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Metabolism as an Integral Cog in the Mammalian Circadian Clockwork

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Abstract

Circadian rhythms are an integral part of life. These rhythms are apparent in virtually all biological processes studies to date, ranging from the individual cell (*e.g.*, DNA synthesis) to the whole organism (*e.g.*, behaviors such as physical activity). Oscillations in metabolism have been characterized extensively in various organisms, including mammals. These metabolic rhythms often parallel behaviors such as sleep/wake and fasting/feeding cycles that occur on a daily basis. What has become increasingly clear over the past several decades is that many metabolic oscillations are driven by cell autonomous circadian clocks, which orchestrate metabolic processes in a temporally appropriate manner. During the process of identifying the mechanisms by which clocks influence metabolism, molecular-based studies have revealed that metabolism should be considered an integral circadian clock component. The implications of such an interrelationship include the establishment of a vicious cycle during cardiometabolic disease states, wherein metabolism-induced perturbations in the circadian clock exacerbate metabolic dysfunction. The purpose of this review is therefore to highlight recent insights gained regarding links between cell autonomous circadian clocks and metabolism, and the implications of clock dysfunction in the pathogenesis of cardiometabolic diseases.

Keywords

Cardiometabolic disease; Chronobiology; homeostasis; energy balance; misalignment

Introduction

In 1865, Claude Bernard published his theories on the '*milieu interieur*', wherein he described that the '*The constancy of the internal environment is the condition for a free and independent life*'. These theories gave birth to the concept of homeostasis, which, in simplistic terms, can be defined as maintenance of the internal environment in a stable/constant state. On the surface, these concepts are in stark contrast to those of the Greek philosopher Heraclitus (535BC - 475BC), who postulated that 'everything flows, nothing stands still'. What is increasingly clear is that the 'truth' lies in a combination of these two theories. Biological processes are indeed in continuous flux, yet are maintained within

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discrete physiologic boundaries, in order to establish a homeostatic internal environment. This is exemplified beautifully by consideration of circadian ('about one day') influences on organisms. Throughout evolution, terrestrial organisms have experienced marked fluctuations in their environment over the course of the day, inclusive of (but not exclusive to) light and UV exposure, temperature, humidity, food availability, and predator locality. It is therefore not surprising that the magnitude of 'activities/fluxes' of discrete biological processes exhibit marked oscillations as a function of the time of day, which routinely trough and peak in proximity to physiology-pathology boundaries. Indeed, in certain instances pathologies can arise due to a temporal suspension of a biological process at the physiology-pathology boundary (*i.e.*, temporal inflexibility). Metabolism is undoubtedly a collection of biological processes which are in constant flux over the course of the day, while metabolic inflexibility is a hallmark of multiple common cardiometabolic disease states (*e.g.*, obesity, diabetes mellitus). The purpose of this review article is therefore to provide a critical overview of the mechanisms responsible for normal time-of-day-dependent oscillations in metabolism in mammals, the physiologic importance of these oscillations, and the pathologic consequences following their disruption.

Time-of-Day-Dependent Rhythms in Metabolism

Oscillations in metabolic fluxes as a function of time-of-day are observed in various organisms, ranging from redox status oscillations in yeast to whole body energy expenditure in humans. For the latter, non-invasive indirect calorimetry has exposed increased energy expenditure during the awake period, and a concomitant dip in this parameter during sleep, which mirror daily oscillations in body temperature (Jung et al., 2011, Kreider et al., 1958, Jequier and Felber, 1987, Ravussin et al., 1982, White and Li, 1985). Similarly, metabolically pertinent processes/functions such as appetite, rate of gastric emptying, and gut motility, all exhibit a time-of-day-dependence (Saito et al., 1980, Saito et al., 1976a, Saito et al., 1976b, Saito et al., 1976c). Such observations, in their own right, are indicative of marked oscillations in metabolism in a normal individual over the course of the day, which fluctuate within a physiological range. Prominent oscillations in clinically relevant metabolic parameters have also been reported in humans, including insulin sensitivity/glucose tolerance and a host of circulating factors (*e.g.*, glucose, non-esterified fatty acids, triglycerides, cholesterol, insulin, leptin) (Nonino-Borges et al., 2007, Gibson and Jarrett, 1972, Biston et al., 1996, Van Cauter et al., 1997, Van Cauter et al., 1992, Schlierf and Dorow, 1973, Barter et al., 1971, Cella et al., 1995). Indeed, insulin resistance (an underlying cause of type 2 diabetes mellitus) is a naturally occurring phenomenon on a daily basis (observed during the night in humans) (Biston et al., 1996, Van Cauter et al., 1992, Gibson and Jarrett, 1972). Time-of-day-dependent oscillations in metabolism have been studied extensively in a number of laboratory models (*e.g.*, rodents). Similar to humans, rodents exhibit marked diurnal variations in metabolic parameters, at the whole body, organ, and cellular levels (Rutter et al., 2002, Wijnen and Young, 2006, Green et al., 2008). Energy expenditure, body temperature, nutrient digestion/absorption, humoral factors, insulin-dependent and independent glucose utilization, isolated tissue metabolic fluxes, steady state levels of tissue metabolites, as well as expression and activity of key metabolic enzymes, have all been reported to oscillate over the course of the day (Bray et al., 2012, Hussain and Pan, 2009, Scheving, 2000, la Fleur et al., 2001, Whichelow et al., 1974, Leighton et al., 1988, Stavinocha et al., 2004).

Classically, fluctuations in metabolism have been attributed to changes in energetic demand and nutrient availability associated with daily sleep/wake and fasting/feeding cycles. During periods of physical activity, muscle (a highly metabolically active tissue that quantitatively impacts whole body substrate utilization due to its mass) tends to increase reliance on glucose as a fuel source, particularly during strenuous exercise (Saltin et al., 1974). Thus,

changes in physical activity potentially contribute towards increased reliance on carbohydrate during the awake hours. Conversely, during the sleep phase, mammals decrease reliance on glucose as a fuel concomitant with an increased reliance on fat, a metabolic switch that is also observed during periods of fasting (Bray et al., 2012, Cahill et al., 1966). Thus, the sleep phase associated fast has been suggested to significantly contribute to daily rhythms in substrate selection. Manipulating the timing of food availability under laboratory settings results in a rapid phase shifting in fuel selection - forcing a rodent to eat during the sleep phase causes the rhythm in the respiratory exchange ratio (RER, a measure of indirect calorimetry indicating substrate selection) to shift by 10.3 hours within 1–2 days, with little impact on sleep/wake cycles (Vollmers et al., 2009, Bray et al., 2012).

It should be noted that not all time-of-day-dependent oscillations in metabolism can be explained exclusively by daily sleep/wake or fasting/feeding cycles. In the latter case, when rodents are subjected to prolonged periods of fasting (*i.e.*, >24 hours), rhythms in some, but not all, metabolic parameters persist, including rhythms in whole body energy expenditure and tissue metabolites (such as liver glycogen levels) (Ishikawa and Shimazu, 1976). Similarly, although restricted feeding causes a rapid re-entrainment of daily rhythms in substrate reliance (10.3 hour phase shift), daily rhythms in energy expenditure only partially re-entrain (5.0 hour phase shift) (Bray et al., 2012). It could be argued that distinct metabolic parameters are sensitive to sleep/wake versus fasting/feeding to varying extents, resulting in a full, partial, or no re-entrainment following manipulation of just one of these parameters. For a scenario in which a metabolic parameter is influenced by sleep/wake and fasting/feeding cycles to equal extents, and that the latter cycles were dissociated such that they become anti-phase, then oscillations in the metabolic parameter would dampen significantly (*i.e.*, become temporally suspended). The pathologic consequence of this scenario will be discussed in subsequent sections.

