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Disruption of circadian rhythm impairs pancreatic islet function and increases susceptibility to beta-cell failure, while pathological complications of Type 2 Diabetes Mellitus have scant effects on the circadian system.

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular, Cellular, and Integrative Physiology

by

Jingyi Qian

2015

ABSTRACT OF THE DISSERTATION

Disruption of circadian rhythm impairs pancreatic islet function and increases susceptibility to beta-cell failure, while pathological complications of Type 2 Diabetes Mellitus have scant effects on the circadian system.

by

Jingyi Qian

Doctor of Philosophy in Molecular, Cellular, and Integrative Physiology

University of California, Los Angeles, 2015

Professor Christopher S. Colwell, Chair

The circadian system plays an essential role in regulation of glucose homeostasis, and the disruption of this system leads to deleterious health consequences, such as Type 2 Diabetes Mellitus (T2DM). Disruption of circadian organization in humans is strongly associated with development of metabolic dysfunction and increases the propensity for development of T2DM. Importantly, the correlation between circadian disruption and T2DM is partly attributed to loss of beta-cell function and mass, called beta-cell failure. To address this association, we first hypothesized that exposure to environmental conditions associated with disruption of circadian rhythms and susceptibility to T2DM in humans disrupts islet clock and beta-cell function. We reported that prolonged exposure to LL disrupts islet circadian clock function through impairment in the amplitude, phase, and interislet synchrony of clock transcriptional oscillations. We also reported that exposure to LL leads to diminished

glucose-stimulated insulin secretion due to a decrease in insulin secretory pulse mass. Second, obesity-mediated insulin resistance is an important contributory factor to induction and progression of T2DM. We, therefore, hypothesized that disruption of circadian rhythms compromises pancreatic beta cell functional and morphological adaptation to diet-induced obesity leading to development of T2DM. We found that concomitant exposure to LL and HFD resulted in development of hyperglycemia characterized by loss of circadian rhythms in insulin secretion, compromised beta cell function, and induction of beta cell apoptosis, suggesting that circadian disruption and diet-induced obesity synergize to promote development of beta cell failure, likely mediated as a consequence of impaired beta cell clock function. Third, as accumulating evidence pointed out that metabolic signals can feed back into the circadian system, we tested whether the impaired glucose homeostasis disturbs the circadian system in return. We examined the expression of circadian rhythms in the human islet amyloid polypeptide transgenic (HIP) rats: a validated non-obese, beta-cell dysfunction model of T2DM. Diurnal and circadian rhythms of locomotor activity, circadian entrainment, and clock gene oscillation in SCN, pancreatic islets and aorta all remains intact in the HIP rats. In summary, these studies examined mechanisms by which circadian disruption predisposes to beta-cell failure in the development of T2DM. We also showed that glucose intolerance caused by beta-cell dysfunction might not be sufficient to cause circadian deficits, which emphasized the causal role of circadian disruption in the development of T2DM.

The dissertation of Jingyi Qian is approved.

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CHAPTER 1

i. Introduction

Overview

To adapt to the light-dark cycle generated by the rotation of earth on its axis, most of the life forms developed an internal system that aligns their behavior, biochemical and physiological processes with the change of light-dark cycle. This intrinsic timekeeping system generates circadian (from the latin 'circa diem', meaning about a day) rhythms which maintain a period of ~ 24 hour even in the absence of a light-dark cycle and are able to be synchronized with environmental cues or Zeitgebers (i.e., time-givers), such as light-dark cycle, ambient temperature cycle and food availability.

Sleep-wake and fasting-feeding cycles are two of the most obvious behavior patterns under the control of the circadian system. Concomitant with these two cycles, energetic demands and nutrient supply oscillate as a function of time-of-day. Thus, it is not surprising to find that marked diurnal fluctuations in metabolism are observed at multiple levels, including body temperature, glucose utilization, lipid metabolism and feeding-related hormones. It has become increasingly clear that rhythms in these metabolic processes are not simply outcomes of behavior-associated factors, but are driven in part by the intrinsic circadian clock. In fact, emerging evidence has suggested that metabolism and circadian clock are tightly interlocked: the clock drives metabolism; and various metabolic parameters feedback to the clocks.

This feedback relationship between the circadian clock and metabolism could provide the organisms with certain survival advantages. For example, it times light-sensitive

processes to avoid sunlight-induced damage; it efficiently separates incompatible metabolic processes in time and space for their successful functioning; it gives the organisms the ability to anticipate and prepare for cyclic environmental changes, such as the availability of food. On the other hand, misalignment between the internal metabolic rhythm and external environmental conditions can lead to severe disadvantages. Accumulating studies in human has revealed that disruption of circadian rhythm is associated with increasing prevalence of metabolic syndromes, such as obesity and type 2 diabetes mellitus (T2DM). Therefore, it is not hard to perceive the importance to understand the mechanisms involved in the disease progression as a consequence of circadian disruption. Furthermore, this knowledge may create new opportunities to develop treatments for the metabolic disorders.

The organization of mammalian circadian system

In mammals, the central circadian clock is located in the suprachiasmatic nuclei (SCN) at the base of the anterior hypothalamus, above the optic chiasm on either side of the third ventricle. This bilaterally paired nucleus is made up of clusters of ~10,000 tightly compacted, small-diameter neurons [1, 2]. These SCN neurons are able to express sustained circadian cycles of electrical activity *in-vitro* [3-6]. The intracellular mechanism underlying circadian rhythmicity is a highly-conserved autoregulatory transcriptional-translational feedback loops generated by a set of interplaying clock proteins. At the core of this molecular clock are the helix-loop-helix transcription factors, CLOCK and BMAL1 [7]. When dimerized, they activate transcription of the Period (PER) genes and Cryptochrome (CRY) genes through a circadian E-box regulatory element. Once translated PER and CRY translocate to the nucleus

where they function as negative regulators of their own transcription by interacting with CLOCK/BMAL1 complex thereby completing the negative feedback loop [8].

The core components of the molecular clock are also presented in most, if not all, peripheral tissues. The circadian oscillation of clock gene expression was first demonstrated in cultured fibroblasts following a serum shock [9]. Later, real-time bioluminescence imaging of the primary tissues (including liver, heart, kidney, pancreatic islets and skeletal muscle) isolated from transgenic rodents in which *Per1* gene promoter or *Per2* gene is fused to a luciferase reporter demonstrated that most major organs contain intrinsic circadian clocks [10-14]. Though these studies indicated that the circadian network in mammal is a multiple oscillator system, the SCN is still believed to be the master pacemaker. It is because, first, the circadian oscillation in clock gene expression in the peripheral tissues was lost in SCN-lesioned animals [15]. Second, the period of activity rhythm restored by SCN grafts was determined by the genotype of the SCN rather than by peripheral phenotype [15]. Third, the peripheral clocks are less robust than the SCN. They generally dampen over 3 to 6 days in culture, while the circadian oscillation in the SCN can last for months. Single cell imaging of fibroblast cultures showed that the dampening of aggregate bioluminescence rhythm in cell cultures is not due to reduced rhythmicity in individual cells, but because of de-synchronization among the cell population [16, 17]. This suggested that the SCN entrains the peripheral clock in part by setting their phase and maintaining their amplitudes.

The next question is how are the SCN and peripheral clocks aligned with external solar time? For the SCN, light is the most potent entrainment signal. It is first detected by melanopsin receptors in the retina and then transmitted to the SCN through the

retinohypothalamic tract (RHT) [18-20]. The SCN innervates other hypothalamic regions which send projections throughout the nervous and endocrine systems [21-23]. This enables the SCN to convey the temporal information to the peripheral tissues and other part of the brain in a tissue-specific manner by multiple pathways, including neural and hormonal signals [24] and indirectly modulating body temperature[25], activity and food intake. Importantly, when the availability of food was restricted to the dark phase, these peripheral oscillators, such as liver and pancreas, could be uncoupled from the SCN [26, 27]. So for these peripheral clocks, feeding time, instead of light, appears to be the dominant zeitgeber. There is some evidence that feeding-related hormones play a role in the feeding-induced entrainment. However, the exact mechanisms are still under investigation.

Just as the electrical rhythm observed in the SCN neurons, a lot of other physiological and metabolic processes also display a circadian rhythm that can sustain in culture. This is because their molecular clock machineries are intertwined with cellular functions. Gene expression profiles obtained from microarrays have revealed that an estimated 5-10% of the transcriptome displays circadian oscillation within the SCN, liver, heart, muscle and adipose tissue [28-32]. These genes which are regulated by the molecular clock are termed clock-controlled genes (CCGs). Considering that very few of these CCGs are overlapped among different tissues, the circadian-controlled transcription is highly tissue- specific. Importantly, it also has been shown that genes involved in the same cellular function or pathway tend to cycle in similar phase [33]. This indicated that circadian regulation may provide a temporal mechanism to coordinate and/or separate a wide range of interdependent biochemical reactions in the cell (Fig. 1-1).

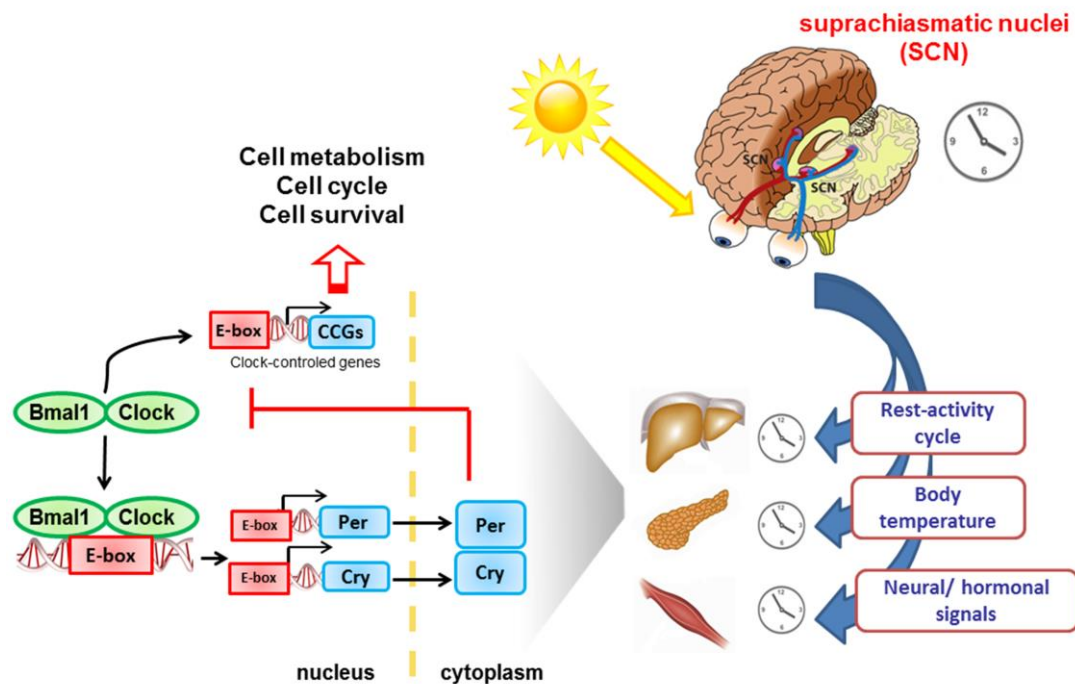


Figure 1-1. Regulation of circadian system in mammals. Light input is perceived by retinal photoreceptors and conveyed to the “master clock” of the circadian system, located in the SCN of the anterior hypothalamus. Moreover, self-sustained circadian oscillators have been shown to be present in multiple peripheral tissues and entrained by rhythmic cues from the SCN.

Circadian regulation of glucose homeostasis

In normal human beings, the plasma glucose homeostasis results from tightly controlled balance between glucose input (food intake and hepatic glucose production) and glucose utilization (uptake by muscle and adipose tissue). Insulin, produced by pancreatic beta-cells, serves as the key regulator of this process by inhibiting hepatic glucose production and stimulating glucose uptake in muscle and fat. While insulin helps to lower the plasma glucose level, the so-called counter-regulatory hormones, mainly glucagon and glucocorticoids, raises blood glucose levels by stimulating hepatic glucose production and/or inhibiting tissue glucose uptake.

The diurnal variation in plasma glucose and insulin level was discovered in early

seventies [34]. However, since in response to food intake, blood glucose increases and stimulates insulin secretion, the rhythms of plasma glucose and insulin levels are highly correlated with feeding rhythm. So for a long time, even after evidence showed that SCN lesion abolished the diurnal variations in plasma glucose and insulin levels[35], people still thought the rhythms of plasma glucose and insulin is secondary to the SCN-driven feeding-fasting cycle. The question remains: does circadian system exerts a direct effect on glucose homeostasis which is independent from the feeding rhythm? Later, several studies have provided evidence indicated that the answer to this question might be “Yes”. First of all, it was the observation of “dawn phenomenon” which is an increase in blood glucose levels in diabetic patients or an increase in insulin secretion in healthy subjects before the beginning of wake period [36-38]. Since this phenomenon occurs at the end of the resting/fasting period (7-9 hours in human), it is very unlikely that the increase in blood glucose/insulin is driven by feeding. In addition, though with smaller amplitudes, rhythms in blood glucose level continued when the rats underwent 36 hours of fasting [39]. Another proof came from studies using the experimental protocols which gave rats identical meals at a same interval throughout a day [40]. Those rats displayed larger postprandial glucose and insulin responses when fed during the dark period than when fed during light period. The feeding rhythm-independent circadian variance in plasma glucose and insulin level has been attributed to a circadian rhythm in glucose tolerance. Actually, it has been repeatedly reported that in healthy human subjects, response to an oral glucose tolerance test was larger in the morning compared to the afternoon and the evening [41-47]. The decreased glucose tolerance towards the evening can be explained by a reduction in insulin sensitivity of peripheral

tissues [48-51] and a decrease in pancreatic beta-cell responsiveness [42, 51-54] during the nighttime.

Glucose tolerance is a manifestation of a number of glucose metabolic processes, such as hepatic glucose production, glucose uptake, insulin secretion, insulin sensitivity, and insulin clearance. So the circadian regulation could happen to different processes on different levels. In addition, as I have mentioned before, besides the master clock in the SCN, almost every peripheral tissue also has a self-sustained intracellular molecular clock. Therefore, the effects of circadian regulation could come from the SCN as well as the peripheral clock themselves.

