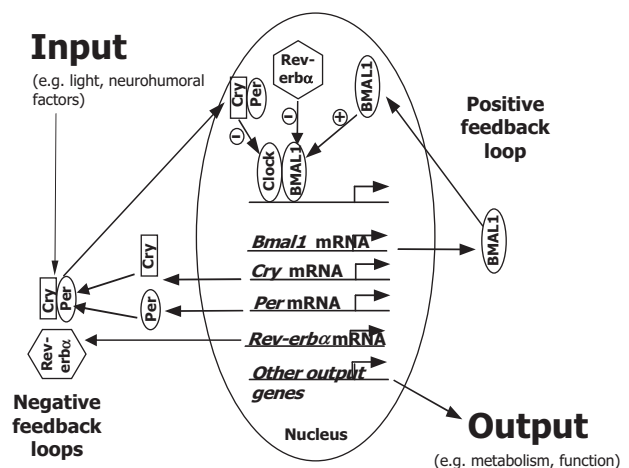


Figure 1 Molecular machinery of the circadian clock.

mechanism is far from complete. Multiple transcriptional, translational and post-translational events are undoubtedly required for the precise operation of this molecular mechanism. Additional factors known to play a role in the circadian clock mechanism include BMAL2 (a homologue of BMAL1), NPAS2 (neuronal PAS domain protein 2; a redox-sensitive homologue of CLOCK), TIMELESS (a *Drosophila* clock component whose true mammalian ortholog has not been identified), GSK3 (glycogen synthase kinase 3; the ortholog of the *Drosophila* clock component SHAGGY) and E4BP4 (adenovirus E4 promoter ATF site binding protein 4; the ortholog of the *Drosophila* protein VRILLE) (32–37). Continued research in the field of molecular chronobiology will undoubtedly assign roles for these, as well as yet-to-be-identified, factors in the circadian clock mechanism.

Roles for the molecular circadian clock

Circadian clock components have been found in all mammalian tissues investigated to date, including adipose (38–40). In order to maintain the selective advantage of anticipation conferred by the circadian clock, central and peripheral clocks must be synchronized with their environment. Zeitgebers (timekeepers) are factors involved in the resetting of circadian clocks. Light is the zeitgeber for the central clock (via specialized ganglia in the retina), while neurohumoral factors are zeitgebers for peripheral clocks (4,5,41,42).

Although the exact identity of the zeitgebers involved in the resetting of peripheral clocks is not known, several candidates have been suggested, including glucocorticoids, retinoic acid and melatonin (38,42–44). Recent studies suggest that one of the strongest entraining influences for peripheral tissues is the timing of feeding (41,45). It has therefore been proposed that the central clock initiates the onset of feeding, and that feeding-induced alterations in neurohumoral factors entrain peripheral clocks (Fig. 2). Although many studies have investigated how alterations in feeding influence both central and peripheral clocks, including adipose (40), relatively little is known regarding how the central and/or peripheral clock mechanisms may, in turn, influence feeding behaviour. Inouye reports that, while the ventral medial hypothalamus (VMH) is involved in the synchronization of rhythms to periodic feeding, the VMH does not appear to contain a self-sustained oscillator (similar to that found in the SCN) associated with food (46). Circadian rhythms in feeding patterns likely arise from, and ultimately may be controlled by, peripheral clocks rather than the central clock, since several satiety factors arising from peripheral tissues, including leptin and ghrelin, have been demonstrated to display a circadian pattern of expression and secretion, independent of food intake (47,48).

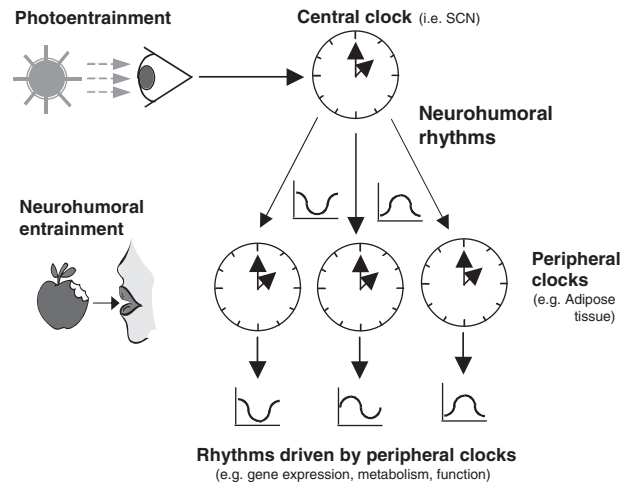


Figure 2 Central and peripheral clock mechanisms. SCN, suprachiasmatic nucleus.