Evidence exists suggesting that mechanism(s) intrinsic to the organism likely contribute towards time-of-day-dependent rhythms in various metabolic parameters. In the 1960's, Aschoff elegantly demonstrated that body temperature, urine excretion, and sleep/wake patterns in humans exhibit a circadian rhythm (*i.e.*, persistence of time-of-day-dependent rhythms when environmental cues remain constant) (Aschoff, 1965). The same is true for animal models, wherein the majority of metabolic parameters investigated to date exhibit persistence of circadian oscillations under constant environmental conditions (*e.g.*, constant lighting, food availability, temperature, *etc.*). However, it should be noted that time-of-day-dependent oscillations in behaviors, such as sleep/wake and fasting/feeding cycles also persist under constant conditions, leading to the possibility that oscillations in metabolic parameters are similarly dependent on fluctuations in physical activity and/or nutrient availability (Moore-Ede et al., 1982). For example, Dallmann has recently shown that 15% of all plasma metabolites exhibit persistent circadian oscillations under constant conditions, including non-esterified fatty acids and glucose (Dallmann et al., 2012). However, it is important to note that evidence exists suggesting that rhythms in sleep/wake and fasting/feeding cycles do not necessarily orchestrate metabolic rhythms. This includes observations that time-of-day-dependent oscillations in distinct metabolic parameters persist in isolated tissues and/or cells in culture. Over 30 years ago, time-of-day differences in glucose utilization (uptake of 2-deoxyglucose) within the suprachiasmatic nucleus (SCN) of animals were observed, independent of the lighting conditions (Schwartz and Gainer, 1977), and 20 years later, these rhythms were shown to persist at the single cell level in immortalized rat SCN cells (Earnest et al., 1999). More recently, rhythms in metabolic/redox state (free radical homeostasis) have been observed in adult SCN slices (Wang et al., 2012). For example, rhythms in nicotinamide adenine dinucleotide (NAD⁺) and flavin adenine dinucleotide (FAD) persist in slices maintained *in vitro* for several cycles such that the SCN

is relatively oxidized in the early night and reduced during the day (Wang et al., 2012). Collectively, observations such as these have led to the suggestion that metabolic oscillations are potentially driven by a mechanism which is intrinsic to the cell (*i.e.*, cell autonomous). One likely candidate mechanism is the circadian clock.

Emergence of Circadian Clocks as Regulators of Metabolism

The Mammalian Circadian Clock

The timing mechanism (the ‘gears’) of the mammalian circadian clock is maintained by a set of proteins that generate self-sustained transcriptional positive and negative feedback loops with a free running period of approximately 24 hours. It has been proposed that these cell autonomous molecular mechanisms confer the selective advantage of anticipation, preparing the cell for an environmental stimulus/stress, thereby enabling a rapid and temporally appropriate response. Figure 1 summarizes interactions of key mammalian circadian clock components (for review see (Takahashi et al., 2008)). At the heart of the mechanism are two transcription factors, CLOCK (circadian locomotor output cycles kaput) and BMAL1 (brain and muscle ARNT-like protein 1), that upon heterodimerization, bind to the E-boxes within the promoters of target genes, resulting in induction. One such target gene is *Bmal1* itself, thereby generating a positive loop in the mechanism. Negative loops include induction of the PER (period; PER1, PER2, PER3) and CRY (cryptochrome; CRY1, CRY2) proteins. Once translated, the PER and CRY proteins form heterodimers, translocate into the nucleus, and inhibit CLOCK/BMAL1 transactivation. A key facet of the PER/CRY mediated negative loop is a specific delay in the accumulation of the PER proteins by approximately 3–6 hours, relative to their mRNA. The PER proteins are targets for ubiquitin-mediated degradation, an event that is promoted by serine phosphorylation (likely mediated by casein kinase 1 [CK1] and possibly glycogen synthase kinase 3 [GSK3]). Upon accumulation and heterodimerization with the CRY proteins, the PER/CRY complex translocates into the nucleus. Subsequent inhibition of CLOCK/BMAL1-mediated transcription decreases expression of the *per* and *cry* genes, ultimately relieving inhibition on CLOCK/BMAL1.

Two additional negative loops of the mammalian circadian clock involve the bHLH (basic Helix-Loop-Helix) transcription factor DEC (of which two isoforms have been identified, DEC1 and DEC2) and the nuclear receptor REV-ERB α . Both the *dec1/2* and *rev-erba* α genes are under direct transcriptional control by the CLOCK/BMAL1 heterodimer (Figure 1). Following translation of the corresponding proteins and translocation into the nucleus, both DEC1/2 and REV-ERB α attenuate CLOCK/BMAL1-mediated transcription; DEC1/2 appear to associate with the CLOCK/BMAL1 heterodimer, impairing transactivational capacity, while REV-ERB α specifically represses *bmal1* transcription. In contrast, CLOCK/BMAL1-mediated induction of *bmal1* expression, via ROR (retinoic acid receptor related orphan receptor), constitutes the positive loop in the mammalian circadian clock (Figure 1).

Although the classic definition of the circadian clock involves an interplay of transcriptional positive and negative feedback loops, multiple post-transcriptional events are essential for complete clock function, including protein synthesis/degradation, reversible phosphorylation, and nucleocytoplasmic translocations. Each of these events represents a potential site of regulation, enabling “fine-tuning” of this molecular clock by extracellular factors (*i.e.*, zeitgebers). CLOCK, BMAL1, CRY, as well as the PER proteins, have all been shown to undergo phosphorylation in a circadian-like fashion; in addition to its influence on protein stability, increasing evidence suggests that phosphorylation status is closely associated with nucleocytoplasmic trafficking of specific circadian clock components (*e.g.*, PER and CLOCK proteins) (Yagita et al., 2002). Multiple protein kinases, including CK1 ϵ , MAPK, AMPK, GSK3 β and PKG (type II), likely play significant roles in the mammalian clock mechanism, thereby providing avenues for modulation of the circadian clock by an

array of extracellular stimuli (Sanada et al., 2002, Vielhaber et al., 2000, Martinek et al., 2001, Tischkau et al., 2004, Hardie et al., 2012). Additional post-translational modifications of circadian clock components include acetylation, polyadenylation, and O-GlcNAcylation (Hardin, 2006, Cardone et al., 2005, Dardente and Cermakian, 2007, Tamaru et al., 2009, Asher et al., 2010, Durgan et al., 2011a). Redox status has also emerged as a critical modulator of the circadian clock; DNA binding of both CLOCK and NPAS2 (neuronal PAS domain protein 2; a CLOCK homolog that can heterodimerize with BMAL1 and induce similar target genes) is influenced by the NAD^+/NADH ratio (Rutter et al., 2001). More recently, studies in enucleated erythrocytes have suggested the existence of a mammalian circadian clock mechanism that operates completely independently of transcriptional events; this putative clock mechanism relies on oscillations in redox status (*i.e.*, peroxiredoxins) (O'Neill and Reddy, 2011).