Here I will start with the pacemaker of the circadian system. It has been shown that electrical stimulation of the SCN- induced hepatic glucose production and led to hyperglycemia [55, 56]. The fact that hepatic sympathectomy in rats eliminated the rhythmicity in plasma glucose [57] indicated that the SCN could regulate hepatic glucose production through the autonomic nerve system. Pancreatic islets are rich innervated by the autonomic nervous system as well [58]. In fact, the sympathetic nervous system and the parasympathetic nervous system have opposing effects on insulin secretion from islet beta-cells [59]. As SCN-pancreas connections have also been identified [60], it is not hard to imagine that the SCN might be able to drive the circadian rhythm in glucose hemostasis through tuning insulin release. Hormonal signaling is another primary pathway that the SCN adopts to carry circadian information. For example, glucocorticoids are well-known adrenal steroid hormones whose secretion is controlled by the SCN through hypothalamus–pituitary–adrenal (HPA) neuroendocrine axis [61]. As one of the main functions of glucocorticoids is to

enhance insulin resistance, glucocorticoids was suggested to be involved in regulation of circadian variation in glucose tolerance. However, this hypothesis remains controversial because high cortisol level coincides with increased insulin sensitivity which is contradictory to the adverse effects of glucocorticoids on insulin sensitivity[62]. To resolve this contradiction, the time duration for glucocorticoids to exert an effect on insulin sensitivity should be investigated. In addition, leptin is an adipocyte hormone which has an inverse circadian relationship with cortisol. Due to its cyclical nature [63] and ability to sensitize most insulin target tissue [64], it was also suggested to modulate the circadian variation in glucose tolerance [65]. Melatonin is another well-known hormone which has a circadian secretory rhythm. It is secreted by the pineal gland and tightly regulated by the SCN. Previous studies have shown that pinealectomy abolished the nocturnal decline in plasma glucose level in rats [66] and altered the daily rhythm of glucose-induced insulin secretion in isolated rat islets [67]. Therefore, melatonin might also participate in this SCN-coordinated neural and hormonal regulation of glucose homeostasis.

At the molecular level, the intracellular mechanism of circadian clock is also essential in glucose homeostasis. The first evidence came from the *Clock* mutant mice. Not only did these mice develop severe circadian deficits, they also exhibit pronounced metabolic phenotype, such as hyperphagic and obese. *Clock* mutant mice also develop a cluster of metabolic disorders, including hyperleptinemia, hyperlipidemia, hepatic steatosis, hyperglycemia, and hypoinsulinemia [68, 69]. Pancreatic islets isolated from *Clock* mutant mice have impaired glucose-stimulated insulin secretion, which partly explains the observed impaired glucose tolerance [14]. *Bmal* knock-out mice, similar to *Clock* mutant, exhibit lower diurnal variance

in blood glucose level, impaired glucose tolerance and insulin resistance. Different from *Clock* mutant, they are hypoglycemic when fasted, and have low plasma insulin level [14, 69, 70]. *Per2* mutant mice have reduced fasting glycemia, altered glycogen accumulation in the liver, enhanced glucose-induced insulin secretion and impaired gluconeogenesis [71-73]. Glucose homeostasis is also altered *Cry*-deficient mice. *Cry1^{-/-}/Cry2^{-/-}* (double knock-out) mice show elevated blood glucose in response to acute feeding after an overnight fast and severely impaired glucose clearance in a glucose tolerance test [74]. Though the results from the whole-body circadian mutants do address the importance of the molecular clock mechanism in glucose homeostasis, they cannot determine to what extent the observed metabolic abnormalities resulted from abnormal behavioral rhythm or which part of the glucose regulation system is affected. Therefore, tissue-specific circadian mutant mice have been generated to elucidate the role of molecular clock in tissue functions independently of other clocks and in a context of normal circadian regulation of feeding behavior and locomotor activity. Data from studies conducted in the tissue-specific mutants have shown that: 1) clock genes are involved in hepatic glucose storage, transport, and export by regulating the circadian expression of glucose transporter 2, glucokinase and pyruvate kinase in the liver [75]; 2) clock genes preserve normal beta-cell function by preventing ROS accumulation via the activation of several antioxidant genes [76]; 3) clock genes prepare the muscle cells for the transition from lipid to glucose metabolism by increase insulin-stimulated glucose uptake [77]; 4) clock genes in adipocytes regulate the plasma concentration of polyunsaturated fatty acids which affect the expression of neurotransmitters responsible for appetite regulation in hypothalamic feeding centers [78].

Circadian disruption, beta-cell failure and T2DM

The worldwide incidence of T2DM has reached an epidemic proportion with estimated 360 million people diagnosed and nearly 5 million yearly deaths. It burdens health care systems with direct global spending estimated at nearly \$500 billion per year. Alarming, the incidence of T2DM worldwide is expected to continue to rise and projected to afflict nearly 600 million people within the next two decades. Undoubtedly, there is an urgent need to develop novel therapeutic and preventative approaches to combat the rise in T2DM prevalence. To meet this objective, it is critical to elucidate the environmental, molecular and physiological mechanisms underlying induction of T2DM.

T2DM is a complex metabolic disorder characterized by fasting and postprandial hyperglycemia in the context of insulin resistance and pancreatic islet failure. Accumulating evidence point out that pancreatic islet failure usually supersedes insulin resistance in inducing diabetes, but both pathological states influence each other and synergistically exacerbate diabetes [79, 80]. Insulin resistance - most commonly caused by obesity- in T2DM primarily manifests in the liver, the adipose tissues and the skeletal muscle resulting in impaired insulin-stimulated glucose uptake and failure to adequately suppress hepatic glucose production [81]. The causes of pancreatic islet failure in T2DM are complex and multifactorial, but can be generally attributed to a defect in beta-cell secretory function and the loss of beta-cell mass [82, 83]. Beta-cell secretory dysfunction is many-sided and manifests as 1) reduced glucose-stimulated insulin secretion [82], 2) diminished post-meal insulin response [84], 3) defects in pulsatile mode of insulin release [85] and 4) impaired

insulin response to non-glucose secretagogues [86]. Beta-cell loss in T2DM can be attributed to increased β -cell apoptosis [83], and probably beta-cell dedifferentiation [87]. The exact molecular pathways contributing to beta-cell apoptosis in T2DM remain unclear, a number of potential mechanisms have been proposed which include cytotoxicity due to prolonged exposure to high glucose levels (glucotoxicity) [88, 89], cytotoxicity due to high concentrations of free fatty acids (lipotoxicity) [90], and cytotoxicity due to intracellular formation of human islet amyloid polypeptide (h-IAPP) toxic oligomers (proteotoxicity) [91].

Many lifestyle factors could raise the risk of T2DM, such as increased caloric intake and physical inactivity [92]. Recently, people have become more aware that circadian disruption is another strong lifestyle factor contributing to the development of T2DM. A number of studies have supported this correlative relationship between circadian disruption and T2DM susceptibility.

First, epidemiological studies suggested an association between susceptibility to T2DM and circadian disruption caused by working conditions such as rotational shift work and extended work schedules [93-96]. It is also reported that humans with disrupted/deficient sleep patterns exhibit an increased risk for T2DM [97-100]; however it is important to note that sleep loss may have circadian-independent effects on glucose metabolism. In addition, a recent study showed that T2DM patients with late chronotype have poorer glycemic control than patients with early chronotype [101]. Moreover, the levels of melatonin, one of the best markers of internal time, were found to be reduced in T2DM patients [102]. Though these studies do provide strong evidence supporting the link between circadian disruption and T2DM, their interpretation is usually limited by confounding environmental, social,

psychological variables. In addition, it is hard to differentiate the mechanism (e.g. insulin secretion vs insulin sensitivity) underlying the increased susceptibility to T2DM in these studies. Human studies using controlled laboratory settings were carried out to reveal effects of circadian misalignment on glucose homeostasis and address the above concerns. It was reported that exposing healthy adult subjects to circadian misalignment (with concurrent sleep restriction) led to impaired glucose tolerance and postprandial hyperglycemia, an effect resulting from inadequate pancreatic insulin secretion [103-105]. More importantly, glucose intolerance and insulin resistance were also observed in subjects went through an experimental protocol introducing circadian misalignment without sleep restriction [106], suggesting that there is a sleep-loss-independent effect of circadian misalignment on glucose homeostasis. The loss of beta-cell function after circadian misalignment and sleep loss was first demonstrated in the classic study by Spiegel and colleagues. Beta-cell function was formally examined here during intravenous glucose tolerance test: first phase insulin and c-peptide secretion were significantly diminished in the sleep-deprived subjects [107]. Moreover, results from GWAS studies also add additional support for role of circadian system in glucose homeostasis and beta-cell health. For example, a variant in a key component of the core circadian clock (*Cry2*) has been shown to be associated with increased fasting glucose levels and a decline in beta-cell function [108]. The link between variance in the melatonin receptor 1B gene (*MTNR1B*) and induction of beta-cell failure in humans has also been identified [109-111].

Taken together, accumulating evidence indicated circadian disruption/misalignment as a novel risk factor for development of T2DM. Thus understanding molecular and physiological

underpinnings this circadian disruption-associated risk of T2DM holds the potential to the development of novel therapeutic and preventative strategies.

Summary

In my studies, I examined various aspects of the association between circadian disruption and the development of T2DM. First, I established and validated methods for continuous longitudinal monitoring of islet circadian clocks *ex vivo* using Per-1:LUC rats. Using this methodology, I demonstrated that islet clock transcriptional oscillations are entrained by changes in the LD cycle and feeding time.

I found that disruption of circadian rhythms due to exposure to LL disturbs islet circadian clock and beta-cell function *in vitro*. This data data suggest that impaired islet clock function caused by circadian system disruption may be responsible for altered islet function and consequent predisposition to T2DM. Because high fat diet is another risk factor of T2DM, which often co-occurs with exposure to light at night and may contribute to the increasing diabetes epidemic, next, I examined whether circadian disruption causes failed adaptation to obese-induced insulin resistance. I found that concomitant exposure to LL and HFD resulted in development of diabetes characterized by loss of circadian rhythms in insulin secretion, compromised beta cell function, and induction of beta cell apoptosis while HFD alone did not adversely augment diurnal glycemia, diurnal insulinemia, beta cell secretory function as well as beta cell survival indicating successful adaptation to increased metabolic demand. Lastly, I explored whether the impaired glucose homeostasis in T2DM disturbs the circadian system and exacerbate the disease in return. Using the human islet amyloid polypeptide transgenic

(HIP) rats, I found that the expression of circadian rhythms of locomotor activity in the 10-month-old diabetic HIP rats is not significantly different from their age-matched wild-type littermates. Moreover, the molecular clockwork in the SCN and several peripheral tissues as measured by *Per1::LUC* was also normal in the diabetic HIP rat, suggesting that glucose intolerance caused by beta-cell dysfunction might be not sufficient to alter the circadian system.

ii. Bibliograph

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CHAPTER 2

i. Introduction

In the published paper entitled “Consequences of Exposure to Light at Night on the Pancreatic Islet Circadian Clock and Function in Rats.” by Qian, J., *et al.* 2013, I assisted with design of the studies, performed studies, and assisted with interpretation of the studies and preparation of the manuscript. This chapter also contains my works on scheduled feeding in the published paper entitled “The Islet Circadian Clock: Entrainment Mechanisms, Function and Role in Glucose Homeostasis.” by Rakshit, K. 2015. Our data show that changes in the light–dark cycle and feeding-fasting cycle *in vivo* entrain the phase of islet clock transcriptional oscillations, whereas prolonged exposure (10 weeks) to constant light (LL) disrupts islet circadian clock function through impairment in the amplitude, phase, and interislet synchrony of clock transcriptional oscillations. We also report that exposure to LL leads to diminished glucose-stimulated insulin secretion due to a decrease in insulin secretory pulse mass.

ii. Consequences of exposure to light at night on the pancreatic islet circadian clock and function in rats

ABSTRACT

There is a correlation between circadian disruption, Type 2 Diabetes (T2DM) and islet failure. However the mechanisms underlying this association are largely unknown. Pancreatic islets express self-sustained circadian clocks essential for proper beta-cell function and survival. We hypothesized that exposure to environmental conditions associated with disruption of circadian rhythms and susceptibility to T2DM in humans disrupts islet clock and beta-cell function. To address this hypothesis, we validated the use of Per-1:LUC transgenic rats for continuous longitudinal assessment of islet circadian clock function ex-vivo. Using this methodology we subsequently examined effects of the continuous exposure to light at night (LL) on islet circadian clock and insulin secretion in-vitro in rat islets. Our data show that changes in the light dark cycle (LD) cycles in-vivo entrain the phase of islet clock transcriptional oscillations, whereas prolonged exposure (10 weeks) to LL disrupts islet circadian clock function through impairment in the amplitude, phase and inter-islet synchrony of clock transcriptional oscillations. We also report that exposure to LL leads to diminished glucose-stimulated insulin secretion due to decrease in insulin secretory pulse mass. Our studies identify potential mechanisms by which disturbances in circadian rhythms common to modern life can predispose to islet failure in T2DM.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is characterized by islet failure due to loss of beta-cell function and mass [1, 2]. The cause of islet failure in T2DM involves an interaction among genetic predisposition and confounding environmental factors [3]. In recent years environmental conditions associated with disruption of circadian rhythms (e.g. rotating shift work, light during the night, sleep loss, etc) have become prevalent and have been reported to augment susceptibility to T2DM [4, 5]. Importantly, the correlation between circadian disruption and T2DM is attributed in part to loss of beta-cell function and mass [6-8]. Despite a strong association between circadian disruption, T2DM and islet failure, the mechanisms underlying this link remain under investigation.

Circadian rhythms allow organisms to align internal metabolism as well as physiological and behavioral attributes to changes in the light-dark (LD) cycle. The “central clock” of the circadian system in mammals is localized in the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN is comprised of molecular oscillators (clocks), operating within individual neurons, governed by precise transcriptional-translational feedback loops [9]. Moreover, molecular oscillators are also present in tissues outside of the SCN, including pancreatic islets [10]. The SCN functions to synchronize peripheral clocks to the LD cycle via a combination of neuronal, behavioral and endocrine outputs [11]. The mechanism driving molecular clocks is governed by a set of core “clock genes”. In short, *CLOCK* and *BMAL1* are two helix-loop-helix transcription factors that are essential components of the core circadian clock [12], and when dimerized activate transcription of the *Period* (PER) genes and *Cryptochrome* (CRY) genes through a circadian E-box regulatory element. Once

translated PER and CRY translocate to the nucleus where they function as negative regulators of their own transcription by interacting with CLOCK/BMAL1 complex thereby completing the negative feedback loop [13]. This complex oscillatory network sustains 24-hour transcriptional oscillations and, importantly, synchronizes transcription of clock effector genes to changes in the LD cycle [14].