Less is known regarding the identity of clock output genes. Studies concentrating in the SCN and liver have identified *vasopressin*, *ldha* (lactate dehydrogenase a) and the family of PAR (rich in proline and acidic amino acid residues) transcription factors as direct clock output genes; the BMAL1/CLOCK heterodimer binds directly to their promoter elements (49–53). These PAR transcription factors include DBP (albumin D-element binding protein), HLF (hepatic leucocyte factor) and TEF (thyrotrophic embryonic factor). Even less is known concerning the identity of the target genes of this family of PAR transcription factors. Although a number of clock output genes have been identified in non-adipose tissues, no targets have been identified for adipose tissue to date.

The role of the circadian clock in the pathogenesis of obesity is just beginning to be explored. It is currently not known whether circadian disturbances precede and/or follow the onset of obesity. Turek *et al.* reported that mutant mice with a ubiquitous loss of CLOCK function develop obesity and demonstrate altered feeding patterns, hyperphagia and hormonal abnormalities reminiscent of the metabolic syndrome, including hyperlipidemia, hyperleptinemia, hyperglycemia and hypoinsulinemia (54). Alternatively, the amplitude of rhythmic gene expression in a subset of clock component genes (*bmal*, *per1*, *per2*, *cry1*, *cry2* and *dbp*) was mildly suppressed in the adipose tissue of obese KK mice and greatly suppressed in the adipose of obese, diabetic (KK-A^y) mice compared with wild type mice (39). Treatment with pioglitazone did not restore the circadian pattern of gene expression in the adipose tissue of these mice, despite an improvement in measures of diabetes (39). What remains to be determined is whether alterations in the circadian clock within the adipocyte can potentiate the onset of obesity (e.g. through changes in metabolic and/or differentiation pathways under the control of this

peripheral circadian clock) and/or whether excess energy balance and the consequent accumulation of adipose tissue associated with obesity can result in disruptions of the circadian clock mechanism within this tissue.

Molecular components of the circadian clock within adipose

Over the course of a 24-h period, the adipocyte must reciprocally adjust rates of triglyceride synthesis (lipogenesis) and storage with rates of triglyceride breakdown (lipolysis). Although diurnal variations in adipose metabolism are undoubtedly influenced by neurohumoral factors, the circadian clock within the adipocyte likely plays a significant role by altering sensitivity of the adipocyte to specific stimuli (e.g. insulin, adrenaline) throughout the day, or by altering the capacity of the adipocyte for triglyceride storage (e.g. influencing expression of lipid-stabilizing proteins, such as perilipin).

We and others have now confirmed the presence of a fully functional circadian clock mechanism within adipose (39,40). Quantitative gene expression patterns of circadian clock components in murine epididymal and subcutaneous

adipose tissue collected every 3 h throughout the course of 24 h are depicted in Fig. 3. Each data point represents the mean of 5–6 animals. Circadian rhythms in the expression of *bmal1* and *clock* exhibit several similarities, including peak expression at the dark-to-light phase transition and similar absolute levels in peak expression. However, trough (i.e. nadir) expression of *bmal1* is noticeably lower compared with *clock*, suggesting that *bmal1* becomes limiting at the light-to-dark phase transition. Consistent with the mechanism described above, *clock* and *bmal1* mRNA circadian rhythms are antiphase to those observed for all three *per* isoforms, as well as *cry1* and *cry2*. The absolute level of expression for the three *per* isoforms is similar among the *per* genes, and *per* gene expression is also similar to that of *cry2* (the dominant *cry* isoform in adipose tissue). Unlike *cry2*, expression of the three *per* isoforms decreases considerably at the dark-to-light transition, consistent with the idea that PER proteins become rate-limiting at this time.

Rev-erba, a newly identified circadian clock component that forms a negative feedback loop also undergoes dramatic circadian rhythms in expression in epididymal and subcutaneous fat. As mentioned previously, the BMAL1/