The CLOCK/BMAL1 heterodimer binds to E-boxes in the promoters of various genes that are not believed to be integral components of the clock mechanism. These clock-controlled genes include a vast number of genes including but not limited to *ldha*, *vasopressin*, *wee1*, *prokineticin2*, and the PAR (proline and acidic amino acid-rich) transcription factors *dbp*, *hlf*, and *tef* (Falvey et al., 1995, Fonjallaz et al., 1996, Ripperger et al., 2000, Jin et al., 1999, Rutter et al., 2001). The latter family of transcription factors, who in turn have the potential to modulate expression of a host of target genes, are antagonized by another bHLH transcription factor, E4BP4 (Mitsui et al., 2001); *e4bp4* is an additional example of a clock-controlled gene, whose expression is likely induced by REV-ERB α . Consistent with their reciprocal function, oscillations in PAR transcription factors and E4BP4 have been reported to be in anti-phase to one another, in various tissues, including the heart (Mitsui et al., 2001, Young et al., 2001b).

Mammalian circadian clocks can be divided into two major classes, depending upon the cell type within which they are found: the primary and secondary circadian clocks. The primary central circadian clock in the brain is located within the suprachiasmatic nucleus (SCN) of the hypothalamus. Conversely, secondary clocks are those clocks found within all non-SCN cells of the organism, including other regions of the central nervous system. SCN neurons are distinctively able to sustain rhythmicity without drifting out of phase with one another as a result of intercellular coupling (Welsh et al., 2010). The SCN is responsible for not only driving (synchronizing) rhythmicity in the primary clock but also for maintaining a proper phase relationship among secondary oscillators, such as the lung, liver, heart, and spleen (Dibner et al., 2010). The humoral and neuronal signals that synchronize peripheral clocks are a result of rhythmic neurophysiological activity - the primary output and a distinguishing characteristic of the SCN network (Herzog, 2007, Colwell, 2011). All clocks are responsive to Zeitgebers, which are factors which reset/entrain central and/or peripheral circadian clocks. The central clock is reset by primarily by light (via electrical signals transmitted along the retinohypothalamic tract), while peripheral circadian clocks are influenced by multiple neurohumoral factors. It is believed that the central clock entrains peripheral clocks via modulation of neurohumoral stimuli, either directly (*i.e.*, innervations between the SCN and specific peripheral tissues) and/or indirectly (*e.g.*, through alterations in feeding behavior) (Dibner et al., 2010). Alternatively, the SCN may entrain peripheral clocks through a releasable factor, as evidenced by early transplant studies in which encapsulated fetal SCN transplants into the ventricles of SCN-lesioned hamsters restored behavioral rhythmicity (Silver et al., 1996).

Circadian Clock Disruption Influences Metabolic Homeostasis

Over the past several decades, circadian clocks have emerged as a fundamental mechanism governing multiple facets of metabolic homeostasis. Both surgical and genetic models have

been employed. For example, ablation of the SCN not only leads to loss of locomotor rhythms but also loss of day-night changes in food intake and increased adiposity (Nagai et al., 1978, Nishio et al., 1979, Coomans et al., 2012). In addition, the SCN-periphery connection is critical for rhythms in glucose (Angeles-Castellanos et al., 2010, Cailotto et al., 2005), insulin (Cailotto et al., 2005), and leptin (Kalsbeek et al., 2001). A role for the SCN in regulating circadian rhythms of whole body glucose homeostasis has also been suggested. Plasma glucose concentrations have been shown to increase just prior to the active phase, for both rats and humans (Trumper et al., 1995, La Fleur et al., 1999, Bolli et al., 1984). This phenomenon (known as the dawn phenomenon) appears to be due to increased hepatic glucose output, rather than decreased glucose utilization; on the contrary, both insulin-dependent and independent glucose utilization increase at this time (la Fleur et al., 2001). Lesioning of the SCN in rats abolishes diurnal variations in both hepatic glucose output and glucose tolerance (La Fleur et al., 1999, la Fleur et al., 2001). More recently, SCN lesions have recently been shown to produce profound insulin resistance (Coomans et al., 2012), and disruption of the SCN rhythmic output signal with constant light impairs energy metabolism and insulin sensitivity (Coomans et al., 2013).

Similar to SCN lesion studies, genetic manipulation of circadian clock components often results in a plethora of metabolic perturbations. This is exemplified by Clock^{Δ19} mutant mice (that harbor an early stop codon mutation, generating a truncated, dominant negative form of the CLOCK protein lacking the transactivation domain encoded by exon 19). These mice exhibit an obesogenic phenotype associated with dyslipidemia and glucose intolerance (a triad of hallmarks for metabolic syndrome) (Turek et al., 2005). Interestingly, glucose intolerance appears to be secondary to abnormalities in insulin secretion, as opposed to impaired insulin sensitivity; Clock^{Δ19} mutant mice actually exhibit increased insulin sensitivity (Rudic et al., 2004), a characteristic not normally observed in metabolic syndrome patients. In terms of time-of-day-dependent rhythms in metabolic/energy balance parameters, Clock^{Δ19} mutant mice have aberrant oscillations in food intake, energy expenditure, digestion/absorption, glucose tolerance, insulin tolerance, as well as a host of metabolically-relevant humoral factors (*e.g.*, leptin), in addition to alterations in sleep/wake cycles (Naylor et al., 2000, Turek et al., 2005, Pan and Hussain, 2009, Rudic et al., 2004). Genetic manipulation of the heterodimerization partner of CLOCK, namely BMAL1, again results in striking alterations in metabolism, exhibiting partial overlap with Clock^{Δ19} mutant mice. For example, Bmal1 knockout mice exhibit impaired insulin secretion, increased insulin sensitivity, as well as abnormalities in gluconeogenesis (Rudic et al., 2004, Marcheva et al., 2010). Interestingly, associated with a critical function of BMAL1 in adipocyte differentiation, Bmal1 knockout mice have a lean, as opposed to obese, phenotype (although at a very young age, these mice may exhibit a slight increase in adiposity, which reverses with age) (Bunger et al., 2005, Shimba et al., 2005, Guo et al., 2012, Lamia et al., 2008). Bmal1 knockout mice also exhibit a dramatic loss in time-of-day-dependent rhythms in all metabolic parameters investigated to date, in addition to sleep/wake and fasting/feeding cycles (Shimba et al., 2005).

Clearly, either SCN ablation or genetic manipulation of circadian clock components in a ubiquitous fashion results in profound perturbations in metabolism/energy balance. Both approaches result in alterations in central and peripheral clock function, disruptions of behavioral rhythms to varying extents, and abnormal oscillations in neurohumoral factors. What is less clear is the relative importance of disruption of oscillations in central clock (*i.e.*, SCN) mediated processes (*e.g.*, behaviors such as sleep/wake and fasting/feeding cycles) or peripheral clock mediated processes. The ‘translational’ (as opposed to purely ‘academic’) relevance of such a question stems from observations that central and peripheral clocks can be easily dissociated by common (daily) environmental/behavioral factors, such as exercise and food intake. A number of strategies have been adopted in attempts to address this issue,

including assessment of time-of-day-dependent metabolic oscillations in: 1) isolated cells; 2) mouse models of tissue/cell type specific clock component genetic manipulation; and 3) 'rescue' of central circadian clock function. As discussed above, SCN cell lines in culture exhibit robust 24hr oscillations in glucose uptake in culture (Earnest et al., 1999), exposing direct regulation of this important metabolic parameter by a cell autonomous circadian clock. Cells lines derived from peripheral tissues have also proven to be useful for investigation of metabolically relevant oscillations in culture. For example, synchronized fibroblasts (*i.e.*, through serum shock) exhibit time-of-day-dependent oscillations in phospholipid synthesis (in addition to expression/activity of key enzymes in phospholipid biosynthesis) (Marquez et al., 2004).