Studies suggest that beta-cell secretory function and survival is under transcriptional control of the circadian clock. Firstly, beta-cell secretory capacity shows a circadian pattern, which is impaired upon exposure to aberrant LD cycles or SCN lesions [15-17]. Circadian disruption in diabetes-prone rodents accelerates hyperglycemia through induction of islet failure [8]. Moreover, clock gene mutants are hyperglycemic, glucose intolerant and lack appropriate insulin secretion [10, 18, 19]. However, despite increased insights into the role of the circadian system in islet function, little data is available on the effects of environmental conditions associated with circadian disruption, common to modern life, on the integrity of the islet circadian clock and function. Consequently, to address this issue, we 1) validated the use of transgenic rats in which *Per-1* promoter was linked to a luciferase reporter (*Per-1:LUC*) for longitudinal assessment of islet circadian clock function *ex-vivo*, 2) tested the hypothesis that changes in the LD cycle *in-vivo* entrain the phase of islet clock transcriptional oscillations, and 3) tested the hypothesis that exposure to environmental conditions associated with circadian disruption (i.e. exposure to light at night), disrupts islet circadian clock and function.

RESEARCH DESIGN AND METHODS

Animal husbandry and behavioral monitoring.

A total of 43 Wild Type (WT) and 37 transgenic rats in which the mouse *Period-1* promoter was linked to a luciferase reporter (*Per-1:LUC*) rats were used. The generation and validation of *Per-1:LUC* rats has been previously described [20]. Rats were housed individually at the University of California, Los Angeles (UCLA) animal facility and kept in environmentally controlled soundproof chambers under standard 12/12 hour LD cycle (lights on at 6:00 hr, lights off at 18:00 hr, all times in Pacific Standard Time). Cages were outfitted with the optical beam sensor system to monitor circadian behavioral rhythms in activity and feeding (Respironics, Murrysville, PA). The UCLA Institutional Animal Care and Use Committee approved all experimental procedures.

Elucidation of behavioral, metabolic and molecular circadian rhythms in WT rat under LD cycle.

To establish diurnal behavioral, metabolic as well as temporal profiles in circadian clock gene and protein expression *in-vivo* we used 2 months-old WT rats. Rats were kept in standard LD cycle for 10 weeks and behavioral rhythms in feeding and activity were monitored. Rats were subsequently euthanized every 4 hours (n=4-5 per time point; at 2:00, 6:00; 10:00, 14:00; 18:00 and 22:00 hr). Blood was immediately collected and pancreas quickly excised with one part preserved for immunohistochemical analysis and another part for laser microdissection of islets for *in-vivo* clock gene expression assessment by real time (rt) PCR.

Laser microdissection of islets for assessment of profiles in islet clock gene expression in WT rats under LD cycle.

Following euthanasia the pancreas was harvested and embedded into OCT solution (Tissue-Tek, Torrance, CA), frozen on dry ice and stored. Subsequently, complete longitudinal sections (8- μ m) of pancreas were obtained using pre-cooled (-20C) cryotome and mounted on UV irradiated nucleic acid free PEN membrane slides (Leica, Germany). Slides were then immediately stained with hemotyxaline to identify islets and subsequently laser microdissected (Leica LMD7000, Germany) with ~ 100 islets per time point per rat. Total islet RNA was isolated using Arcturus PicoPure extraction method (Applied Biosystems, Foster City, CA) and cDNA synthesis performed using the SuperScript III First-Strand synthesis kit (Invitrogen, Carlsbad, CA). The rtPCR was carried with validated gene-specific primers for core clock genes (*Bmal-1* and *Per-1*) according to the manufactures instructions (7900HT Applied Biosystems, Foster City, CA).

Immunofluorescent staining for assessment of profiles in beta-cell clock protein expression in WT rats under LD cycle.

Following the euthanasia pancreas was immediately harvested and fixed in 4% paraformaldehyde. Paraffin embedded pancreatic sections were co-stained by immunofluorescence for markers of islet endocrine cell types insulin (18-0067; Invitrogen, Carlsbad, CA), glucagon (G2654, Sigma, St. Louis, MO), pancreatic polypeptide (NB100-1793, Novus Biologicals, Littleton, CO), somatostatin (MAB354, Millipore,

Billerica, MA) and core clock proteins BMAL-1 (sc-8550), PER-1 (sc-25362) and PER-2 (sc-25363) obtained from Santa Cruz Biotechnology. Slides were viewed using a Leica DM6000 microscope (Leica Microsystems, Bannockburn, IL, USA) and images acquired using OpenLab 5 (PerkinElmer, Waltham, MA).

Longitudinal assessment of rat islet circadian clock ex-vivo using islets isolated from Per-1:LUC rats.

Following euthanasia, rat islets were isolated from 2 months old *Per-1:LUC* rats using standard collagenase method (performed always at 9:00 PST time). Batches of 50 islets matched by size and diameter (using ocular measuring device) placed on PTFE membranes (Millipore, Billerica, MA) in culture dishes containing culture medium [serum-free, no sodium bicarbonate, no phenol red, Dulbecco's modified Eagle's medium (DMEM; D5030-10L, Sigma–Aldrich; St. Louis, MO)] supplemented with 10 mM Hepes (pH 7.2), 2mM glutamine, B27 (2%, GIBCO), and 0.1 mM luciferin. To test glucose sensitivity of the islet clock (Fig. 2-2) culture medium was supplemented with 2 mM, 5mM, 11mM or 25mM D-glucose. For all subsequent studies (Fig. 2-3) islets were cultured in standard 11mM glucose rodent medium. Culture dishes containing islets were immediately placed into a luminometer (LumiCycle, Actimetrics, Wilmette, IL) inside a light-resistant 37°C chamber with bioluminescence recorder photomultiplier tube (PMT) detector. The bioluminescence signal was counted in 1-min bins every 10 min for at least 5 days and data normalized by subtraction of the 24 hour running average from the raw data and then smoothed with a 2 hour running average (Lumicycle Data Analysis, Actimetrics) (Suppl Fig. 2-2). The peak was

calculated as the highest point of smoothed data, and the free-running period was computed as the mean between the peaks in each cycle. The amplitude was summed by the highest point and the lowest point of the each cycle [21]. *Per-1*-driven luciferase rhythms emanating from individual islets were imaged using XR/MEGA-10Z cooled intensified charge-coupled device (ICCD) camera (Stanford Photonics, Palo Alto CA). Isolated islets were cultured as previously described. Photon counts were integrated over 3-minutes with Piper control (Stanford Photonics, INC. CA) and processed with Image-J (National Institute of Health, Bethesda, MD). Amplitude, period and the phase of *Per*-driven luciferase rhythms emanating from individual islets were calculated using Lumicycle Analysis software (Actimetrics, Wilmette, IL). Phase coherence of islets was determined using Rayleigh plots which were constructed by El-Temps software (Antoni D éz-Noguera, Univ. Barcelona, Spain).

Entrainment of the islet circadian clock by changes in the LD cycle.

To test whether changes in the LD cycle *in-vivo* entrain the phase of islet clock transcriptional oscillations, we studied *Per-1*:LUC rats acclimatized for 2 weeks to standard 12/12 hour LD cycle. Behavioral recording were performed as described to confirm robust circadian behavioral rhythms. Subsequently, we inverted the phase of the LD cycle by 12 hours to DL (lights on at 18:00 hr, lights off 6:00 hr) for 4 weeks and confirmed entrainment to the new DL cycle using behavioral recordings. At the end of the 4 week period DL rats were euthanized, islets isolated, and the amplitude, phase and period of islet-specific *Per*-driven luciferase rhythms was determined as described.

Effects of circadian disruption due to light at night (LL) on behavioral, metabolic and molecular circadian rhythms in WT rats.

2 months-old WT rats were kept under LL for 10 weeks and induction of circadian arrhythmicity was confirmed utilizing diurnal feeding and activity recordings. Subsequently rats were euthanized every 4 hours (n=4-5 per time point; at 2:00, 6:00; 10:00, 14:00: 18:00 and 22:00 hr) across the 24 hour day and blood and pancreas was immediately collected for subsequent metabolic analysis.

To test the hypothesis that chronic exposure to LL results in disruption of the islet clock function we studied 9 *Per-1*:LUC rats. All rats were first acclimatized for 2 weeks to standard LD cycle and then exposed to 10 weeks of either control LD cycle (LD; n=5) or 24 hour constant light exposure (LL; n=4). Behavioral rhythms were monitored at baseline and during the study to confirm disruption of circadian rhythms in LL rats. At the end of the 10 week experimental period, all LD and LL rats were euthanized, islets isolated, and *Per-1*-driven bioluminescence rhythms were used to assess the amplitude, period and the phase of the islet circadian clock, both in batches of 50 islets (Fig. 2-4), and in individual islets (Fig. 2-5).

Islet isolations and assessment of beta-cell function in-vitro.

Pancreatic islets were isolated using standard collagenase procedure. To assess the dose-dependent glucose responsiveness, batches of 20 islets were statically incubated in 4, 8,

12 and 16 mM glucose supplemented RPMI solution for 30 minutes at each glucose concentration. Different batches of 20 islets were also stimulated with either 10nM Exendin-4 or 250µM Tolbutamide for 30 minutes for assessment of non-glucose dependent insulin release. Supernatant and lysed islets were assayed by insulin ELISA. To assess the pulsatile mode of insulin secretion islet perfusion experiments were performed (ACUSYST-S, Cellex Biosciences, Inc., Minneapolis, MN) [22]. Batches of 20 islets were first exposed to 40 minutes of 4mM glucose in Krebs Ringer bicarbonate buffer supplemented with 0.2% serum albumin, preheated to 37 C, and oxygenized with 95% O₂ and 5% CO₂ followed by 40 minutes of hyperglycemic perfusate (16 mM glucose). Effluent was collected in 1-min intervals and assayed for insulin by ELISA for subsequent determination of total insulin secretory rate, insulin pulse mass and insulin pulse interval [22].

Analytical procedures.

Plasma glucose was measured by the glucose oxidase method (YSI Glucose Analyzer, Yellow Springs, OH). Insulin and c-peptide were measured using ELIZA assay (Alpco, Salem NH).

Statistical analysis and calculations.

Activity and feeding recordings were analyzed using ClockLab software (Actimetrics, Chicago, IL). Circadian rhythms in beta-cell function and insulin sensitivity were calculated using homeostasis model assessment indices (HOMA) of insulin sensitivity ($HOMA_{IR} = [\text{glucose}] \times [\text{insulin}] / 405$; insulin in mU/L and glucose in mg/dl) and beta-cell function ($HOMA_{\beta} = [20 \times \text{insulin}] / [\text{glucose} - 3.5]$; insulin in mU/L and glucose in mmol/l) [23].

Statistical analysis was performed using ANOVA analysis with Fisher's post-hoc were appropriate (Statistica, Statsoft, Tulsa, OK). Data in graphs are presented as means \pm SEM and assumed statistically significant at $P < 0.05$.

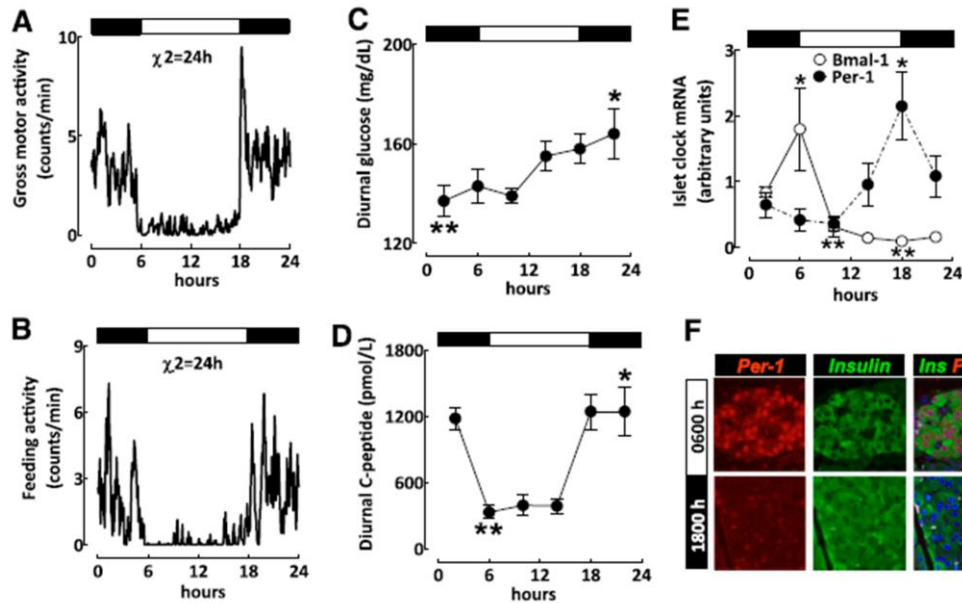


Figure 2-1. Robust behavioral, metabolic and islet molecular circadian rhythms in rats maintained under standard LD cycle conditions. (A, B) Representative mean daily measurements of diurnal gross motor and feeding activity in rats exposed to typical light regiment (12:12 hr, LD). Note rats exhibit robust diurnal 24-hour behavioral rhythms. χ^2 denotes diurnal oscillation period and black and white bars reflect the dark and light phase of the daily cycle. (C, D) Diurnal profiles in plasma glucose and c-peptide secretion measured in rats at 4-hr intervals ($n=4-5$ rats per time point) across the 24-hr day under typical LD cycle. (E) Diurnal transcriptional profiles in *BMAL-1* (open circles) and *Per-1* (black circles) mRNA obtained from laser-microdissected islets from rats at 4-hr intervals ($n=4-5$ per time point) across the 24-hr day under typical LD cycle. Note distinct antiphase relationship among *Bmal-1* and *Per-1* transcripts. (F) Examples of pancreatic islets stained by immunofluorescence for Insulin (green), *Per-1* (red) and Nuclei (blue) in rats euthanized either during the “lights on” (6:00 hr) or lights off (18:00 hr) period. Data expressed as mean \pm SEM, * $P < 0.05$ denotes statistical significance for peak value related to 24 hour LD cycle. ** $P < 0.05$ denotes statistical significance for trough value related to 24 hour LD cycle.