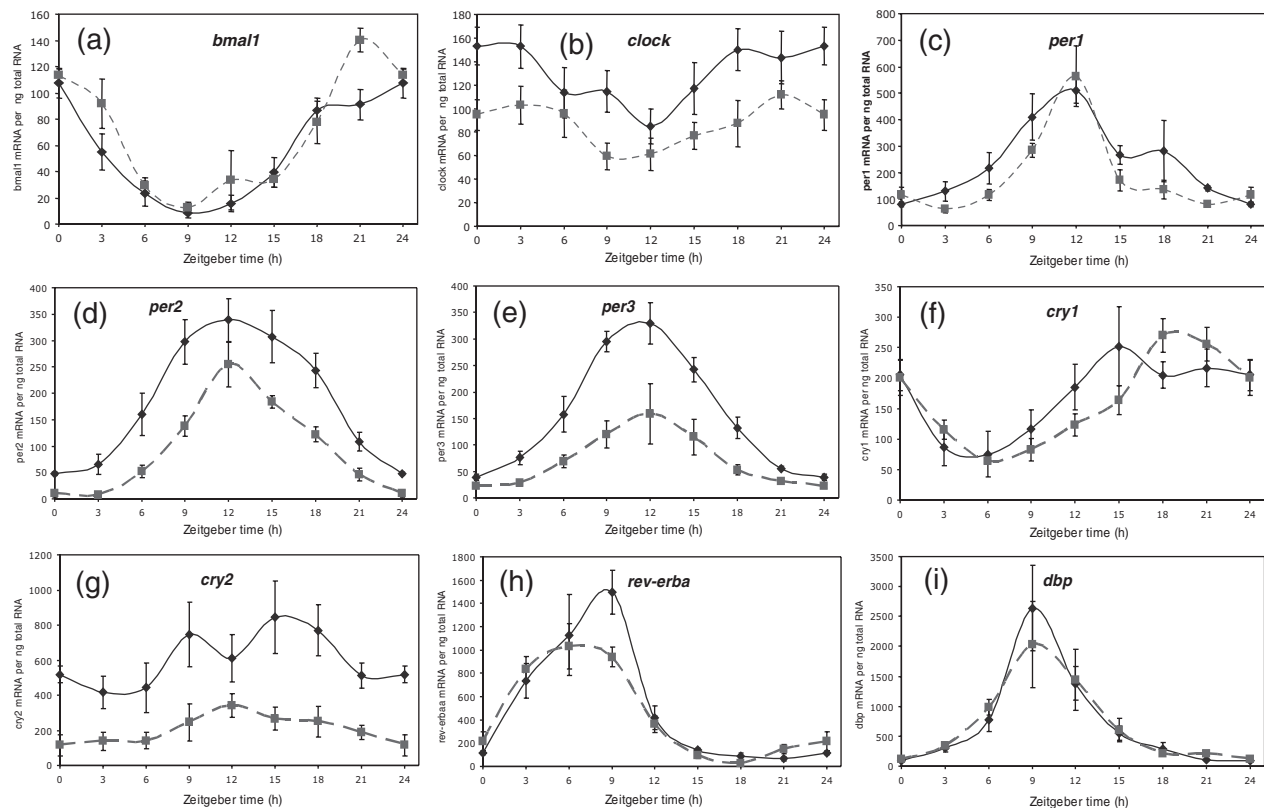


Figure 3 Rhythmic expression of *bmal1* (a), *clock* (b), *per1* (c), *per2* (d), *per3* (e), *cry1* (f), *cry2* (g), *rev-erba* (h) and *dbp* (i) mRNA in mouse epididymal (solid lines) and subcutaneous (dashed lines) adipose. Male Sv1mJ mice were housed in a 12-h light/12-h dark cycle (lights on at zeitgeber time 0). Adipose depots were isolated at 3-h intervals over the course of the day. RNA isolation and real-time RT-PCR were performed as described previously (111,112). Values are shown as the mean \pm SEM for either 5 or 6 observations. Data are represented as mRNA molecules per ng total RNA.

CLOCK heterodimer, when transcriptionally active, induces the expression of several clock output genes. These genes include those encoding for the family of PAR transcription factors, DBP, HLF and TEF (49–51). Similar circadian patterns of gene expression are observed for *dbp*, compared with the *per* genes, in murine epididymal and subcutaneous adipose. To date, no targets have been identified for the PAR transcription factors in adipose tissue. These transcription factors, whose level of expression fluctuates approximately 28-fold within one day (in the case of DBP) are likely to have physiologically relevant targets in adipocytes.

While the clock mechanism is considered to be universal, distinct tissue-specific differences in gene expression of the clock components have been observed. Subtle differences between peripheral clocks within distinct cell types may provide important insight not only into tissue-specific roles for peripheral circadian clocks, but also into the association between alterations in circadian clocks and different disease states. As shown in Fig. 3, differences in gene expression for clock-related genes can be seen even between specific adipose depots. The overall amplitude of gene expression across 24 h was significantly lower in subcutaneous vs. epididymal adipose tissue, for *clock*, *per2*, *per3* and *cry2*. In addition, peak expression was lower for *rev-erba* and *dbp* and phase-shifted for *cry1* in subcutaneous adipose, compared with epididymal adipose tissue. Differences in the timing of peripheral circadian clocks may reflect distinct zeitgebers acting in a tissue-specific manner. Indeed, although the timing of feeding appears to be one of the strongest zeitgebers for peripheral clocks, the neurohumoral factors mediating the effects of feeding are potentially numerous.