Cell Autonomous Circadian Clocks Regulate Metabolism

More recently, a genetic approach has been taken to disrupt peripheral circadian clocks in a cell type specific manner. In doing so, rhythms in behaviors such as sleep/wake and fasting/feeding cycles remain intact, allowing the function of a distinct cell autonomous circadian clock to be revealed. For example, Lamia *et al* have utilized such an approach to investigate the role of the hepatocyte circadian clock in glucose tolerance. The investigators reported that ubiquitous Bmal1 null mice exhibit decreased glucose tolerance, whereas the opposite is observed in liver-specific Bmal1 null mice (*i.e.*, increased glucose tolerance is observed) (Lamia et al., 2008). Using a different liver-specific model, in which the negative loop component Rev-erba was overexpressed in hepatocytes, Kornmann *et al* revealed a host of genes that are regulated by the liver circadian clock, many of which encoded for critical enzymes involved in gluconeogenesis, glycogen turnover, and *de novo* fatty acid synthesis (Kornmann et al., 2007). An adipose-specific Bmal1 knockout mouse model has been reported recently, which has a pro-obesity phenotype (Paschos et al., 2012). This phenotype is comparable to that reported for both drosophila and mouse models expressing the Clock^{Δ19} mutant protein specifically in adipocytes (Bray and Young, 2009, Xu et al., 2008). In all three cases, increased food intake is observed specifically at the end of the active period, a time of the day at which food consumption has been shown to promote adiposity. This clearly illustrates a role for the adipocyte circadian clock in regulation of time-of-day-dependent oscillations in food intake, an important parameter in energy balance. The Clock^{Δ19} mutant protein has been expressed in various additional metabolically active tissues, including cardiac and skeletal muscle. In the case of cardiac-specific Clock^{Δ19} mutant mouse hearts, time-of-day-dependent oscillations in myocardial glucose utilization and triglyceride turnover are completely abolished (Durgan et al., 2011b, Tsai et al., 2010), revealing that the cardiomyocyte circadian clock mediates these metabolic rhythms.

Closing the loop – Metabolic Signals Influence the Circadian Clock

Impact of Feeding/Exercise on the Clock Mechanism

As highlighted in the previous section, circadian clocks undoubtedly regulate various aspects of metabolism. What is becoming increasingly apparent is that metabolism in turn is able to influence the timing of the circadian clock mechanism. One of the first examples of this included the observation that the timing of food intake can dissociate peripheral clocks from the central clock. More specifically, forcing a rodent to consume its daily calories during the light/sleep phase (*i.e.*, restricted feeding) results in an approximate 12 hour phase shift of the circadian clock in the liver, with little or no effect on the central SCN oscillator (which remains entrained to the light/dark cycle) (Damiola et al., 2000), although some studies have shown that the SCN is sensitive to restricted feeding paradigms (Escobar et al., 2007). Recent studies have shown that this food-induced dyssynchrony extends beyond the central versus peripheral clock relationship, but causes dyssynchrony between peripheral clocks (Bray et al., 2012, Reznick et al., 2013). For example, although the liver clock

entrains by approximately 8 hours during a restricted feeding paradigm, clocks within both skeletal and cardiac muscles phase shift by only approximately 4 hours (Bray et al., 2012). This likely reflects the relative contribution of SCN-dependent versus independent zeitgebers between distinct peripheral tissues; that being feeding-derived zeitgebers dominate entrainment of the liver clock, whereas organs such as the heart and skeletal muscle receive mixed signals from SCN- and feeding-derived zeitgebers. Consistent with this concept, not only are clock gene oscillations phase shifted by a lesser extent in muscle during restricted feeding, but the amplitude of the oscillations are diminished (Bray et al., 2012).

In addition to restricted feeding, overall meal timing and frequency can have a significant effect on the phase of peripheral clocks. Using an *in vivo* PER2 reporter mouse model, recent evidence suggests that the size of the last meal before a fast greatly influences peripheral clock phase (Kuroda et al., 2012). These results have implications for people who eat a large dinner late in the evening. Studies on meal timing in humans agree. For example, consuming a carbohydrate-rich meal in the evening decreases melatonin and increases core body temperature and heart rate in people isolated within a controlled constant routine environment (Krauchi et al., 2002). Likewise, skipping breakfast has been shown to increase energy intake, fasting cholesterol levels, as well as postprandial insulin resistance (Farshchi et al., 2005).

Another important component of energy homeostasis is energy expenditure. As mentioned earlier, the primary Zeitgeber for resetting the central circadian clock is light; however, other 'nonphotic' stimuli such as exercise can also produce phase advances in activity rhythms (Edgar et al., 1991a, Edgar et al., 1991b, Sack et al., 1997, Laemle and Ottenweller, 1999, Edgar et al., 1997, Marchant and Mistlberger, 1996). In a human laboratory study that utilized a constant liquid diet, a 1-h session of vigorous evening exercise was shown to advance melatonin rhythms by ~30 min (Buxton et al., 2012). In addition to effects on the central circadian clock, exercise may also influence the plasticity of peripheral oscillators. One recent study in mice demonstrated that when a weak photic Zeitgeber (*i.e.*, a dim light-dark cycle) is in conflict with a nonphotic Zeitgeber (restricted running wheel access during the inactive period), both central and peripheral circadian oscillators are sluggish to re-adjust their phase to a 12-h reversal of the light-dark cycle (Castillo et al., 2011). However, wheel access (and/or exercise) can also alleviate dampened rhythmicity (in SCN physiology, locomotor behavior, and clock gene expression) of genetically altered mice that have a disrupted circadian system (*i.e.*, vasoactive intestinal polypeptide-deficient mice) in a phase-specific manner (Schroeder et al., 2012). These results suggest that energy expenditure feeds back to affect central and peripheral clock phase in a way that is time-of-day-specific.