RESULTS

Rats exhibit robust behavioral, metabolic and molecular circadian rhythms under LD

cycle. Non-invasive monitoring of locomotor activity and feeding was used to evaluate the integrity of the circadian system [24]. As expected, WT rats kept in standard LD cycle show robust 24-hour behavioral rhythms (Fig. 2-1A, B). In addition, rats also display robust circadian rhythms in glycemia (peak: 22:00hr, trough: 2:00hr, $P < 0.05$, Fig. 2-1C) and c-peptide secretion (peak: 22:00, trough: 6:00, $P < 0.05$, Fig. 2-1C). To assess islet clock gene expression *in-vivo*, we performed laser microdissection of islets from pancreatic tissue

sections collected during the 24 hour LD cycle. Key circadian clock transcriptional activator *Bmal-1* (peak: 6:00, $p < 0.05$) and negative feedback regulator *Per-1* (peak: 18:00, $P < 0.05$) showed robust antiphase expression consistent with proposed model of the mammalian circadian clockwork (Fig. 2-1E). Furthermore, immunofluorescence confirmed diurnal oscillations in clock protein expression localized to beta-cells (Fig. 2-1F, Suppl Fig. 2-1).

Longitudinal assessment of rat islet circadian clock using islets isolated from *Per-1*:

LUC rats. Tracking of cell bioluminescence with a clock gene luciferase fusion construct was employed for assessment of islet circadian oscillators *ex-vivo* [25]. Islets cultured in standard islet media (11mM glucose) displayed sustained circadian rhythms in *Per-1* bioluminescence with a robust amplitude, oscillatory period, and the phase of circadian oscillations reflected the temporal profile of *in-vivo Per-1* mRNA expression (peak: 20.2 ± 0.6 hr) (Fig. 2A-D, Suppl Fig. 2-2). Islet circadian clocks also demonstrated glucose sensitivity (Fig. 2A-D). Specifically, islets cultured at low glucose (2 or 5 mM) showed a reduction in the amplitude (3.7-fold, $P < 0.05$ vs. 11mM), lengthening of the oscillatory period (27.5 ± 0.5 vs. 22.3 ± 0.1 hours, $P < 0.01$ vs. 11mM), and altered phase (peak: 2.5 ± 0.3 vs. 20.2 ± 0.6 hr, $P < 0.05$ vs. 11mM) of *Per-1* bioluminescence oscillations (Fig. 2A-D). Exposure to hyperglycemic conditions (25mM) did not alter the amplitude, period or the phase of the islet circadian clock (Fig. 2-2).

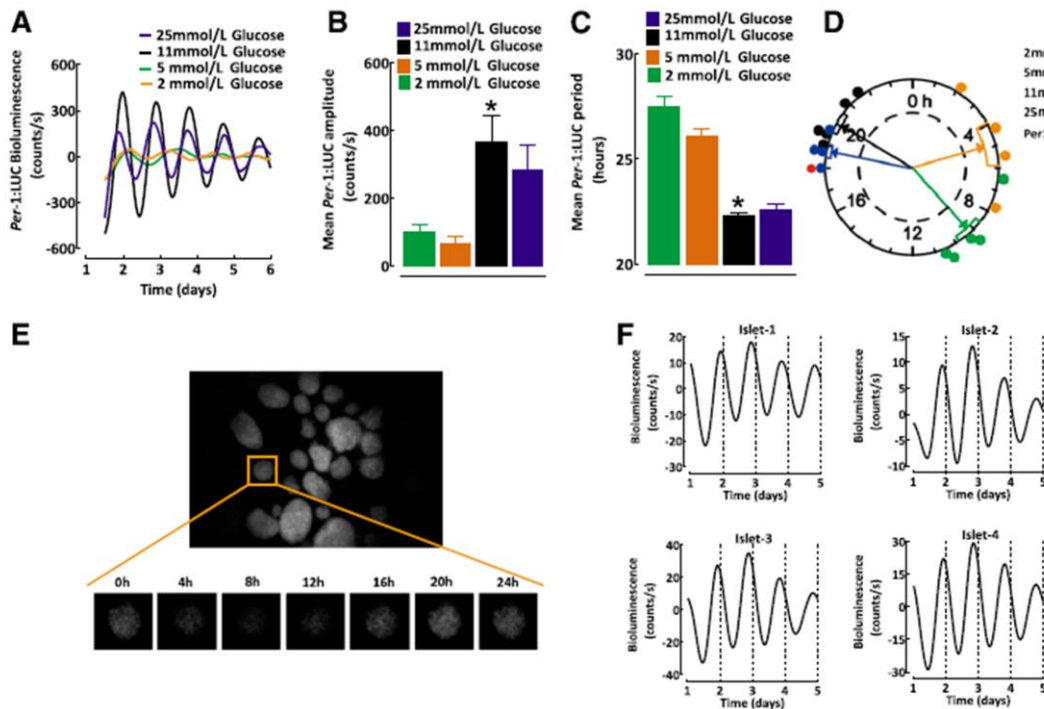


Figure 2-2. Continuous longitudinal monitoring of the islet circadian clock using *Period-1: luciferase* transgenic rats (*Per-1: luc*). (A) Representative examples of *Per*-driven diurnal bioluminescence rhythms in batches of 50 islets isolated from *Per-1: luc* rats cultured in standard islet media for 7 days at 2 mM (orange), 5mM (green), 11 mM (black) or 25 mM (blue) glucose concentrations. Corresponding mean amplitude (B) and period (C) of *Per*-driven diurnal bioluminescence rhythms in islets isolated from *Per-1:luc* rats cultured for 7 days at 2, 5, 11 or 25 mM glucose concentrations ($n=4-5$ per condition). (D) Raleigh vector plot with individual time points representing the timing of the peak *Per-1: luc* expression relative to the 24-hr day LD cycle ($n=4-5$ independent experiments) in batches of 50 islets cultured at 2, 5, 11 and 25mM glucose concentrations. Rayleigh vector plots were used to assess the significance of phase clustering of peak phases of *Per-1* expression in relation to circadian time. Each filled circle indicates the phase (time) of peak *Per-1: luc* bioluminescence in individual cultures of isolated islets under various glucose levels. The direction of an arrow indicates the mean phase vector and its length indicates the significance relative to the ($p<0.05$) with significance threshold indicated by the inner broken circle. Rectangle boxes surrounding arrow heads show variance (standard error) of phase between individual experiments. Note that the peak *Per-1* mRNA expression obtained through laser microdissection of islets (described in Fig. 2-1) is shown (red circle) to reflect the phase of *Per-1* diurnal oscillations in-vivo. (E) Whole-field image (10X) of islets isolated from *Per-1:luc* transgenic rats under standard LD condition taken by intensified charge-coupled device (ICCD) camera. An individual islet image collected over 24 hours from the islet in the orange square in the figure. (F) Examples of corresponding *Per*-driven bioluminescence rhythms obtained from 4 individual islets shown in (E) isolated from *Per-1: luc* rats under standard LD cycle cultured in 11mM glucose. Note the robust in phase oscillations among individual islets. Data are expressed as mean \pm SEM, * $P<0.05$ denotes statistical significance for 11mM vs. 2mM and 5mM glucose.

The amplitude of *Per-1* bioluminescence in islets gradually dampened over time as would be expected in the absence of the input from the SCN [21]. However, activation of cAMP-response element binding protein (a mediator of circadian clock entrainment) with

Forskolin (10uM) restarted *Per*-driven bioluminescence oscillations confirming that dampening was not due to tissue attrition (Suppl Fig. 2-3). Moreover, circadian oscillations in *Per-1* bioluminescence were also detected from single islets which displayed robust in-phase oscillations (Fig. 2-2E, F).

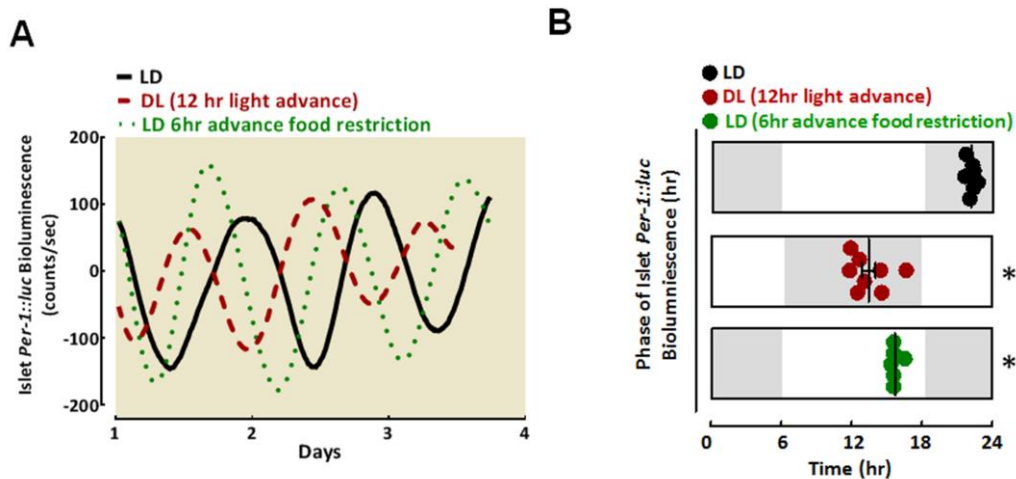


Figure 2-3. Photoperiod and feeding entrains the phase of the islet circadian clock. (A) Representative examples of *Per*-driven diurnal bioluminescence rhythms in batches of 50 islets isolated from *Per1:Luc* rats housed under 1) standard LD (lights on at 6:00 h, lights off 18:00 h) cycle and given ad-libitum food intake (black lines), 2) 12 h advance in the light cycle DL (lights on at 18:00 h, lights off 6:00 h) and given ad-libitum food intake (red lines), and 3) standard LD (lights on at 6:00 h, lights off 18:00 h) cycle with food intake restricted to 6 h period (12:00 h to 18:00 h) during the light phase of the LD cycle for 7 days (green lines). (B) Peak phase (mean \pm S.E.M) of *Per*-driven bioluminescence rhythms in ($n=6-8$) independent batches of 50 islets isolated from *Per1:Luc* rats housed under 1) standard LD cycle and given ad-libitum food intake (black circles), 2) 12 h advance in the light cycle DL and given ad-libitum food intake (red circles), and 3) standard LD cycle with food intake restricted to 6 h period (12:00 h to 18:00 h) during the light phase of the LD cycle (green circles). For clarity, data are plotted against the x-axis showing circadian time (0-24 h) with dark and light phases of LD cycle indicated by white vs. grey area shading.

Feeding is the dominate Zeitgeber for islet circadian clock. To investigate potential mechanisms mediating entrainment of islet circadian clocks, we first examined whether sudden changes in photoperiod can modulate and reset the phase of transcriptional oscillations in islet clocks. Subsequently, inversion of the phase of the LD cycle in *Per1:LUC* transgenic rats by 12 h from LD (lights on at 6:00 h, lights off 18:00 h) to DL (lights on at

18:00 h, lights off 6:00 h) resulted in near 12 h phase reversal of the islet *Per1:LUC* bioluminescence signal with no change in oscillatory period or the amplitude (Fig. 2-2, red). Feeding has been previously shown to entrain peripheral clocks and uncouple peripheral oscillators from the SCN pacemaker, an observation particularly evident in organs responsive to nutritional load, such as the liver. Thus *Per1:LUC* transgenic rats were used to assess the ability of feeding to entrain islet clocks. Restricting food availability for 6 h during the light phase of standard LD cycle entrained the islet clocks as evident by rapid 6 h shift in phase of islet *Per1:LUC* bioluminescence rhythm (Fig. 2-2, green). Similar to sudden changes in photoperiod, restricting feeding time appears to reset the phase of islet clocks, suggesting that peripheral oscillators in the islet may be coupled to the SCN via modulation of feeding rhythmicity.

Circadian disruption due to light at night (LL) disrupts islet circadian clock. Increased exposure to light at night disrupts circadian rhythms and augments susceptibility to T2DM in humans [26, 27]. Thus we next set out to address whether chronic exposure to LL disrupts islet circadian clock function. We exposed 2-month-old *Per1:LUC* rats to 10 weeks of either normal LD cycle or nightly light exposure (LL) regimen. As expected, exposure to LL in rats resulted in behavioral arrhythmicity (Fig. 2-4A, B) and abolished circadian rhythms in calculated indices of insulin sensitivity and beta-cell function derived from homeostasis model assessment, $HOMA_{ir}$ and $HOMA_{\beta}$ (Fig. 2-4C, D). Importantly, exposure to LL led to the dampening in the amplitude of *Per1*-driven luciferase oscillations in isolated islets, but did not impact the oscillatory period of *Per1*-driven oscillations (Fig. 2-4E, F). We

further confirmed this observation at the single islet level utilizing ICCD camera recordings (Fig. 2-5).

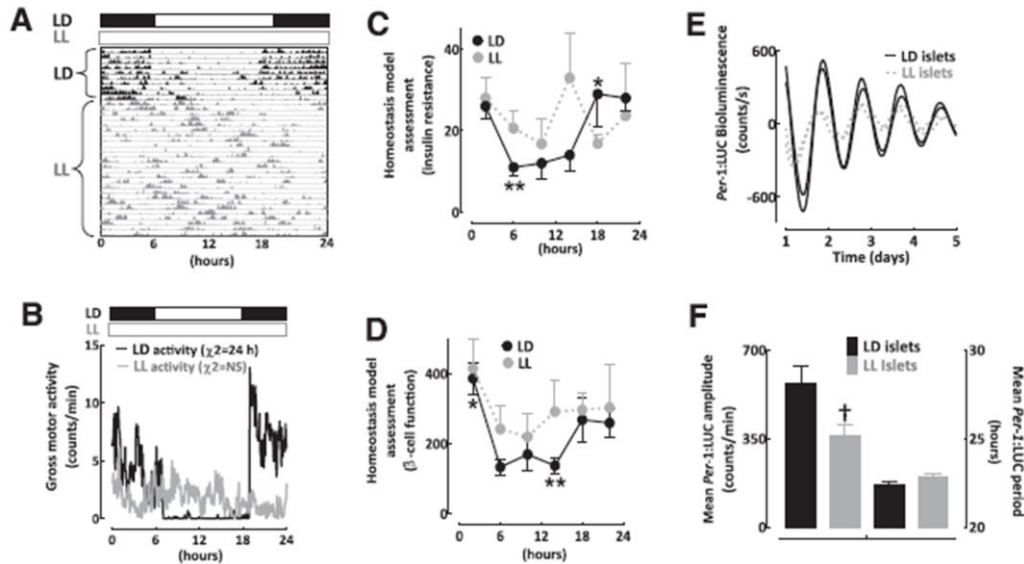


Figure 2-4. Exposure to light at night (LL) disrupts behavioral, metabolic and islet molecular circadian rhythms in rats. (A) An actogram recording of gross motor activity in a representative *Per-1: luc* rat exposed to 7 days of standard LD cycle (black) followed by exposure to light at night LL (grey) for the next 10 weeks (4 weeks of LL is shown). (B) Mean diurnal measurements of gross motor in rats exposed to 10 weeks of either standard LD (black lines) or LL (grey lines) cycle. χ^2 denotes diurnal oscillation period and black and white bars reflect the dark and light phase of the daily cycle. Note complete behavioral arrhythmicity in LL rats. (C, D) Diurnal profiles in the calculated indices of insulin sensitivity and beta-cell function derived from homeostasis model assessment, HOMA_{IR} and HOMA _{β} (see methods for calculations) in rats obtained at 4-hr intervals ($n=4-5$ per time point) across the 24-hr day under standard LD (black circles) and disrupted LL (grey circles) cycles. (E) Representative examples of diurnal bioluminescence rhythms in 2 independent batches of 50 islets isolated from *Per-1: luc* rats exposed to either 30 days of LD cycle (black lines) versus LL cycle (grey lines). (F) Mean amplitude and period of diurnal bioluminescence rhythms in batches of 50 islets isolated from *Per-1: luc* rats exposed to either 10 weeks of LD (open bars) versus LL (grey bars) cycle ($n=4-5$ per condition). Data are expressed as mean \pm SEM. * $P<0.05$ denotes statistical significance for peak value related to 24 hour LD cycle. ** $P<0.05$ denotes statistical significance for trough value related to 24 hour LD cycle. † $P<0.05$ denotes statistical significance for LD vs. LL

Whereas individual islets isolated from LD rats showed well-defined, in-phase high-amplitude circadian cycles of *Per1*-driven luciferase expression, LL rats exhibited dampened amplitude (80%, LD vs. LL, $P<0.05$ Fig. 2-5C) of circadian oscillations. Furthermore, *Per-1* bioluminescence recorded from LL islets showed impaired phase and the

synchrony of *Per*-driven luciferase oscillations among individual islets (peak: 22.5 ± 0.1 vs. 17.6 ± 0.5 hours for LD vs. LL, $P < 0.05$, Fig. 2-5E). Interestingly, LL-treated islets still maintained robust period of clock oscillations (Fig. 2-5D).