We have initiated studies comparing rhythmic expression of circadian clock components between heart, soleus muscle and epididymal adipose isolated from the same mice. Figure 4 illustrates the relative differences in *bmal1* circadian rhythms between these three peripheral tissues. The greatest fluctuations in *bmal1* gene expression are observed in the heart, as compared with soleus muscle and adipose. The peak in *bmal1* expression appears to be in the same phase for heart and adipose tissue (i.e. ZT21), whereas the *bmal1* peak is approximately 3 h later for soleus muscle (i.e. ZT24). The lowest level of *bmal1* expression was observed at ZT9 for adipose, while for heart and soleus muscle, the nadir in *bmal1* expression was observed at ZT12. These subtle tissue-specific differences in rhythmic expression of circadian clock components likely reflect differential influences by distinct zeitgebers. In addition, both neural and neurohumoral inputs may be necessary for the regulation of some peripheral circadian clocks. In parabiosis experiments between SCN-lesioned and non-lesioned rodents recently conducted by Guo *et al.*, neurohumoral and behavioural factors were sufficient to maintain peripheral

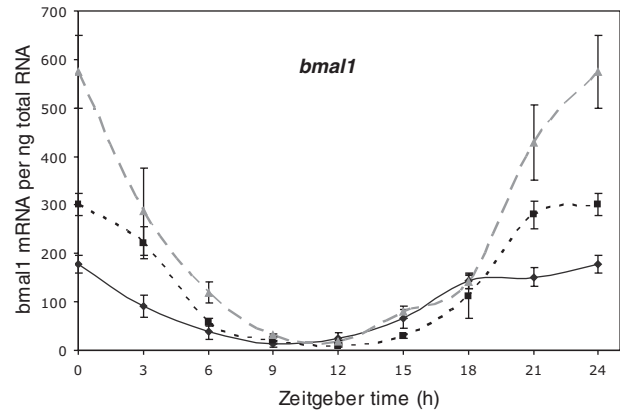


Figure 4 Tissue-specific differences in *bmal1* expression in soleus (dashed line), heart (dotted line) and epididymal fat (solid line). Male Sv1mJ mice were housed in a 12-h light/12-h dark cycle (lights on at zeitgeber time 0). Solei, hearts and epididymal fat were isolated at 3-h intervals over the course of the day. RNA isolation and real-time RT-PCR were performed as described previously (111). Values are shown as the mean \pm SEM for either five or six observations. Data are represented as mRNA molecules per ng total RNA.

eral circadian rhythms in liver and kidney, but not in the heart, spleen or skeletal muscle, suggesting that coordination between the SCN and other zeitgebers may be critical in the regulation of selected peripheral clocks (55).

Circadian rhythms and adipocyte proliferation

Numerous environmental influences promote adipocyte proliferation and differentiation, and the early stages of these processes are governed by a complex interaction of adipocyte-enriched transcription factors. An early signal of adipocyte differentiation is the expression of the C/EBP family of transcription factors (56), which are induced by pro-adipogenic signals, such as insulin, glucocorticoids, IGF-1 and other growth factors, as well as fatty acids. Mice lacking both C/EBP- β and C/EBP- δ show a poor development of adipose tissue, while mice lacking either of the two transcription factors alone have well-differentiated fat cells, suggesting a level of redundancy between C/EBP family members (57). C/EBP- β has also been shown previously to undergo light entrainable circadian rhythms in the eye (58), suggestive of regulation by the circadian clock. Consistent with these observations, we find that *c/ebp β* exhibits dramatic diurnal variations in expression in murine epididymal adipose. Taken together, these observations suggest that C/EBP- β , a critical factor in adipocyte differentiation and a putative clock-controlled gene, may be a novel link between the circadian clock, adipocyte biology and global adiposity.