Potential Metabolic Mediators of Circadian Clock Regulation

The underlying molecular mechanisms for entrainment of peripheral clock phase by metabolic zeitgebers are currently unknown. Putative candidates include various signaling molecules that play integral roles in energy and metabolic homeostasis. These include, but are not limited to, GSK3 β , AMPK, cAMP, NAD, and the NAD⁺/NADH ratio (*i.e.*, redox status). For example, Rutter *et al* revealed that both CLOCK and NPAS2 are redox sensitive transcription factors, such that the ratio of NAD⁺/NADH directly modifies CLOCK/BMAL1 and NPAS2/BMAL1 heterodimer DNA binding (Rutter et al., 2001). Absolute NAD⁺ levels also affect the timing the mammalian clock mechanism. NAD⁺ is a critical substrate utilized in several post-translational modifications, including those catalyzed by sirtuins (NAD⁺-dependent deacetylases) and PARP (NAD⁺-dependent poly ADP ribose polymerase) (Haigis and Sinclair, 2010, Hassa et al., 2006). CLOCK has been shown to directly acetylate BMAL1 in a time-of-day-dependent manner, which in turn influences CLOCK/BMAL1

transcriptional activity (Doi et al., 2006). Conversely, sirtuin 1 (SIRT1) deacetylates BMAL1 in a time-of-day-dependent manner and pharmacological activation of SIRT1 dampens circadian clock gene amplitude (Bellet et al., 2013). SIRT1 deacetylation has been shown to be dependent upon oscillations in cellular NAD⁺ levels (Ramsey et al., 2009). The latter are driven by circadian clock dependent regulation of the NAD⁺ salvage pathway (Ramsey and Bass, 2009, Ramsey et al., 2009). Thus, NAD⁺ is a classic example of an integral metabolic cog in circadian clock, being regulated by the clock mechanism, and in turn generating a feedback loop. A second integral metabolic in the mammalian circadian clock appears to be heme. Heme biosynthesis oscillates in a time-of-day-dependent manner, due to regulation of aminolevulinic acid synthase (the rate limiting step in this process) by the cell autonomous clock (Kaasik and Lee, 2004). Conversely, multiple circadian clock components directly bind heme, including CLOCK, NPAS2, PER2, and REVERB α (Lukat-Rodgers et al., 2010). In the latter case, Yin *et al* have reported that reversible heme binding by REV-ERB α influences the DNA binding capacity of this clock transcription factor, which in turn potentially influences processes such as gluconeogenesis and lipogenesis (Yin et al., 2007).

More recently, GSK3 β has emerged as a critical signaling component that interlinks metabolism and the circadian clock. Glycogen synthase kinase 3 is a serine/threonine kinase that is an established modulator of insulin sensitivity. For example, genetic loss of GSK3 α results in increased glucose tolerance and insulin sensitivity as well as reduced body fat (MacAulay et al., 2007). The two mammalian isoforms (α and β) of the serine/threonine kinase GSK3 are normally in the de-phosphorylated, active form. Phosphorylation on regulatory serine residues (S21-GSK3 α and S9-GSK3 β) inhibits kinase activity by formation of a pseudosubstrate that competes with GSK3 substrates (Frame et al., 2001, Woodgett, 1990). GSK3 is phosphorylated by the PI3K/Akt pathway and de-phosphorylated by PP1 (Hur and Zhou, 2010). When activated, this kinase has a variety of substrates: nearly 50 metabolic substrates have been identified thus far (Kockeritz et al., 2006). GSK3 β has been shown to phosphorylate various circadian clock components, including BMAL1. Pharmacological inhibition of GSK3 modulates the period (Dokucu et al., 2005, Hirota et al., 2008, Kurabayashi et al., 2010, Li et al., 2012, Martinek et al., 2001, Osland et al., 2010), and chronic activation decreases amplitude of the circadian clock (Paul et al., 2012, Sahar et al., 2010), highlighting the importance of this signaling molecule in clock control. Importantly, phospho-GSK3 β (an inverse marker of activity) is rhythmic in the liver, heart, and brain and these rhythms are abolished following genetic disruption of the circadian clock (Iitaka et al., 2005, Durgan et al., 2010). Although these studies clearly establish a role for GSK3 β in the clock-metabolism interrelationship, no studies have examined how circadian variation in GSK3 β activity affects daily metabolic plasticity. Similar to GSK3 β , AMPK activity oscillates in a time-of-day- and circadian clock-dependent manner in multiple mammalian tissues, and has been shown to phosphorylate (and affect the activity of) multiple circadian clock components (*e.g.*, CK1 ϵ and Cry1) (Lamia et al., 2009, Um et al., 2007).

Metabolic Desynchrony as a Cause for Cardiometabolic Diseases

The concept of multiple endogenous oscillators is not a new one, and historically, evidence supporting this concept arose from studies of human subjects kept in isolation from environmental time cues for many weeks (Moore-Ede et al., 1982) In these situations, “internal desynchronization” was observed when the subject exhibited a sleep-wake rhythm with a drastically longer period (*e.g.*, 33 hours) from the period of the core body temperature rhythm (*e.g.*, 25 hours), although a later study showed that this sleep-wake pattern was greatly influenced by naps aligned to a particular phase of the temperature rhythm. Regardless, this mismatch among different circadian oscillators or the oscillators and the

environment (“circadian misalignment”) (Dibner et al., 2010) can also be experimentally-induced by use of an enforced sleep/wake schedule on a very short day (20-h) or long day (28-h). Just three cycles of a 28-h schedule produces blunted leptin rhythms, increased postprandial glucose and insulin, and cortisol rhythms that are 180° out of phase with the behavioral rhythm. Some participants showed evidence of a pre-diabetic or diabetic state (Scheer et al., 2009), suggesting that desynchronization of central and peripheral clocks with the environment or behavior is an ideal candidate mediator of increased risk for developing metabolic and cardiovascular diseases.(Morris et al., 2012)

Shift Work

A real-world environment in which circadian misalignment undoubtedly occurs is the shift work environment (see illustration in Figure 2). For example, melatonin phase in night shifters exhibits large inter-individual variability even when measured after the last night shift worked in permanent night shifters (Weibel et al., 1997) or after 12 days of night shift (Boivin and James, 2002). In one report, cortisol rhythms took five consecutive shifts to adapt to the new diurnal sleep phase, and even then, 25% of the workers’ rhythms never adapted (Hennig et al., 1998). This misalignment of central-clock controlled rhythms (melatonin and cortisol) with sleep-wake behavior may have important cardiometabolic consequences (reviewed in (Morgan et al., 2003, Morris et al., 2012)). In fact, shift workers have an increased risk of developing cancer, gastrointestinal diseases, and cardiometabolic disorders (Boivin et al., 2007, Foster and Wulff, 2005, Scheer et al., 2009, Petroiusti et al., 2010) such as ischemic heart disease (Knutsson et al., 1986b) which are major causes of morbidity and mortality (Petroiusti et al., 2010, Kroenke et al., 2007). More specifically, female shift workers have a higher incidence of obesity and high blood pressure (Chen et al., 2010) and are more likely to develop cardiometabolic syndrome over a 4/5-year period than day-shift controls. (Petroiusti et al., 2010). In Antarctica, male and female shift workers have increased postprandial levels of insulin, glucose, and triacylglycerol (TAG) during the night shift and do not recover even after two days of day shift (Lund et al., 2001). Female night shift workers show increased energy intake, insulin insensitivity, increased triglycerides, and reduced post-prandial ghrelin compared to day shift controls (Schiavo-Cardozo et al., 2012). Given that approximately 15% of the workforce includes evening, night, or rotating shifts (U.S.D.O.L., 2005), shift-work induced circadian misalignment is an important environmental risk factor for cardiometabolic disease.