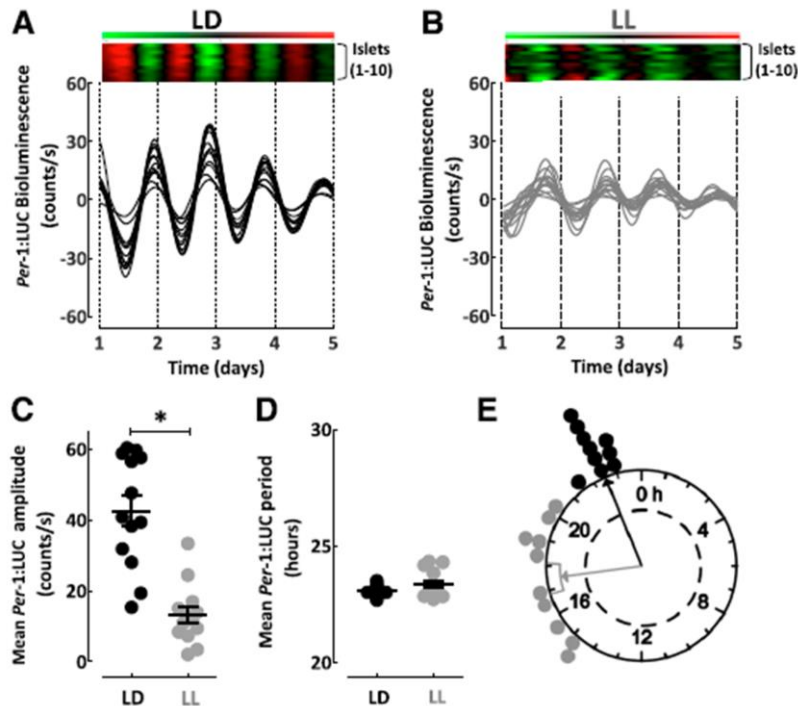


Figure 2-5. Exposure to light at night disrupts the amplitude, phase and coordination of molecular circadian rhythms in individual pancreatic islets. (A, B) Representative examples of *Per*-driven diurnal bioluminescence rhythms imaged by ICCD camera at the level of individual islets ($n=13$) from *Per-1:luc* rats exposed to either 10 weeks of LD cycle (black lines) or aberrant LL cycle (grey lines). Each black or grey line represents *Per*-driven bioluminescence signal from an individual islet. Raster plots shown on top of the each graph represent corresponding diurnal *Per-1:luc* expression in 10 individual islets across the 5 day recording period with green color representing the peak and red color the trough expression. Note the apparent loss of the amplitude and synchrony in *Per-1:luc* expression among individual islets in LL rats. Mean amplitude (C) and period (D) of *Per-1:luc* oscillations in individual islets from rats exposed to 10 weeks of either LD (black) vs. LL (grey). (E) Raleigh plot with each filled circle indicating the phase (time) of peak *Per-1:luc* bioluminescence in an individual islet from rats exposed to 10 weeks of either LD cycle (black) or LL (grey) cycle. The direction of an arrow indicates the mean phase vector and its length indicates the significance relative to the ($p < 0.05$) with significance threshold indicated by the inner broken circle. Rectangle boxes surrounding arrow heads show variance (standard error) of phase between individual LD or LL islets.

Effects of circadian disruption due to light at night (LL) on beta-cell function in-vitro. To

assess whether prolonged exposure to LL has deleterious effects on beta-cell secretory capacity, we exposed 2-month-old rats to 10 weeks of either LD or LL cycle, confirmed

disruption of circadian rhythms in LL via activity monitoring, and assessed insulin secretion in-vitro in islets by static incubation and islet perfusion (Fig. 2-6). In islets isolated from LD animals, dose-dependent glucose stimulation induced by 8, 12 and 16mM glucose led to a robust increase in insulin release (~3, 4 and 5 fold vs. 4mM respectively, $P<0.05$ at each glucose, Fig. 2-6A, B).

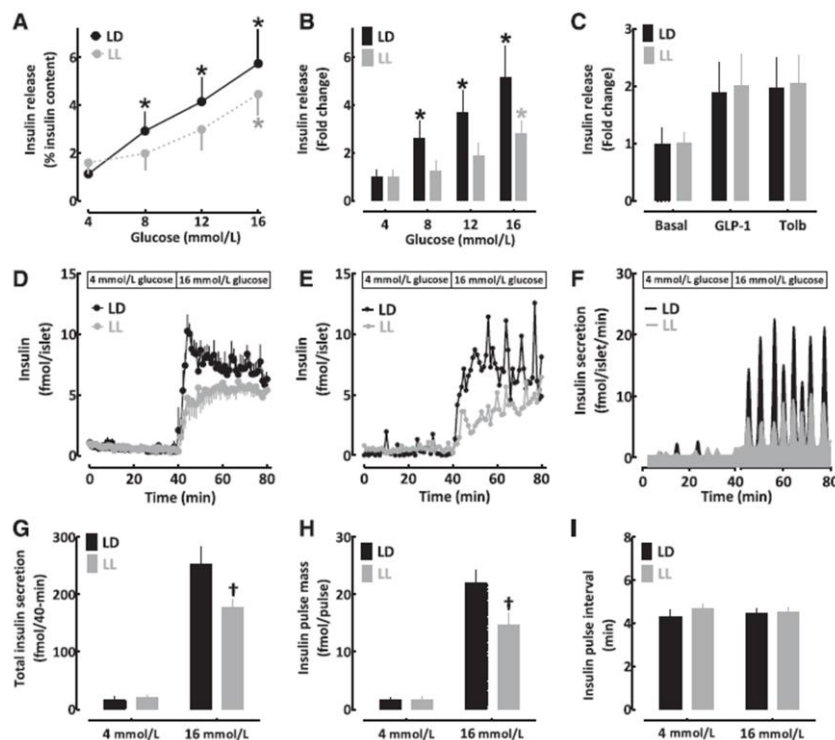


Figure 2-6. Effects of circadian disruption due to light at night (LL) on glucose-stimulated insulin secretion in isolated islets. Glucose-stimulated insulin release expressed either as percent of insulin content (A) or fold change above basal (B) in isolated islets from rats exposed to 10 weeks of either standard LD (black lines/bars) or LL (grey lines/bars) cycle ($n=7$ per condition). (C) Insulin release following stimulation with non-glucose secretory stimuli GLP-1 (10nM Exendin-4) and Sulphonylurea (250 μ M Tolbutamide) in isolated islets from rats exposed to 10 weeks of either standard LD (black bars) or LL (grey bars) cycle ($n=5$ per condition). (D) Mean insulin concentration profiles during islet perfusion at 4mM (0-40 minutes) and 16 mM glucose (40-80 minutes) in isolated islets from rats exposed to 10 weeks of either standard LD (black lines) or LL (grey lines) cycle ($n=5$ per condition). Representative islet perfusion insulin concentration (E) and derived insulin secretion (F) rates during islet perfusion at 4mM (0-40 minutes) and 16 mM glucose (40-80 minutes) in isolated islets from a rat exposed to 10 weeks of either standard LD (black lines) or LL (grey lines) cycle. Mean insulin secretion (G), insulin secretory pulse mass (H), and insulin pulse interval (I) during islet perfusion at 4mM (0-40 minutes) and 16 mM glucose (40-80 minutes) in isolated islets from rats exposed to 10 weeks of either standard LD (black bars) or LL (grey bars) cycle ($n=5$ per condition). Data are expressed as mean \pm SEM, * $P<0.05$ denotes statistical significance vs. 4 mM glucose, † $P<0.05$ denotes statistical significance vs. LD.

In contrast, LL-islets showed blunted glucose-responsiveness with only 16mM glucose eliciting a significant increase in insulin release from baseline (Fig. 2-6A, B). Insulin response to non-glucose secretory stimuli such as Exendin-4 and Tolbutamide remained intact in LL animals suggesting a primary defect in glucose metabolism and/or coupling to oxidative phosphorylation (Fig. 2-6C). To elucidate the mechanism underlying diminished glucose-stimulated insulin release we performed islet perfusions to assess the pulsatile mode of insulin secretion (Fig. 2-6D-I). LL-treated islets demonstrated blunted glucose-stimulated insulin secretion (~40% vs. LD, $P < 0.05$) which was due to ~40% deficit in insulin secretory pulse mass with no alteration in the frequency of pulsatile insulin release (Fig. 2-6D-I).

DISCUSSION

Mammalian circadian system is organized as a multi-level oscillator network. The main SCN oscillator is synchronized to the LD cycle through specialized retinal ganglion cells with light serving as the principal entrainment stimuli [28]. The SCN, in turn, plays a central role in synchronizing the rhythms of the peripheral circadian clocks to the 24 hour LD cycle [29]. This multi-level circadian oscillator system undoubtedly provides an evolutionary advantage for human health. However, because the circadian system is sensitive to changes in the LD cycle, disruption of circadian rhythms as a result of shift work, sleep loss, exposure to light at night and others has been associated with deleterious health consequences [30, 31].

Specifically, circadian disruption is associated with development of T2DM, a relationship attributed in part to islet failure [5-8, 10, 18, 19]. This implicates disturbances in the islet circadian clock as a potential molecular mechanism underlying the association between circadian disruption, T2DM and islet failure. Thus, in the current study, we sought to elucidate effects of the environmental conditions associated with circadian rhythm disruption common to modern life on the islet circadian clock and function. To address this, we first validated the use of *Per-1:LUC* rats for assessment of islet clock function *ex-vivo*. Secondly, we established that changes in the LD cycles *in-vivo* entrain the phase of islet clock transcriptional oscillations. Thirdly, we demonstrated that exposure to LL disrupts islet circadian clock function through impairment in the amplitude, phase and inter-islet synchrony of clock transcriptional oscillations. Finally, we also report that islets isolated from rats exposed to LL exhibit diminished glucose-stimulated insulin secretion attributed to the deficit

in insulin secretory pulse mass.

Evidence supports a role for the beta-cell circadian clock in regulation of insulin secretion and beta-cell survival. Circadian rhythms in glucose tolerance and beta-cell secretory capacity have been observed under fasting, glucose-infusion and meal conditions [15, 32, 33]. Importantly, lesioning of the SCN in rats or exposure to LL abolishes circadian rhythms in glucose tolerance and insulin release and impairs beta-cell function [16]. This suggests that the central clock regulates diurnal insulin secretion, plausibly through influencing beta-cell circadian clock function. In support of this hypothesis pancreas-specific CLOCK and BMAL-1 mutant mice demonstrate a loss of diurnal rhythms in glucose tolerance and insulin secretion and exhibit diminished beta-cell function [10, 18]. Furthermore, pancreas-specific clock mutants also show impaired expression of genes regulating islet growth, survival and proliferation emphasizing that islet circadian clock may also be significant in regulation of beta-cell survival [10]. Indeed, cellular response to oxidative and endoplasmic reticulum (ER) stress is under transcriptional control of the circadian clock [34]. This is significant because both of these features contribute to islet failure in diabetes [35, 36]. Interestingly, circadian disruption due to prolonged exposure to LL in rats increases beta-cell vulnerability to apoptosis associated with overexpression of human islet amyloid polypeptide, a known inducer of beta-cell ER stress [8].

One of the key observations in our study is that exposure to LL leads to dampening, alterations in the phase and desynchrony of islet clock transcriptional rhythms. This can be attributed to disruption or altered function of the SCN clock resulting in impaired entrainment of circadian oscillators in islets. Indeed prolonged exposure to LL (of similar duration and light intensity employed in our study) leads to desynchronization of circadian oscillators in the SCN resulting in the loss of behavioral and endocrine rhythms [37]. Similar abolishment of circadian behavioral and endocrine rhythms is observed following SCN

lesioning in rodents which can be partially restored upon SCN transplantation [38, 39]. Importantly, SCN lesions are associated with damping of the amplitude of circadian clock oscillations in peripheral tissues (e.g. liver and kidney) [40, 41], albeit with some studies only noting alterations in the phase of transcriptional oscillations [42]. Consistent with our findings, these studies suggest that surgical or light-induced disruption of the SCN circadian clock can result in impaired cyclical expression, dampened amplitude and altered phase of circadian clock transcriptional oscillations in peripheral tissue. Moreover, our work also shows that in some parallels to the results obtained by lesioning the SCN [37], exposure to light at night results in impaired coupling among individual circadian oscillators in pancreatic islets. The functional significance of uncoupling individual circadian oscillators in islets is unknown; however, synchronization of individual neuronal clocks in the SCN is essential for proper clock function [43].