A second potential link between the circadian clock and adipocyte differentiation is afforded by the core clock gene

rev-erba. This pro-adipogenic transcription factor also exhibits striking diurnal variations in expression in murine adipose tissue (Fig. 3), and others have demonstrated that *rev-erba* gene expression oscillates in human primary hepatocytes and in rat liver and fibroblasts (59). During adipocyte differentiation, *rev-erba* has been shown to act as a repressor of anti-adipogenic genes, and to act downstream of differentiation factors such as peroxisome proliferator receptor- γ (PPAR γ) by facilitating gene expression of PPAR γ target genes, including *ap2* and *cebpa* (60). Higher levels of *rev-erba* expression during the light phase in rodents suggest increased propensity for adipocyte differentiation while the animal is at rest. Whether PPAR γ or other factors in the differentiation process are themselves circadian clock-regulated genes is not known, but these observations suggest that the adipocyte-specific circadian clock may modulate the effect of diurnal variations in external influences (e.g. dietary fatty acids) that influence adipocyte proliferation and differentiation.

Diurnal variations in adipose tissue metabolism

Central and peripheral circadian clocks have been linked to both whole body and organ-specific energy metabolism. For example, lesioning of the SCN in rats abolishes diurnal variations in whole body glucose homeostasis (61), altering not only rhythms in glucose utilization rates but also endogenous hepatic glucose production. In the case of the liver, glycogen synthase activity has been shown to exhibit a marked circadian rhythm. Preservation of diurnal variation in hepatic glycogen synthase activity in fasted rats suggests mediation by intrinsic (i.e. circadian clock) rather

than extrinsic (i.e. fasting/feeding cycles) influences (62). In addition to these metabolic rhythms, dramatic diurnal variations in adipocyte lipolysis and lipogenesis occur in mammals. When an animal sleeps, rates of lipolysis increase, resulting in increased release of non-esterified fatty acids into the circulation. In contrast, when an animal is awake, rates of lipolysis decrease, with a concomitant increase in lipogenesis. Classically, diurnal variations in adipose triglyceride turnover have been explained primarily in terms of reciprocal changes in neurohumoral influences promoting lipolysis (e.g. sympathetic activity) and lipogenesis (e.g. insulin). However, Suzuki *et al.* reported that diurnal variations in the sensitivity of adipose to adrenaline-induced lipolysis persisted *ex vivo*, suggesting that the intrinsic nature of the adipocyte exhibits a diurnal variation (63).

We have examined the circadian gene expression patterns for key components of lipid metabolism in mouse epididymal adipose. These initial studies investigated genes encoding for enzymes involved in fatty acid entry into the cell (*lipoprotein lipase [lpl]*, *fatty acid translocase [cd36]*, *fatty acid transport protein 1 [fatp1]*), fatty acyl-CoA synthesis (*fatty acyl-CoA synthetase 1 [acs1]*), lipid droplet stability (*adipocyte differentiation-related protein [adrp]*, *perilipin [plin]*) and initiation of lipolysis (*adipocyte triglyceride lipase [atgl]*) (see Fig. 5). Of the seven metabolic genes investigated, three (*fatp1*, *acs1* and *adrp*) exhibited diurnal variations in expression with fluctuations greater than two-fold (nadir-to-zenith). Assuming these rhythms in mRNA result in similar diurnal variations for the corresponding protein, it is possible that FATP1 and ACS1 promote higher rates of fatty acid uptake and activation during the first 6 h of the dark (awake/feeding) phase (i.e. postprandial phase),