In addition to the many studies documenting the hazards of shift work, some studies have sought to determine whether certain interventions improve adaptation to shift work. For example, simulated night shift studies report that bright light, in combination with sunglasses or fixed sleep schedules following “work” nights, result in a greater likelihood of re-entrainment (as measured by dim light melatonin onset) (Crowley et al., 2004, Horowitz et al., 2001). A similar intervention study for shift workers in their normal environment reported that bright-light exposure during 6 h of a night shift along with light-shielding during the commute home restores an appropriate phase relationship between bed time and melatonin onset compared to controls (Boivin and James, 2002). These intervention studies have focused on aligning circadian phase to work days on the night shift without taking into account that most night shift workers (*e.g.*, 97% of night shift nurses (Gamble et al., 2011)) prefer to revert to nocturnal sleep on days off. An intervention that has shown some success in a night shift simulation study is one in which an intermediate phase is induced during days off by scheduling sleep to occur between 3:00am and 12:00pm. This strategy results in a significantly later circadian phase, improved cognitive performance, and reduced fatigue and mood disturbance (Smith and Eastman, 2008, Smith et al., 2009).

In a field study of hospital shift work nurses (Gamble et al., 2011), we found that nurses choose to follow different behavioral sleep patterns for days off. For example, the majority of nurses (97%) switch from diurnal to nocturnal sleep on days off, and nearly have do so by using a strictly enforced schedule but without giving up substantial sleep time (e.g., they sleep late or nap on the day they will start night-shift work). Surprisingly, one of four nurses use sleep deprivation as a means to switch from days to nights and vice versa by choosing a > 24-hr period to stay awake entirely. Therefore, many shift workers are likely to suffer from both sleep deprivation and circadian misalignment, two known contributors to metabolic impairment, which can also interact (see below). Other less common sleep strategies for night shift included taking daily naps on off days, switching half way between days and nights, and continuing to sleep during the day on days off. An important area of future investigation is how these sleep strategies affect timing of food intake as well as metabolic function during shift work.

Several studies have employed animal models of shift work and restricted feeding paradigms to explore the effects of circadian misalignment on metabolic function. As discussed in previous sections, restricted feeding paradigms in mice (*i.e.*, food access only during the light phase) result in circadian misalignment not only between the SCN and peripheral tissues, but also between various distinct peripheral tissues. This experimental feeding strategy also leads to metabolism dyssynchrony between peripheral tissues, as well as between metabolic parameters (*e.g.*, RER phase shifts by 10 hours, while energy expenditure phase shifts by 5 hours). These misalignments are associated with increased adiposity. Interestingly, distinct studies have highlighted that high fat feeding at the end of the active phase or during the sleep phase are both associated with increased adiposity, as well as elevations in various additional cardiometabolic parameters (*e.g.*, glucose intolerance, dyslipidemia). In a work-for-food paradigm, mice fed a hypocaloric diet shift the primary activity bout into the light period in an energy conservation effort (Hut et al., 2011). Interestingly, mice rapidly revert to a nocturnal activity pattern (predicted from the prior light-dark cycle) upon release into *ad libitum* feeding in constant darkness, suggesting that the central clock (SCN) remained entrained to the LD cycle. Whether or not this happens to people in shift-work environments is yet to be determined. However, a rat model of shift work suggests that a consistent metabolic strategy of restricting food intake to the normal active period rescues metabolic impairment induced by shift work (Salgado-Delgado et al., 2010b). Specifically, rats were forced to live in slowly rotating drums five days per week during the rest (lights on) period followed by two *ad libitum* days off for several weeks. This shift work paradigm resulted in abdominal obesity, flattened glucose rhythms, and internal desynchrony between the SCN and extra-SCN brain regions (Salgado-Delgado et al., 2010a, Salgado-Delgado et al., 2010b). Food restriction to only the dark period (normally active phase for nocturnal animals) on every day of the week (including *ad libitum* days) rescued the metabolic phenotype. Although information regarding food intake timing in human shift workers are limited, two studies have found that night shift workers have flattened food intake rhythms primarily due to increased food intake during the night time (Reinberg et al., 1979, Pasqua and Moreno, 2004). Thus, a potential strategy to be explored is whether nocturnal food restriction reduces metabolic impairment associated with shift work.

Sleep Disruption

In addition to effects of circadian desynchrony on metabolic function, sleep disruption may also lead to metabolic impairment. Both human and animal model studies of restricted sleep have shown that sleep loss can result in reduced glucose tolerance, insulin insensitivity, and weight gain (for review, see (Laposky et al., 2008, Spiegel et al., 2009)). For example, restriction of total sleep to 4 hours per night for 2–6 nights results in decreased glucose

tolerance and a decrease in insulin sensitivity, culminating in a decreased disposition index (quantifies the risk of diabetes as the relationship between β -cell sensitivity and insulin sensitivity) (Spiegel et al., 2005, Spiegel et al., 1999). However, the loss in total sleep duration may not be as important as loss in slow wave sleep. When slow wave sleep (but not total sleep duration) is reduced by 90%, similar effects result: decreased insulin sensitivity, decreased disposition index, and reduced glucose tolerance (Tasali et al., 2008). The satiety hormone leptin is an attractive candidate to explain this relationship between sleep and metabolism because peak leptin levels normally reached during the night are blunted in a dose-dependent manner to the amount of sleep restriction (Taheri et al., 2004). In fact, leptin levels are reduced in people who habitually restrict their sleep (Chaput et al., 2007). Reduced leptin is also observed in sleep deprived rats that also exhibit hyperphagia, decreased insulin, and impaired glucose (Bodoss et al., 2004, Koban and Swinson, 2005, Laposky et al., 2008). At the CNS level, sleep deprivation also increases hypothalamic expression of neuropeptide y (orexigenic) and decreases expression of pro-opiomelanocortin (anorectic) (Koban et al., 2006).

The connection between circadian misalignment and sleep deprivation makes differentiating the distinct contributions of these parameters to metabolic dysfunction difficult. However, one study suggests that the combination of circadian desynchrony and sleep disruption can compound diabetic risk (Buxton et al., 2012). Specifically, a three-week protocol of combined circadian misalignment (28-h day) and sleep restriction (5.6 h/day) reduces overall leptin levels and increases ghrelin levels, similar to acute misalignment or sleep deprivation studies. However, the combination of the two treatments reduces resting metabolic rate, increases post-prandial glucose, and reduces post-prandial insulin. Importantly, these measures were made at the same circadian phase (after realignment) so that any effects were due to the recent history of misalignment and sleep deprivation. These results suggest that circadian misalignment plus sleep restriction not only induces insulin insensitivity but also causes insufficient insulin secretion by pancreatic β cells, an effect not previously observed in acute sleep deprivation or acute misalignment studies. Altogether, these symptoms could explain increased risk of obesity and diabetes in those who routinely sleep short durations (Cappuccio et al., 2010).