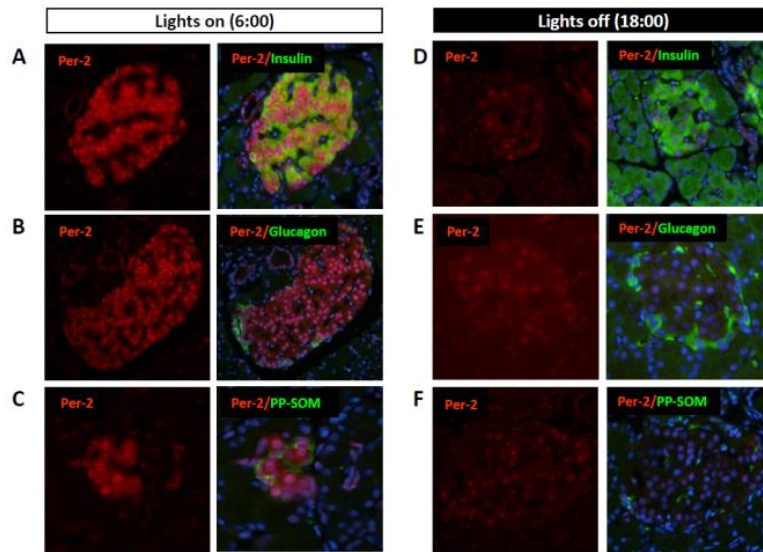
Mechanisms have been proposed to mediate entrainment of peripheral oscillators by the SCN [29]. However, it is currently unknown which mechanism(s) regulate entrainment of islet circadian clocks. The SCN has been shown to modulate peripheral clock expression through autonomic nervous system (ANS) [44]. Pancreatic islets receive extensive innervation by the ANS and the SCN has been shown to innervate the pancreas via the parasympathetic neuronal pathway [45]. The SCN also has been shown to modulate entrainment of peripheral clocks through circadian regulation of hormonal release (e.g. corticosterone and melatonin) [29]. Recent work on the role of melatonin receptor signaling in the regulation of islet failure in T2DM suggests that the significance of diurnal melatonin secretion for entrainment of islet clocks merits further investigation [46]. In addition, the SCN also controls diurnal feeding, the timing of which has been shown to entrain the phase and the amplitude of peripheral clocks, particularly in organs responsive to nutrient

availability such the liver and the pancreas [20, 47]. In our study, exposure to LL was associated with the loss of circadian rhythms in hormonal release (melatonin and corticosterone) as well as feeding behavior. However, further work is needed to delineate exact mechanisms regulating entrainment of clocks in islets.

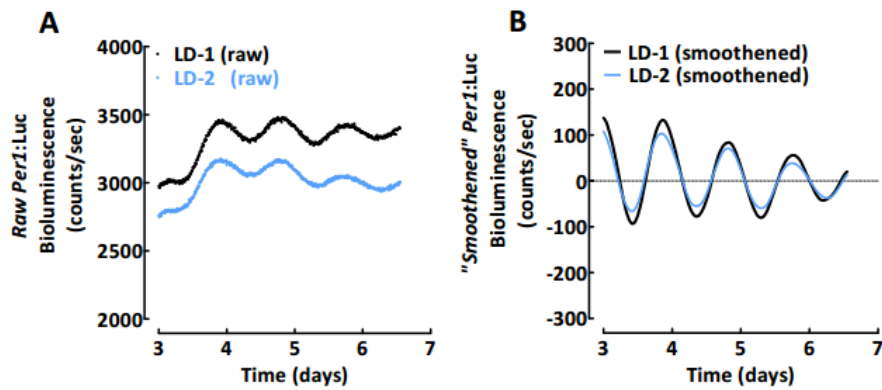
This report also examined effects of prolonged exposure to LL on beta-cell function in isolated islets. Our observation that prior exposure to LL results in diminished beta-cell function is consistent with findings in humans that report induction of glucose intolerance and diminished insulin response to glucose following circadian misalignment [7, 17]. Our data is also consistent with findings reported in rodents with genetic mutations in the core components of the circadian clock genes which also demonstrate diminished beta-cell glucose-responsiveness hypothesized to be associated with either defective insulin exocytosis and/or mitochondrial uncoupling leading to diminished glucose-induced mitochondrial potential and ATP production [10, 48].

In conclusion, we established and validated methods for continuous longitudinal monitoring of islet circadian clocks *ex-vivo* using *Per-1:LUC* rats. Utilizing this methodology, we demonstrated that islet clock transcriptional oscillations are entrained by changes in the LD cycle and found that disruption of circadian rhythms due to exposure to light at night profoundly disturbs islet circadian clock and beta-cell function *in-vitro*. Our data suggests that impaired islet clock function caused by circadian system disruption may be responsible for altered islet function and survival and consequent predisposition to T2DM.

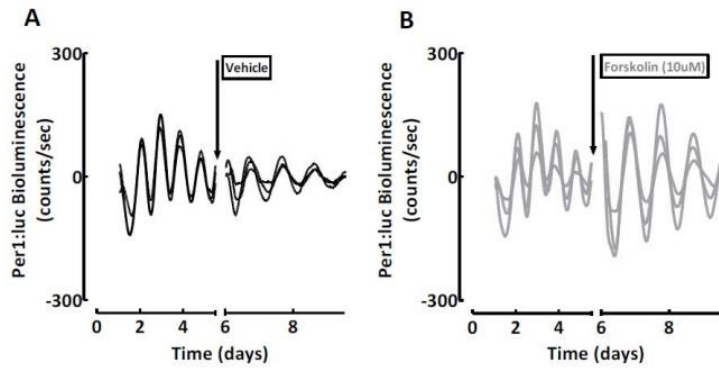
iii. Supplemental Data



Supplementary Figure 2-1. Diurnal clock protein expression in islet endocrine cell types in rats. Examples of rat pancreatic islets stained by immunofluorescence for (A, D) Insulin (green), Per-2 (red) and Nuclei (blue); (B, E) Glucagon (green), Per-2 (red) and Nuclei (blue); (C, F) Pancreatic Polypeptide (PP) and Somatostatin (SOM) (green), Per-2 (red) and Nuclei (blue) in rats euthanized either during the “lights on” (left; 6:00hr) or lights off (right; 18:00hr) period.



Supplementary Figure 2-2. Comparison between “raw” and “smoothened” (with baseline subtraction) islet Per-1 bioluminescence curves from rats kept under standard LD cycle. (A) Individual raw data points of Per-1: luc bioluminescence recorded from two separate batches of 50 isolated islets (black and blue) recorded at 30 second intervals over 4 day period under standard 11mM glucose culturing conditions. (B) Corresponding “smoothened” and baseline subtracted curves of Per-1:luc bioluminescence derived from raw Per-1:luc recordings shown in (A).



Supplementary Figure 2-3. Forskolin treatment reentrains islet *Per-1* bioluminescence rhythms. Representative examples of diurnal bioluminescence rhythms in 3 independent batches of 50 islets. Isolated from *Per-1: luc* rats raised in standard LD cycle. Vehicle (A) or a cAMP-response element binding protein activator Forskolin (B) was administered after 5 days of culture under standard conditions. Note that administration of Forskolin (10uM) restarted *Per*-driven bioluminescence oscillations confirming that dampening in *Per*-driven bioluminescence that occurs after 4-5 days of culture is not attributed to islet attrition.

iv. Bibliography

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CHAPTER 3

i. Introduction

In the submitted manuscript entitled “Circadian disruption and diet-induced obesity synergize to promote development of beta cell failure and diabetes in rats.” by Qian, J. *et al.*, we used constant light (LL) and high fat diet (HFD) to examine the effects of circadian disruption on beta-cell adaptation to obesity-induced insulin resistance. We found that whereas exposure to HFD under LD leads to appropriate adaptive beta cell response and preservation of normal glucose homeostasis, HFD under LL results in development of diabetes characterized by loss of circadian rhythms in insulin secretion, impaired beta cell function, and increased rate of beta cell apoptosis.

I need to thank Bonnie Yeh and Kevin Hsu for their assistance with the in-vitro islet function assay, immunohistochemistry and immunofluorescence.

ii. Circadian disruption and diet-induced obesity synergize to promote development of beta cell failure and diabetes in rats.

ABSTRACT

There are clear epidemiological associations between circadian disruption, obesity and pathogenesis of Type 2 Diabetes (T2DM). The mechanisms driving these associations are under investigation. In the current study we hypothesized that disruption of circadian rhythms compromises metabolic and pancreatic beta cell functional and morphological adaptation to diet-induced obesity leading to development of T2DM. To address this hypothesis we studied Sprague Dawley (SD) as well as *Per1:LUC* transgenic rats for 10 weeks under standard light dark cycle (LD) or circadian disruption (LL, constant light) with concomitant ad libitum access to either standard chow or 60% high fat diet (HFD). Exposure to HFD led to a comparable increase in food intake, body weight and body adiposity in both LD and LL-treated rats. However, LL rats displayed profound loss of global circadian rhythms as well as disrupted pancreatic islet clock function characterized by the impairment in the amplitude and the phase islet clock transcriptional oscillations. Under LD cycle, HFD did not adversely augment diurnal glycemia, diurnal insulinemia, beta cell secretory function as well as beta cell survival indicating successful adaptation to increased metabolic demand. In contrast, concomitant exposure to LL and HFD resulted in development of diabetes characterized by loss of circadian rhythms in insulin secretion, compromised beta cell function, and induction of beta cell apoptosis. This study suggests that circadian disruption and diet-induced obesity synergize to promote development of beta cell failure leading to development of T2DM.

INTRODUCTION

The incidence of Type 2 diabetes (T2DM) has reached epidemic proportions raising the significance of understanding pathophysiology of this common disease. Pancreatic beta cell failure is a key feature of T2DM, characterized by compromised beta cell insulin secretory function and survival [1, 2]. Mechanisms underlying beta cell dysfunction are complex, but are largely attributed to a decline in glucose-stimulated insulin secretion [3] as well as a loss of circadian control of insulin release [4, 5]. Compromised beta cell survival in T2DM is associated with increased frequency of beta cell apoptosis attributed in part to induction of endoplasmic reticulum (ER) and/or oxidative stress [6].

Obesity has long been described as an important contributory factor to induction and progression of T2DM [7]. This is largely attributed to obesity-mediated insulin resistance which places an increased metabolic demand on the beta cells to produce and secrete appropriate amounts of insulin to maintain normoglycemia [8, 9]. Indeed, non-diabetic obese insulin resistant individuals remain normoglycemic due to compensatory increase in beta cell function while retaining appropriate beta cell survival [10, 11]. In contrast, beta cell failure and subsequent hyperglycemia occurs in a subset of individuals who fail to maintain adequate beta cell function and survival in lieu of increased metabolic demand associated with obesity [2, 12]. Thus, understanding molecular and physiological mechanisms underlying failed beta cell adaptation to obesity is critical for prevention and treatment of T2DM.

It is becoming increasingly apparent that circadian system plays an important role in regulation of normal metabolic function and glucose homeostasis [13]. Circadian system is

a fundamental property of nearly all living organisms and provides physiological advantage by adapting internal metabolism to changes in the light dark cycle (LD). The “master circadian clock” in mammals is localized in the suprachiasmatic nucleus (SCN) of the hypothalamus governed by a highly conserved set of core “clock” genes (molecular oscillators) regulated by a transcriptional-translational feedback loops [14]. The SCN clock synchronizes molecular oscillators in multiple peripheral tissue subtypes accomplished thru combination of neuronal, behavioral and endocrine mechanisms [15]. Robust circadian organization is particularly essential for pancreatic beta cells, given critical importance to restrain insulin production and secretion during the sleep phase (to prevent hypoglycemia), and activate insulin production and secretion during the active phase (to avoid hyperglycemia) [16]. Accordingly, beta cell molecular clocks have been shown to be essential for transcriptional regulation of insulin release, production, as well as control of beta cell survival and stress response [17-19]. Consistent with these observations, disruption of circadian organization in human populations has been demonstrated to promote development of metabolic dysfunction, and particularly, increase propensity for development of T2DM [20-22]. However, the mechanisms underlying increased susceptibility to T2DM in individuals exposed to circadian disruption remain to be elucidated.

These observations raise the question whether circadian disruption accelerates development of T2DM by compromising normal beta cell functional and morphological adaptation to obesity-induced insulin resistance, mediated in part through loss of beta cell clock function? To address this hypothesis in the current study we examined effects of biological interactions between circadian disruption (accomplished by prolonged exposure to

constant light, LL) and diet-induced obesity *in-vivo* in rats by detailed assessment of 1) global circadian rhythms, 2) islet circadian clock function, 3) glucose homeostasis, 4) islet function as well as 5) islet turnover and survival.

RESEARCH DESIGN AND METHODS

Study design. A total of 46 male Sprague Dawley rats and 19 male Wistar rats transgenic for the mouse *Period-1* promoter linked to a luciferase reporter (*Per1:LUC*) were used in the current study. The generation and validation of *Per-1:LUC* rats for the study of islet clock function have been previously described [19, 23]. All rats were bred and housed individually before the study at the University of California Los Angeles animal housing facility and subjected to standard 12/12 hour LD (lights on at 6:00hr, lights off 18:00hr, PST) cycle. Two weeks prior to initiation of study protocols, all rats were synchronized to standard living conditions with lights on at 6:00hr and lights off at 18:00hr in a custom made environmentally controlled soundproof chambers outfitted with the optical beam sensor system to monitor circadian behavioral rhythms in locomotor activity (Respironics, Murrysville, PA). At 3 months of age rats were randomly assigned into four experimental protocols for 10 weeks: (1) *LD-Chow*; 10 weeks of standard LD cycle (lights on at 6:00hr, lights off 18:00hr) on standard laboratory chow diet (14% Fat, 32% protein and 54% carbohydrates, Harlan Laboratories), (2) *LD-HFD*; 10 weeks of standard LD cycle on high fat diet (HFD) (60% Fat, 20% protein and 20% carbohydrates, Research Diets Inc, New Brunswick, NJ) (3) *LL-Chow*; 10 weeks of 24 hour constant light exposure (25 watt fluorescent tubes 12" above the cage at >100 Lux light intensity) on standard laboratory chow diet, and 4) *LL-HFD*; 10 weeks of 24 hour constant light exposure on HFD. The University of California Los Angeles Institutional Animal Care and Use Committee approved all experimental procedures described in the study.

Circadian activity analyses. Rats exposed to the four experimental protocols (LD-Chow, LD-HFD, LL-Chow, and LL-HFD) were housed individually in standard rat cages equipped with infrared detectors, and 24hr locomotor activity was recorded in 3-min intervals for duration of the study. Circadian activity data was analyzed and plotted by using the normalized format in ClockLab software (Actimetrics, Wilmette, IL, USA). Data from last 14 days of each experimental protocol was used for final data analysis.