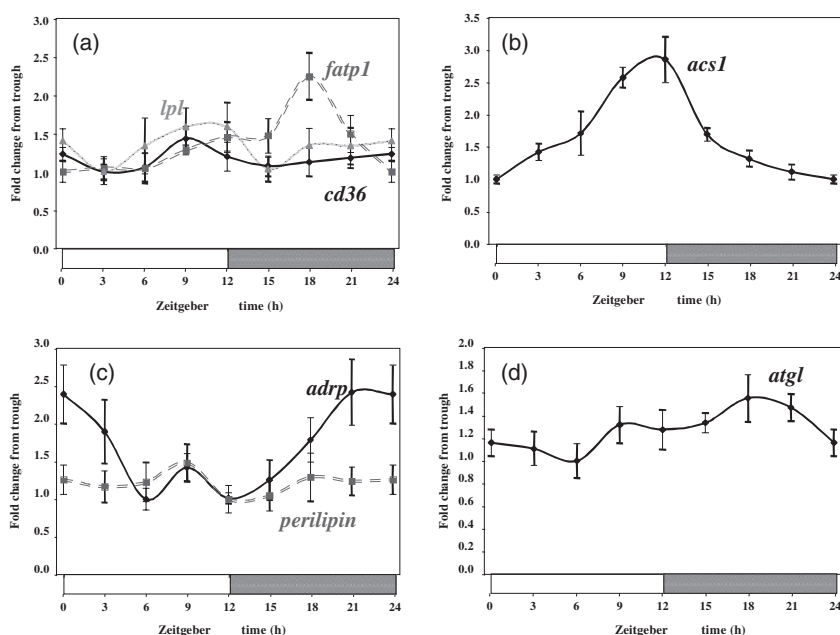


Figure 5 Diurnal variations in mouse epididymal adipose gene expression of enzymes involved in fatty acid uptake (*lpl* [dotted line], *cd36* [solid line], *fatp1* [dashed line]; [a]), fatty acyl-CoA synthesis (*acs1*; [b]), lipid droplet stability (*adrp* [solid line], *perilipin* [dashed line]; [c]), and lipolysis (*atgl*; [d]). Male Sv1mJ mice were housed in a 12-h light/12-h dark cycle (lights on at zeitgeber time 0). Epididymal fat was isolated at 3-h intervals over the course of the day. RNA isolation and real-time RT-PCR were performed as described previously (113). Values are shown as the mean \pm SEM for either five or six observations. Data are represented as mRNA molecules per ng total RNA.

while induction of ADRP as the dark phase continues will promote storage of triglyceride. Conversely, decreased expression of ADRP while the animal rests would promote lipolysis. These observations are the first to suggest that diurnal variations in adipocyte lipid turnover are transcriptionally mediated, potentially by the intra-adipocyte circadian clock.

Circadian rhythms and the regulation of body mass and fat

Since the discovery of the leptin gene in 1994 by Zhang *et al.* (64), much has been learned about its role in the regulation of feeding behaviour, basal metabolism and adipocyte formation. Leptin is expressed and secreted almost exclusively from mature adipocytes and has been shown to influence numerous metabolic pathways, revealing the adipocyte as a central factor in whole body endocrine regulation. Many studies have provided support for leptin as a central indicator in a feedback system in which an 'adiposity signal' provides information to the brain regarding the quantity and quality of stored energy (in the form of adipose) within the organism. Leptin levels have been shown to correlate with the amount of stored body fat in both humans and animals, and leptin levels rise and fall concurrently with changes in body fat stores induced by caloric restriction or overfeeding (65–67).

Leptin exhibits striking circadian patterns in both gene expression and protein secretion, with peaks in leptin expression occurring during the sleep phase of the sleep-wake cycle in humans (47). Ablation of the SCN has been shown to eliminate leptin circadian rhythmicity in rodents, suggesting that leptin is regulated at the level of the central circadian clock, since neither timing of feeding nor adrenalectomy affected the rhythmicity of leptin release (48). Nevertheless, ablation of the central circadian clock mechanism also disrupts peripheral clocks, and it is currently not known whether adipocyte-specific circadian clocks play a role in the regulation of leptin expression and secretion.

Leptin retains diurnal variation in release even in altered metabolic states such as obesity, although the amplitude of peak release is lower in obese subjects (68). In adolescents, the nocturnal rise in leptin was paralleled by a similar nocturnal rise in growth hormone and free fatty acid levels, with the amplitude of leptin variation being greater in obese individuals (69). Leptin 24-h excursion was lower in obese vs. non-obese girls, suggesting that blunted diurnal variation may play a role in leptin resistance and obesity. Circadian patterns of leptin concentration were distinctly different between adult women with upper-body or lower-body obesity, with a delay in peak values of leptin of approximately 3 h in women with upper-body obesity (70). A delay in leptin peaking may be associated with decreased suppression of night-time appetite. In addition, altering

sleep patterns also has a profound effect on leptin plasma levels. Mullington *et al.* studied the effect of severe sleep deprivation on 24-h leptin concentrations in 10 healthy men who were sleep-deprived for 88 h, followed by three nights of recovery sleep for 7 h or 14 h per night. The amplitude of leptin levels was significantly decreased during the sleep-deprived period, returning to normal after one night of sleep recovery (71).

The strongest entraining influences for the circadian clock mechanism within peripheral tissues are feeding-induced alterations in neurohumoral factors (41,45), providing a putatively important link between leptin, the peripheral circadian clock within the adipocyte, and body fat regulation. Expression of the leptin receptor in the brain has been colocalized with neurones expressing factors that control feeding behaviour and energy metabolism, including neuropeptide Y (NPY), agouti-related protein (AGRP), pro-opiomelanocortin (POMC), cocaine- and amphetamine-related transcript (CART), orexin (OX), ghrelin (GHRL) and others (72–74). Mutant mice in which *clock* function is impaired exhibit significantly higher energy intake and almost complete ablation of rhythmic expression in *cart*, *ox* and *ghrl* (54).