Gene-Environment Interactions

Not only does sleep/circadian rhythm dysfunction contribute to increases in metabolic disease risk factors, but metabolic disease is marked by altered circadian rhythms and sleep. For example, haplotypes in three common polymorphisms in the *CLOCK* gene significantly confer risk of cardiometabolic syndrome (Scott et al., 2008). In another study, people with one of these polymorphisms (rs1554483 in combination with rs4864548) were 1.8 times more likely to be obese or overweight (Sookoian et al., 2008). Although insulin sensitivity is relatively low during the night in healthy people, subjects with diabetes show increased sensitivity to insulin at this phase (Boden et al., 1996). Moreover, diabetic subjects also have diminished rhythmicity of circadian clock gene transcriptional rhythms (*BMAL1*, *PER1*, and *PER3*) in white blood cells (Ando et al., 2009). Animal models of metabolic dysfunction (Zucker rats, diabetic db/db mice, and leptin knockout mice) agree with these human studies, and in general, sleep organization is disrupted and activity rhythms are dampened (primarily due to increased nocturnal sleep and diurnal activity) in these animal models (Sans-Fuentes et al., 2010, Danguir, 1989, Megirian et al., 1998, Kudo et al., 2004, Laposky et al., 2006). Importantly, animal studies have the advantage of being able to examine disease progression, and one study suggests that dampened clock gene rhythms in the liver are evident in 3-week old mice, prior to onset of obesity (Ando et al., 2011). Consistent with this idea, high fat diet has been shown to disrupt photic entrainment at the level of the SCN (Mendoza et al., 2008) as well as molecular rhythms of central and peripheral oscillators

(Kohsaka et al., 2007). Moreover, liver (but not SCN) clock phase is altered in as little as 1 week of high fat diet exposure (Pendergast et al., 2013). In sum, these findings suggest that environmental exposure and/or genetic factors may result in dampened circadian rhythms in peripheral metabolic clocks leading to central-peripheral dyssynchrony (as illustrated in Figure 2) and that this chronic condition may induce metabolic disease states.

Cardiovascular Disease

Cardiovascular disease (CVD) is the leading cause of death in the United States (A.H.A., 2010). It has been estimated that one in every four Americans has some form of CVD (2010). As with many common diseases, CVD results from a complex gene-environment interaction; genetic polymorphisms in susceptibility genes influence the responsiveness of individuals to CVD risk factors, such as nutrients (quantity and quality), physical activity (duration and intensity), and sleep (quality and duration). Epidemiological studies have identified a host of environmental factors that correlate with the staggering rise in cardiometabolic diseases during the last several decades; two of the strongest associations are increased food availability and light exposure/sleep duration (Keith et al., 2006). Biological mechanisms that significantly influence responsiveness of an organism to common environmental CVD risk factors, *and* are themselves modulated by these stimuli/stressors (*e.g.*, during disease states), could therefore be considered candidate mediators in the etiology of CVD. One such mechanism is the cell autonomous circadian clock.

As with numerous physiologic cardiovascular parameters (*e.g.*, heart rate, blood pressure), clinically-relevant adverse cardiovascular events exhibit a time-of-day-dependence (*e.g.*, myocardial infarctions, sudden cardiac death) (Durgan and Young, 2010, Young, 2006, Young, 2009, Muller et al., 1989, Degaute et al., 1991, Carson et al., 2000). Classically, oscillations in these parameters have been attributed to fluctuations in stimuli/stressors that are extrinsic to the cardiovascular system (*e.g.*, neurohumoral factors, sheer stress, posture, *etc*) (Muller et al., 1989). Vascular resistance, blood pressure, and sheer stress are increased when the organism is awake, co-incident with bouts of physical activity/exercise (Degaute et al., 1991). These normal rhythms in pro-hypertrophic stimuli do not induce adverse remodeling of the myocardium. In contrast, exercise training (most often performed during the awake period) improves cardiac output through induction of a physiologic hypertrophic program (Weber et al., 1987). Non-dipping hypertensive patients (whose blood pressure drops <10% during sleep, and therefore exhibit greater stress on the heart during the sleep phase) exhibit increased left ventricular hypertrophy and are at increased risk of cardiovascular and renal disease (compared to dipping hypertensive subjects) (Bianchi et al., 1994, Palatini et al., 1992). Similarly, obstructive sleep apnea is associated with increased sympathetic stimulation on the heart during the sleep phase, adverse cardiac remodeling and increased risk of heart failure (Bradley and Floras, 2003, Kario, 2009). Collectively, these observations suggest that the time of day at which the myocardium is challenged with pro-hypertrophic stimuli markedly influences the remodeling process. Emphasizing further the clinical significance of this concept are recent findings from the MAPEC study (large prospective ambulatory blood pressure monitoring study), which reported a reduction in adverse cardiovascular events when nighttime blood pressure was specifically targeted (Hermida et al., 2010).

There is increasing appreciation that circadian clocks potentially influence both cardiovascular physiology and pathophysiology. For example, time-of-day-dependent oscillations in both heart rate and blood pressure are abolished in Clock^{Δ19} mutant and Bmal1 null mice. The significance of maintaining normal circadian clock function, in terms of cardiovascular health, has been highlighted recently through phenotypic analysis of genetic mouse models of disrupted clock function. For example, heterozygous tau hamsters

(that harbor a mutation in CK1 ϵ , resulting in a 22 hour circadian clock) develop hypertrophic cardiomyopathy (Martino et al., 2008). A cardiomyopathic phenotype is also observed when the clock output genes *Dbp*, *Hlf*, and *Tef* are genetically deleted (Wang et al., 2010). Lefta *et al* observed that targeted ablation of the core clock component *Bmal1* in a ubiquitous manner results in severe cardiac dysfunction (in the absence of cardiac hypertrophy) (Lefta et al., 2012). It is important to remember that circadian clock disruption is not limited to genetic models. As a consequence of the marked plasticity exhibited by the clock mechanism, alterations in this mechanism are both extremely rapid and common. Indeed, circadian clocks are altered during a number of cardiometabolic disease states, including obesity, diabetes mellitus, hypertension/hypertrophy, myocardial infarction, sleep apnea, and aging (Young et al., 2001a, Young et al., 2002, Hsieh et al., 2009, Kung et al., 2007, Burioka et al., 2008, Kunieda et al., 2006). As mentioned above, an obvious example of clock disruption being associated with CVD in humans is that of shift work; night shift workers exhibit a concomitant increased incidence in cardiometabolic diseases, including obesity, diabetes mellitus, and CVD (relative to day shift co-workers) (Knutson et al., 2007, Knutsson et al., 1986a, Harma and Ilmarinen, 1999, Koller, 1983). Circadian clocks therefore meet criteria as likely mediators of CVD pathogenesis. Specifically: 1) clocks regulate critical cardiovascular functions; 2) clocks are modulated by common CVD risk factors; 3) clocks are altered in CVD states; and 4) genetic disruption of clocks leads to CVD.

Why Might Metabolism have Evolved as an Integral Circadian Clock Component?