Islet Per1:LUC Bioluminescence Recording and Data Analysis. *Per1:LUC* rats were exposed to LD-Chow, LD-HFD, LL-Chow, and LL-HFD conditions for 10 weeks and subsequently euthanized. Following euthanasia, pancreatic islets were isolated using standard collagenase method (performed always at 9:00 PST time). Subsequently, 50 isolated islets per animal were handpicked carefully matched by size and diameter (using an ocular measuring device) and plated onto PTFE membranes (Millipore, Billerica, MA) in 35mm culture dishes containing culture medium [serum-free, no sodium bicarbonate, no phenol red, Dulbecco's modified Eagle's medium (DMEM; D5030-10L, Sigma-Aldrich; St. Louis, MO)] supplemented with 10 mM HEPES (pH 7.2), 2mM glutamine, B27 (2%, GIBCO), and 0.1 mM luciferin. Dishes were sealed and placed into a LumiCycle luminometer (Actimetrics, Inc.), which was kept inside a standard light-resistant tissue culture incubator at 37 °C. Bioluminescence was measured by photomultiplier tube for 1 min at intervals of 10 min and continuously recorded for at least 7 days. For analysis of rhythm parameters, original data were detrended by subtraction of the 24 h running average from the raw data, and then smoothed with a 3 h running average¹ (Lumicycle Data Analysis, Actimetrics). Peak

was defined as the highest point of smoothed data and the 1st peak after 24 h in vitro was used as a phase marker. Amplitude was calculated as the difference between the highest and lowest 12-h means for each circadian cycle. Period is calculated as the average time difference between adjacent peaks as previously described [19].

Assessment of plasma metabolic parameters. Body weights and food intake were measured at the start of each week just prior to the previously maintained dark/active phase. During the final week (week 10) of LD-Chow, LD-HFD, LL-Chow or LL-HFD protocols diurnal blood samples were collected at circadian times 4 (10:00 PST), 10 (16:00 PST), 16 (22:00 PST), and 22 (4:00 PST) with at least 12 hour gaps between individual blood draws on the same rat to avoid confounding effects of stress and blood loss. Blood was collected via lateral saphenous venipuncture using sterile 21-gauge, 1-inch syringe needle into chilled, EDTA-treated microcentrifuge tubes and immediately centrifuged at 4 °C for subsequent collection of plasma. Plasma glucose was measured immediately using the YSI 2300 STAT Plus Glucose & Lactate Analyzer (YSI Life Sciences) and plasma aliquots were frozen at -80 °C for future analyses for insulin (Alpco Diagnostics, Salem, NH) and triglycerides (WAKO, Richmond, VA).

Assessment of beta cell function in-vitro. Pancreatic islets were isolated using standard collagenase procedure from rats exposed to the four experimental protocols (LD-Chow, LD-HFD, LL-Chow, and LL-HFD). To assess basal and glucose-stimulated insulin secretion islet perfusion experiments were performed (ACUSYST-S, Cellex Biosciences,

Inc., Minneapolis, MN) as previously validated for the study of insulin secretion [24]. Batches of 20 islets were first exposed to 40 minutes of 4mM glucose in Krebs Ringer bicarbonate buffer supplemented with 0.2% serum albumin, preheated to 37 C, and oxygenized with 95% O₂ and 5% CO₂ followed by 40 minutes of hyperglycemic perfusate (16 mM glucose). Effluent was collected in 1-min intervals and assayed for insulin by ELISA (Alpco diagnostics) for subsequent determination of basal and glucose-stimulated insulin release.

Assessment of beta cell turnover by immunohistochemistry and immunofluorescence.

Rats exposed to the four experimental protocols for 10 weeks were euthanized and pancreas was quickly excised and weighed, then fixed in 4% paraformaldehyde overnight at 4 °C and subsequently embedded in paraffin and sectioned through the maximal length. Paraffin embedded pancreatic sections (3 sections per animal spaced through the width of the pancreas) were stained first for hematoxylin/eosin, and insulin (guinea-pig anti-insulin, Abcam) for determination of insulin positive area and beta cell mass was measured by first quantifying the pancreatic cross-sectional area positive for insulin and multiplying this by the pancreatic weight. In addition, sections were co-stained by immunofluorescence for insulin (guinea-pig anti-insulin, Abcam) and terminal deoxynucleotidyl transferase biotin-dUTP nick end-labelling (TUNEL method, Roche Diagnostics, Mannheim, Germany) for quantification of beta-cell apoptosis, and insulin and Ki-67 (mouse anti Ki-67, Sigma) for determination of beta-cell replication. All islets per pancreatic section (~200 cells per section) were examined in detail and counted at ×200 magnification (×20 objectives, ×10ocular) for the

total number of apoptotic and proliferating beta cells. Slides were visualized using Leica DM600 microscope (Leica Microsystems, Wetzlar, Germany) and images acquired using OpenLab software (Improvision) and analyzed using ImagePro Plus software.

Statistical analysis. Statistical analysis was performed using ANOVA with post hoc tests wherever appropriate (GraphPad Prism v.6.0, San Diego, CA). Data in graphs are presented as means \pm SEM and assumed statistically significant at $p < 0.05$.

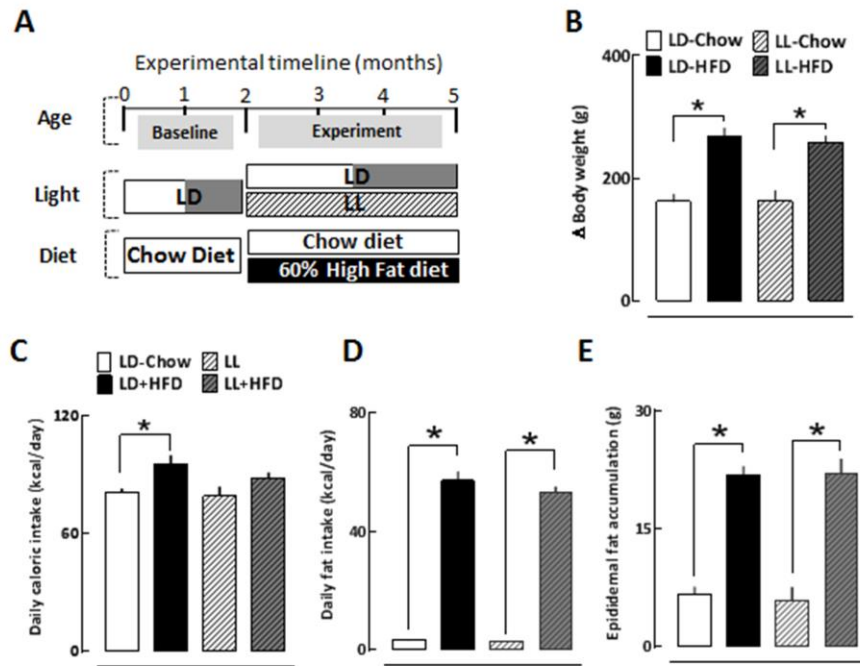


Figure 3-1. Induction of diet-induced obesity in rats exposed to 10 weeks of standard LD cycle or constant light (LL). (A) A diagram of experimental timeline. (B) Mean body mass gain, (C) daily caloric intake, (D) daily fat intake and (E) mass of epididymal fat depots in rats exposed for 10 weeks to standard LD cycle on chow diet (LD-Chow; open bars; $n=11$), 2) LD cycle on 60% high fat diet (LD-HFD; black bars; $n=15$), 3) LL cycle on chow (LL-Chow; open striped bars; $n=8$) and 4) LL cycle on HFD (LL-HFD; shaded striped bars; $n=15$). Bar graphs represent mean \pm SEM. * $P<0.05$.

RESULTS

Body weight, total calorie, and fat-derived calorie intake increased comparably in LD and LL housed rats in response to 10 weeks of HFD treatment (Fig 3-1B-D, $P>0.05$ for LD-HFD vs. LL-HFD). In addition, exposure to HFD led to a comparable epididymal fat accumulation (Fig. 3-1E) and equal elevations in diurnal triglycerides (data not shown) in LD and LL suggesting effective and equivalent induction of diet-induced obesity in both light treatment groups. As expected, when housed under LD cycle both chow and HFD rats displayed robust 24hr circadian locomotor activity rhythms (Fig. 3-2A, B) with no significant differences in the power of circadian rhythm denoted by Fast Fourier Transform (FFT) values ($p>0.05$, Fig. 3-2C) or average daily locomotor activity ($p>0.05$, Fig. 3-2D). In contrast,

exposure to LL (in both LL-Chow and LL-HFD) led to a profound loss of circadian rhythmicity characterized by loss of circadian activity period and a ~ 90% decline in FFT values compared to both LD-Chow and LD-HFD groups (Fig. 3-2C). Importantly, no significant difference between all 4 groups were observed in respect to average daily activity implying that employed experimentations did not alter total daily activity (Fig. 3-2D).

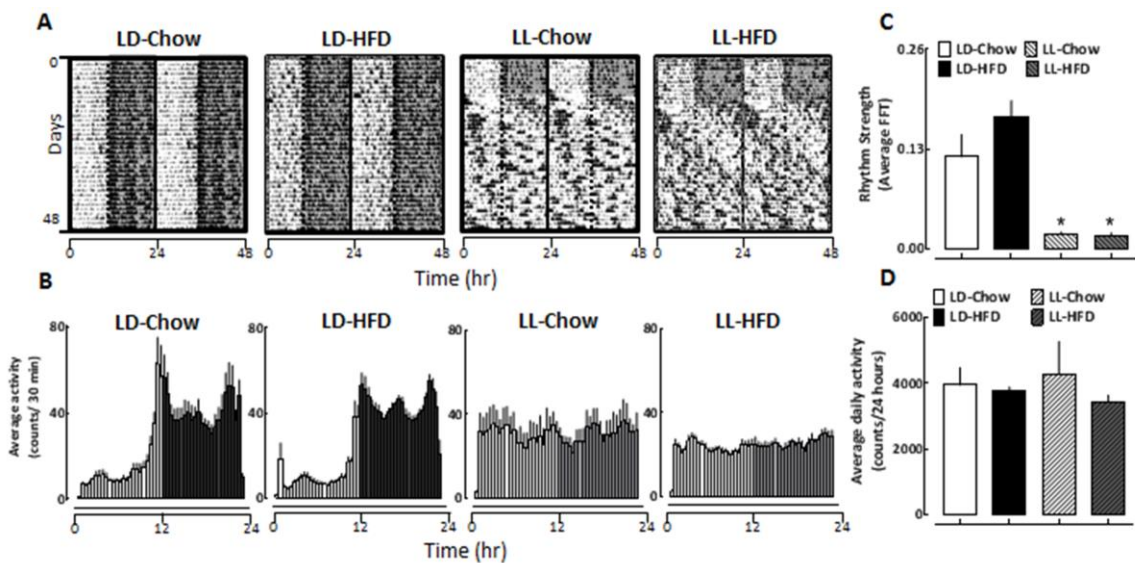


Figure 3-2. Induction of global circadian disruption in rats exposed to 10 weeks of standard LD cycle or constant light (LL) fed either regular chow or 60% high fat diet (HFD). (A) Representative behavioral profiles of gross motor activity in rats (double plotted) monitored for 1 week at baseline under standard LD cycle fed regular chow diet followed by 6 weeks of recordings in LD-Chow, LD-HFD, LL-Chow or LL-HFD. Shaded areas represent periods of dark cycle. (B) Mean circadian activity (expressed as counts/30 min) shown across the 24 hour circadian day in rats exposed for 10 weeks to LD-Chow, LD-HFD, LL-Chow or LL-HFD. Each bar represents mean \pm SEM ($n=4-7$ per group, per time point). Shaded areas represent periods of darkness under LD, and corresponding time periods under LL. (C) A measure of circadian rhythm strength denoted by Fast Fourier Transform (FFT) values, and (D) average daily (24 hr) activity of respective groups. Bar graphs represent mean \pm SEM ($n=4-7$ per group). * $P<0.05$ vs. LD-Chow and LD-HFD.

We next examined effects of biological interactions between LD, LL and Chow, HFD on the function of the islet circadian clock employing continuous tracking of islet cell bioluminescence with a clock gene luciferase fusion construct utilizing *Per1:LUC* transgenic rats (Fig. 3-3).

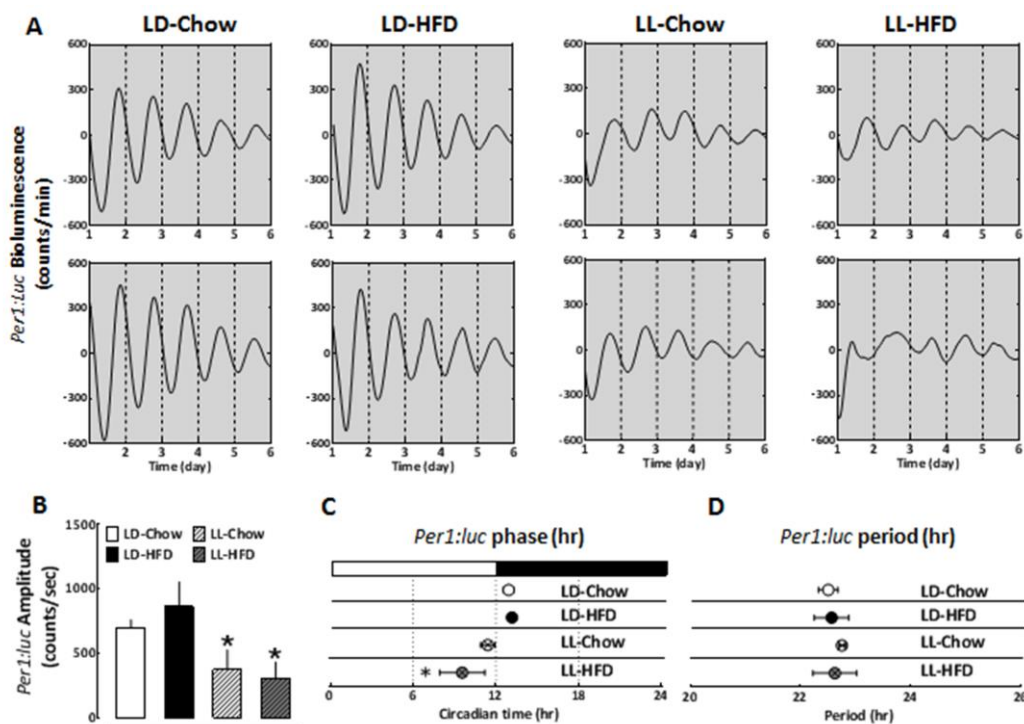


Figure 3-3. Real-time bioluminescence monitoring of islets isolated from *Per1:LUC* transgenic rats exposed to 10 weeks of standard LD cycle or constant light (LL) fed either regular chow or 60% high fat diet (HFD). (A) Representative examples of 5 days of continuous recordings of *Per*-driven bioluminescence rhythms in islets isolated from *Per1:LUC* transgenic rats exposed for 10 weeks to LD-Chow, LD-HFD, LL-Chow or LL-HFD. (B) Mean amplitude, (C) phase and (D) period of circadian clock oscillations in islets isolated from *Per1:LUC* transgenic rats exposed for 10 weeks to LD-Chow, LD-HFD, LL-Chow or LL-HFD conditions. Bar graphs represent mean \pm SEM ($n=4-6$ per group). * $P<0.05$ vs. LD-Chow and LD-HFD.