Fu *et al.* reported that expression of clock genes in osteoblasts is regulated by leptin via the sympathetic nervous system (75). In our studies of *leptin* expression in mouse epididymal and subcutaneous adipose, *leptin* exhibits a dramatic diurnal variation in expression in epididymal adipose extracted from chow-fed mice, with highest levels of expression in the middle of the dark phase. Surprisingly, the magnitude and rhythmic pattern of leptin expression in subcutaneous adipose tissue were much lower and phase-shifted compared with that of epididymal tissue (Fig. 6).

Along with leptin, a number of additional adipocyte-specific factors have been demonstrated to exhibit rhythmic expression, including acylation stimulating protein (*asp*), adipisin (d.f), resistin (*rstin*), adiponectin (*apm1*) and visfatin (*pbef1*). Ando *et al.* report that, although C57BL/6J wild type mice exhibited circadian rhythms in *apm1*, *rstin*, *pbef1* and *lep* expression, the rhythmic expression of *apm1* and *rstin* was greatly blunted in obese (KK) and obese, diabetic (KK-A^y) mice (39). In humans, circulating adiponectin levels exhibit both ultradian pulsatility and a diurnal variation. In the latter case, the pattern of adiponectin release is out of phase with leptin with a significant decline at night, reaching a nadir in the early morning (76). In obese subjects, adiponectin levels were significantly lower than lean controls, although the obese group had significantly higher average pulse height and valley concentrations (77). Despite the fact that numerous reports have documented diurnal variations in leptin and related peptides, it is currently not known whether the circadian clock mechanism intrinsic to the adipocyte directly influences these circadian patterns of expression and secretion.

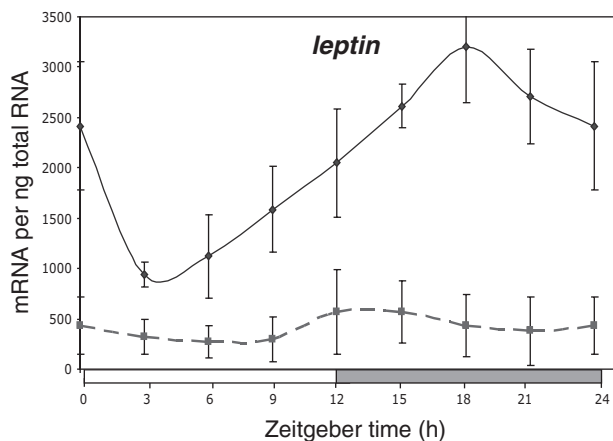


Figure 6 Leptin expression in murine epididymal (solid line) and subcutaneous (dashed line) adipose tissue. Male Sv1mJ mice were housed in a 12-h light/12-h dark cycle (lights on at zeitgeber time 0). Adipose depots were isolated at 3-h intervals over the course of the day. RNA isolation and real-time RT-PCR were performed as described previously (113). Values are shown as the mean \pm SEM for either five or six observations. Data are represented as mRNA molecules per ng total RNA.

Genetic variation, obesity and the circadian clock

Numerous studies of families, twins and adoptees have investigated the role of genes in the determination of body mass, body composition and fat topography. These studies estimate the heritability, or the amount of variance in a trait that can be accounted for by variation in genes, of body weight, body circumferences and BMI to range from 0.38 to 0.90 (78–85), suggesting that even though environmental factors contribute substantially to the development of obesity, genetic variation is also a significant contributor to this process.

Although studies of related individuals provide evidence that genes are important in the determination of obesity, identification of a causative gene or gene defect for human obesity has proven challenging. Nevertheless, recent years have been an exciting time in the field of obesity research with the discovery of several genes in which mutations that eliminate or greatly diminish the function of their protein products give rise to syndromic forms of obesity in humans very similar to that of the animal models in which they were discovered. Nearly all of the genes identified in these animal and human models produce obesity both through stimulation of feeding and impaired energy metabolism. Rare, recessive mutations in leptin (*LEP*) and the leptin receptor (*LEPR*) have been demonstrated to produce morbid, early onset obesity, hypoleptinemia, hyperphagia, hyperinsulinemia, hyperglycemia and reproductive abnormalities (86–88). The melanocortin pathway, previously unknown to be associated with obesity, also appears to play a critical role in the regulation of feeding