In general terms, the role of cell autonomous circadian clocks is to confer the selective advantage of anticipation, thereby allowing the cell/organ/organism to respond to environmental stimuli/stresses in a temporally appropriate manner. At the whole organism level, mammals have two ‘major’ behavioral oscillations to contend with on a daily basis. These are sleep/wake and fasting/feeding rhythms. In terms of evolutionary selective pressures, we hypothesize that sleep/wake cycles are more predictable on a daily basis (fasting/feeding rhythms are hypothesized to be less predictable due to potential food availability limitations). It is important to note that during the awake period, foraging for food, avoidance of predation, and reproduction are energetically demanding, and that intuitively these energetic demands remain high during the active period even if the animal in the wild is not successful in its forage for food. We hypothesize that organisms that anticipate this scenario (*i.e.*, physical activity/energetic demand rhythms independent of feeding status) would have an evolutionary selective advantage. At a cellular and molecular level, increased energetic demand could potentially be anticipated by increased ATP generation at that time and/or concomitant decreases in ATP utilization by ‘non-essential’ processes (*i.e.*, not required *immediately* for physical activity). Regarding the latter, one such set of processes would include growth and repair. Synthesis of cellular components (*e.g.*, protein, phospholipids, organelles, *etc.*) is an energetically demanding process, which could potentially compete with contractile processes during the active period, in terms of ATP utilization. It is noteworthy that during the awake period the likelihood of protein damage (*e.g.*, through oxidative stress and/or pollutants/toxins due to increased physical activity, increased respiration rate, and ingestion) is increased. We speculate that growth and repair immediately following the awake phase (*i.e.*, the sleep phase) would undoubtedly facilitate preparation of the organism for the subsequent awake period. Consistent with this hypothesis, both protein and phospholipid synthesis are increased in the heart during the sleep phase in rodents (Durgan et al., 2007, Rau and Meyer, 1975). This concept is akin to the well-established temporal gating of DNA repair and DNA synthesis (DNA repair occurs during the daylight hours when greater UV exposure occurs, while DNA synthesis occurs

during the dark phase, thereby reducing the number of mutations transmitted from parent to daughter cells); the mechanism responsible for the latter temporal gating is the cell autonomous circadian clock (Mitra, 2011). This logic has led us to hypothesize that a critical function of mammalian circadian clocks is to temporally partition processes promoting physical activity (*e.g.*, glucose utilization) versus growth/repair (*e.g.*, protein synthesis, autophagy). Clearly, direct regulation of metabolism by the cell autonomous circadian clock would be critical to confer anticipation of sleep/wake cycles.

Considerable evidence exists in support of the hypothesis that cell autonomous circadian clocks confer a selective advantage for anticipation of fasting/feeding cycles. During periods of fasting, blood glucose levels are maintained within a physiologic range through various counter-regulatory mechanisms, including increased rates of gluconeogenesis, lipolysis and fatty acid β -oxidation, protein turnover and amino acid utilization, as well as ketone body utilization (relative contributions to homeostasis vary depending on the duration of the fast). As highlighted in preceding sections, cell autonomous circadian clocks appear to influence all these processes. It is therefore not surprising that differential responses to fasting have been observed in multiple models of circadian clock manipulation. For example, *Cry1* knockout mice exhibit markedly lower blood glucose levels during acute (6 hour) fasting, in comparison to wild-type counterparts, likely due to impaired gluconeogenesis (Zhang et al., 2010).

Following the above rationale, lucid arguments can be contextualized regarding why cell autonomous circadian clocks evolved to directly regulate metabolic processes. The converse question relates to why circadian clocks are exquisitely sensitive to perturbations in metabolism. By definition, circadian clocks are comprised of positive and negative feedback loops. As such, one could hypothesize that metabolism is an integral clock component, forming a series of interconnecting feedback loops within the mammalian circadian clockwork. One selective advantage of this scenario would be the fine tuning (*i.e.*, entrainment) of the clock mechanism in response to metabolically-relevant signals. In doing so, the clock would be highly sensitive to the same factors that it anticipates, such as physical activity and feeding (*e.g.*, breaking of the sleep phase fast). In doing so, the resetting of the clock mechanism at the time of actual physical activity and/or feeding would ensure appropriate anticipation of the same processes exactly 24 hours later, thereby increasing efficiency of metabolic processes.

Summary

The circadian system is comprised of three levels of synchronous clocks: (1) the basic 24-h mechanism within the cellular clock, (2) synchrony of endogenous tissue-level neural/peripheral clocks within an organism, and (3) synchrony of an individual's behavior with the environment (as illustrated in Figure 2). Each of these levels involve metabolism, and metabolic processes have emerged as integral clock components, temporally gating energetically costly processes. For example, at the cellular level, flux through distinct metabolic processes should be regulated such that they occur in a temporally appropriate manner (*e.g.*, protein turnover at a period of time after inducers of protein damage have subsided). At the tissue level, neural regions and peripheral organs involved in controlling processes such as satiety/hunger, lipid breakdown/storage, glucose production/storage, are synchronized in a fashion that allows whole body homeostasis. At the organism level, it is important that somewhat predictable environmental/behavioral parameters, such as physical activity and/or food availability, are anticipated so that key physiologic factors (such as increased heart rate upon awakening) are modulated appropriately in a time-of-day-dependent manner. Together, synchrony at all three levels provides a selective advantage, improving the likelihood of survival. Disruption of these systems undoubtedly leads to

impaired cellular signaling, accumulation of damaged proteins/lipids, energetic imbalance (energy intake – energy expenditure), and ultimately to cardiometabolic diseases such as obesity, diabetes, and cardiovascular disease. Although significant advances have been made recently with regards to improvement of our fundamental understanding of the importance of metabolic rhythms, many deficiencies in our knowledge remain. Several critical unanswered questions remain, which focus around: 1) the exact mechanisms by which meal timing influences circadian alignment in mammals; 2) dissecting all inter-connecting loops between the circadian clock and metabolism; 3) defining the roles of cell autonomous clocks in various aspects of whole body metabolic homeostasis; and 4) determining whether circadian re-alignment is an amenable strategy for the treatment of cardiometabolic diseases.

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None

Declaration of Interest

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Figure 1. Molecular Gears of the Mammalian Clockwork

The 24-h timing of the molecular clock is orchestrated by a set of clock genes and proteins that form a positive feedback loop when CLOCK dimerizes with BMAL1 and binds to the E-box elements of *PER* and *CRY* genes, activating transcription. Negative control of transcription occurs when *PER/CRY* dimerize, and this complex translocates into the nucleus where it interacts with CLOCK-BMAL1 and inhibits the transcription of its own genes. Post-translational modifications of the clock components are critical for precise 24-h timing. Specifically, phosphorylation of the negative regulators of the molecular clock (by kinases such as CK1 and GSK3) can either stabilize the protein and promote nuclear entry (speeding up the clock) or target the proteins for proteasomal degradation (slowing the clock). In addition, a secondary feedback loop is formed when CLOCK-BMAL1 activate transcription of a nuclear orphan receptor *Reverba* whose protein product feeds back to transcriptionally repress *Bmal1* when it binds to the *Reverba* Response element (RRE) in the *Bmal1* promoter.

Figure 2. Schematic representing circadian alignment and misalignment

Circadian oscillators (including cellular and tissue levels) must remain synchronized among each other and with the environment. Under physiological conditions (top), the light-sensitive, primary circadian clock in the suprachiasmatic nucleus (SCN) entrains other secondary oscillators in the body (adipose, muscle, and liver clocks are shown for simplicity). These peripheral clocks exhibit differential sensitivity to environmental Zeitgebers such as food, and exercise. These tissue clocks consist of cellular clocks containing the core molecular clock gene components (represented by the blue gears; see Figure 1). Importantly, the SCN additionally guides food intake and exercise (as well as the physiological responses to these behaviors), keeping all of the oscillators in synch. When

sleep/wake, food intake, and physical exertion takes place outside of the SCN-permissive periods, these Zeitgebers pull the weaker, secondary oscillators away from the synchronizing signal originating from the primary clock in the SCN. This circadian misalignment often characterizes individuals suffering from shift work, jet lag, or cardiometabolic dysfunction.