behaviour. Rare mutations in pro-opiomelanocortin (*POMC*) have been associated with altered pigmentation, severe hyperphagia and morbid obesity in humans, while alterations in the proconvertase 1 (*PC1*) gene produced increased levels of prohormones along with hyperphagia and early onset obesity (89,90). Of all genetic defects identified to date in human obesity, the most compelling are found within the melanocortin 4 receptor (*MC4R*) gene. Recent studies of the *MC4R* gene in humans have shown it to be highly variable. Vaisse *et al.* and Yeo *et al.* identified the first two families with mutations in the *MC4R* gene, and subsequent studies have since reported numerous mutations in the *MC4R* gene leading to obesity (91–93). Of these mutations, many have been shown to produce alterations in binding affinity or receptor activation, and most are associated with morbid obesity and overeating resulting from haploinsufficiency of the receptor protein (94,95). Based on these reports, it is estimated that up to 5% of the variation in human obesity may be accounted for by these common variants in the *MC4R* gene. This finding is remarkable, given that a similar amount of variation is accounted for by the *BRCA1* gene in predicting familial forms of breast cancer, and suggesting that a common variant may be responsible for a substantial proportion of human obesity. Importantly, prior to these discoveries, the melanocortin pathway was completely unexplored for its role in human obesity, and thus, gene variation in the components of the circadian clock mechanism may be a similarly unexplored pathway that may lead to obesity.

Interindividual variation in morning–evening (ME) preference and other markers of circadian rhythm has been well established in humans, and recent studies have demonstrated that several markers of circadian rhythms have a significant heritable component. Duffy *et al.* reported a wide range of response in measures of the circadian phase of core body temperature and plasma melatonin rhythms, as well as the phase relationship between the timing of these physiologic measures and the timing of sleep–wake and associated light–dark cycle, among both young and old individuals; this variability in circadian phase was significantly correlated with self-reported morningness–eveningness (96). In twin studies, heritability of ME was estimated at 44% among adolescents (mean age = 17.8 years) and 47% among adults (mean age = 46.5 years), and the genetic correlation between generations was 0.30, suggesting that both similar and different genes influence circadian rhythms in the two generations (97). In another study, narrow-sense heritability of ME was approximately 24% in a population of Canadian Hutterite families (98). For self-reported measures of sleep timing, quality and duration, heritability was estimated at 29% for stability of bedtime, 26% for duration in bed and 20% for time awake after sleep onset, with the lowest estimates of heritability

for wake time and stability of wake time, both approximately 12% (98). Thus, ME preference and sleeping behaviour appear to be intrinsic traits that are controlled, in part, by genetic factors.

Studies in both animals and humans have indicated that alterations in the rhythmic expression and release of adipocyte-specific molecules (putatively under the control of the circadian clock mechanism) can both precede and follow the onset of obesity, and it is not surprising that genes for obesity and the circadian clock mechanism may be mutual. For example, a recent linkage scan for leptin levels in obese families segregating for sleep apnea identified a linkage region on chromosome 3 containing the glycogen synthase kinase 3 beta (GSK3 β) gene (99), which has been demonstrated to play an important role in the circadian clock mechanism via the phosphorylation and regulation of nuclear translocation of PER2 (100). These results provide a putative link between the circadian clock, obesity and sleeping disorders. Several genetic polymorphisms in humans have been described to date in genes comprising the circadian clock mechanism. DNA sequence variants in *CLOCK*, casein kinase I epsilon (*CK1E*), *GSK3 β* , *PER2* and *PER3* have been associated with sleep and mood disorders, bipolar disease, seasonal affective disorder, advanced sleep phase syndrome, depression and ME preference (31,101–110). While obesity has been associated with a number of these conditions, no studies specifically investigating the role of variation within circadian clock genes in human obesity have been reported to date.

Conclusions

The role of the peripheral circadian clock mechanism within the adipocyte represents an exciting new field of study in pursuit of the causes of increasing obesity prevalence. Elucidation of the link between the adipocyte-specific circadian clock and obesity may have profound implications on the timing of obesity therapies. By identifying new genes and processes that are important in the aetiology of obesity, we can then investigate whether variation in the genes controlling these processes has an impact on both susceptibility to obesity and susceptibility to the development of the potentially debilitating comorbidities associated with obesity.

Conflict of Interest Statement

No conflict of interest was declared.

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Note Added in Proof

Our very recently published study using a dominant negative cardiomyocyte-specific disruption of the circadian clock reveals that the triglyceride metabolism genes adiponutrin (*adpn*) and diacylglycerol acyltransferase 2 (*dgat2*) are directly regulated by the circadian clock within the cardiomyocyte (114). In addition, we find that loss of circadian clock function specifically within the cardiomyocytes of the hearts alters myocardial triglyceride metabolism during fasting (114). These studies suggest that the circadian clock within the adipocyte may also be a potential regulator of triglyceride metabolism, and that impairment of this molecular mechanism may contribute towards adiposity.

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