Islets isolated from LD-Chow and LD-HFD rats displayed comparable sustained circadian rhythms in *Per1* bioluminescence with robust amplitude, near 24 hr oscillatory period, and the phase of circadian oscillations not significantly impacted by 10 week exposure to HFD (Fig. 3-3). In contrast, exposure of rats to LL (independent of diet) led to the dampening (~50%) in the amplitude of *Per1*-driven luciferase oscillations evident in both LL-Chow and LL-HFD rats (Fig. 3-3, $P<0.05$, vs. LD-Chow and LD-HFD). Interestingly, LL-HFD animals demonstrated a significantly altered phase (peak: 13.17 ± 0.29 vs. 9.56 ± 4.02 CT for LD-HFD vs. LL-HFD, $P<0.05$, Fig. 3-3C) and increased variance in the phase of

Per1-driven luciferase oscillations in individual animals. All 4 groups exhibited comparable near 24 hr period of islet clock oscillations.

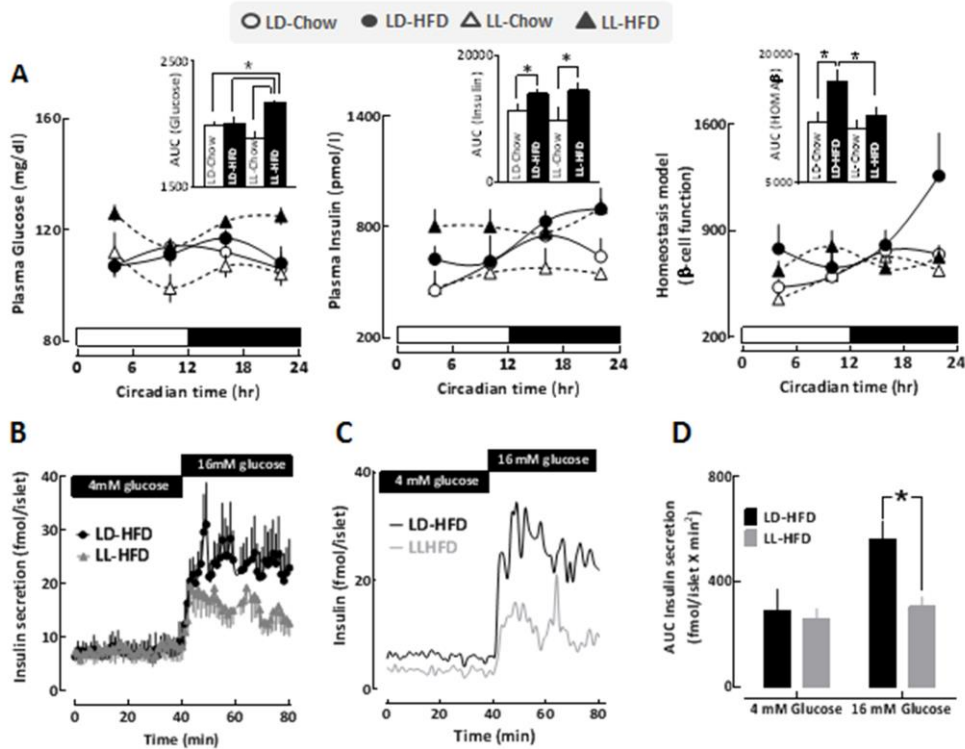


Figure 3-4. Regulation of glucose homeostasis and beta cell function in rats exposed to 10 weeks of standard LD cycle or constant light (LL) fed either regular chow or 60% high fat diet (HFD). (A) Circadian profiles in plasma glucose (left), insulin (middle) and calculated index of beta-cell function (right) derived from homeostasis model assessment ($HOMA_{\beta}$) in rats. Samples were obtained at 6-hr intervals across the 24-hr circadian day in rats exposed to 10 weeks of LD-Chow (open circles), LD-HFD (black circles), LL-Chow (open triangles) and LL-HFD (black triangles). Insert within each graph represents mean area under the curve (AUC) for each respective measure calculated across the 24 hr circadian day. Bar graphs represent mean \pm SEM (n=5-9 per group). * $P < 0.05$. (B) Mean insulin concentration profiles during islet perfusion at low basal glucose 4mM (0-40 minutes) and hyperglycemic 16 mM glucose (40-80 minutes) in isolated islets obtained from rats exposed to 10 weeks of either LD-HFD (black circles, n=5) or LL-HFD (gray triangles; n=5). (C) Example of a representative pulsatile insulin secretion profile sampled at 1min intervals at 4mM (0-40 minutes) and 16 mM glucose (40-80 minutes) in isolated islets from rats exposed to 10 weeks of either LD-HFD (black lines) or LL-HFD (gray lines). (D) Mean rates of insulin secretion calculated as area under the curve (AUC) from insulin concentrations obtained during islet perfusion at 4mM (0-40 minutes) and 16 mM glucose (40-80 minutes) in isolated islets from rats exposed to 10 weeks of either LD-HFD (black bars, n=5) or LL-HFD (gray bars; n=5).

To experimentally assess interactions between LD, LL and diet-induced obesity on diurnal glucose homeostasis we first examined circadian profiles of plasma glucose, insulin,

as well as calculated index of beta cell function (HOMA_β) (Fig. 3-4A). Under LD, exposure to HFD did not augment diurnal glycemia ($P>0.05$, LD-Chow vs. LD-HFD) suggestive of successful metabolic adaptation to diet-induced obesity. In contrast, under LL exposure to HFD led to induction of overt hyperglycemia evident across the 24 hr cycle compared to all experimental groups ($p<0.05$ for LL-HFD vs. LD-Chow, LD-HFD and LL-Chow, Fig. 3-4A). Under LD, insulin levels exhibited robust circadian variations with peak insulin restricted to the dark (feeding) phase of the circadian cycle (CT16 for LD and CT22 for LD-HFD, $P<0.05$, Fig 3-4A). On the other hand, LL led to loss of circadian rhythms in insulin secretion, particularly evident under LL-HFD condition (Fig. 3-4A). Importantly, HFD led to comparable increase in total insulin secretory output (measured as area under the curve for insulin concentrations across the 24 hr day) under both LD and LL conditions (Fig. 3-4A). However, prevailing insulin levels in LL-HFD animals, albeit elevated compared to LL-Chow, were not appropriate for given level of hyperglycemia, indicating beta cell dysfunction. Consistent with this, HFD resulted in a robust increase in a measure of beta-cell function (derived from homeostasis model assessment, HOMA_β) under LD, but not under LL (Fig. 3-4A). To confirm deleterious effects of LL and HFD on beta-cell secretory capacity, we performed isolated islet perfusion experiments in islets from LD-HFD vs. LL-HFD rats (Fig. 3-4B-D). LL-HFD islets demonstrated significantly blunted glucose-stimulated insulin secretion (Fig. 3-4B-D, $P<0.05$ for LD-HFD vs. LL-HFD) attributed to the decline in the amplitude of insulin secretory pulses in response to hyperglycemia (Fig. 3-4B-D), thus confirming induction of beta cell dysfunction.

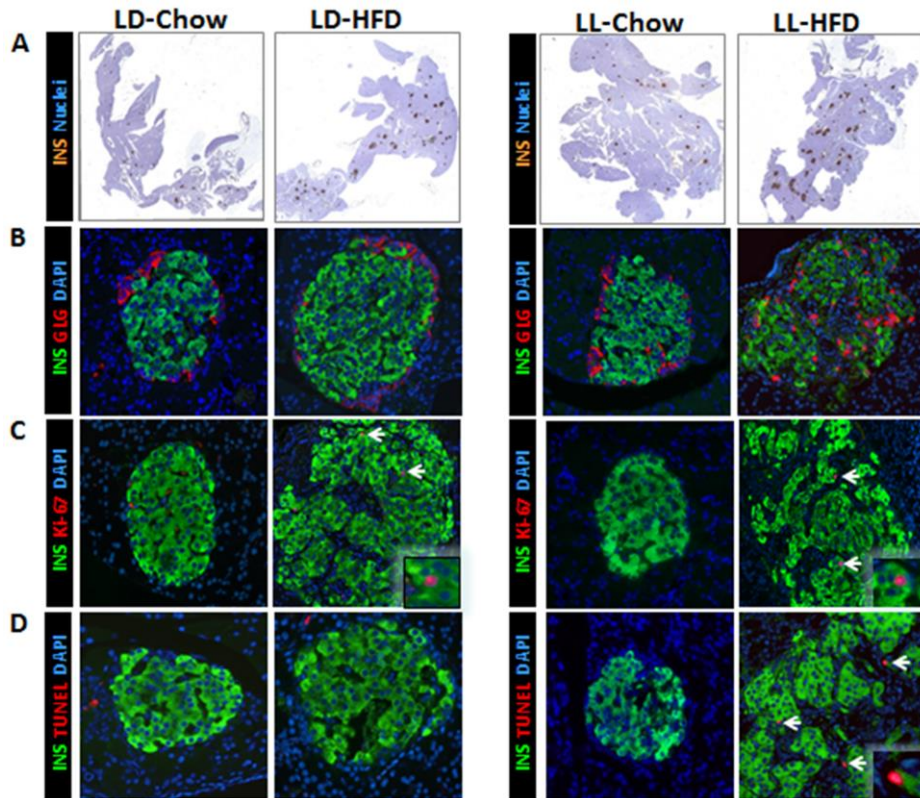


Figure 3-5. Islet morphology in rats exposed to 10 weeks of standard LD cycle or constant light (LL) fed either regular chow or 60% high fat diet (HFD). (A) Representative examples of pancreatic sections imaged at 4X stained for insulin (brown) and hematoxylin (blue) in rats after 10 weeks of LD-Chow, LD-HFD, LL-Chow or LL-HFD. (B-D) Representative examples of islets stained by immunofluorescence for insulin (green) and counterstained with either glucagon (B), replication marker Ki-67 (C), or apoptosis marker TUNEL (D) and nuclear stain Dapi (blue) imaged at 20X in rats after 10 weeks of LD-Chow, LD-HFD, LL-Chow or LL-HFD conditions.

We next examined effects of light and diet interactions on beta cell turnover (Fig. 3-6). No changes were observed among the 4 experimental groups with respect to pancreatic mass (Fig. 3-6). As expected, HFD provoked a 46% increase in β -cell mass in LD rats (13.6 ± 3 vs. 20 ± 2 mg, $p < 0.05$ for LD-Chow vs. LD-HFD), which corresponded with beta-cell hyperplasia characterized by 4.2 fold increase in the frequency of beta cell proliferation (Fig. 3-5 and 3-6, $P < 0.05$ for LD-Chow vs. LD-HFD). Interestingly, HFD induced a similar effect on beta cell expansion under LL, evident by 58% increase in beta cell mass and a 4.4 fold increase in the frequency of beta cell proliferation (Fig. 3-5 and 3-6, $P < 0.05$ for

LL-Chow vs. LL-HFD). However, while there was no significant increase ($p=0.46$) in beta cell apoptosis in LD rats on a HFD, the frequency of beta cell apoptosis increased 2.8 fold in LL rats following exposure to diet-induced obesity indicating compromised beta cell survival (Fig. 3-5 and 3-6, $P<0.05$ for LL-Chow vs. LL-HFD).

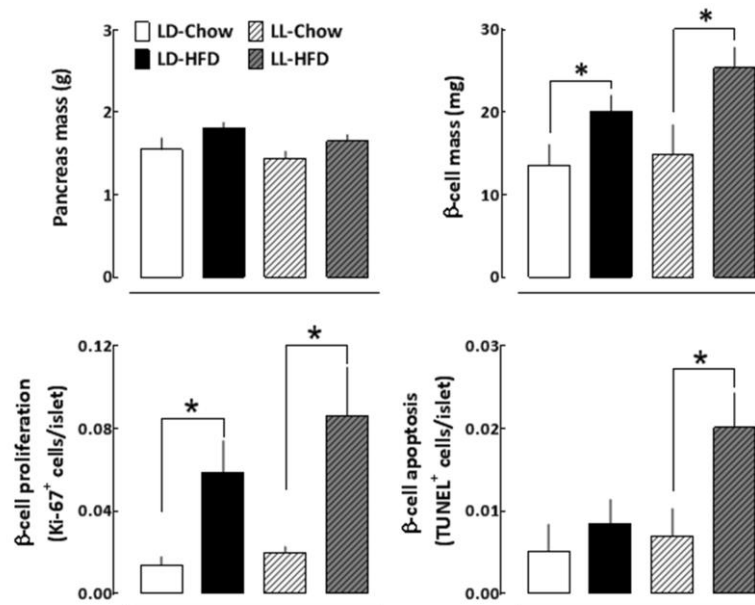


Figure 3-6. Quantification of beta cell turnover in rats exposed to 10 weeks of standard LD cycle or constant light (LL) fed either regular chow or 60% high fat diet (HFD). Mean pancreatic weight (upper left), beta cell mass (upper right), frequency of beta cell proliferation (lower left) and frequency of beta cell apoptosis (lower right) in rats exposed to 10 weeks of LD-Chow, LD-HFD, LL-Chow or LL-HFD. Bar graphs represent mean \pm SEM ($n=5-6$ per group). * $P<0.05$.

DISCUSSION

Obesity and corresponding insulin resistance are known to increase the risk of T2DM [25]. However, non-diabetic obese individuals compensate for onset of insulin resistance by elevating insulin production and secretion and thus remain normoglycemic [8, 26]. In those vulnerable to T2DM, this compensation fails due to beta cell failure characterized by beta cell secretory dysfunction and increased beta cell apoptosis [2, 12]. In the current study, we investigated whether disruption of circadian rhythms (achieved by continuous exposure to LL) promotes development of T2DM by compromising normal beta cell functional and morphological adaptation to diet-induced obesity. We also addressed whether maladaptive response to obesity is mediated through loss of islet clock function? We report that concomitant exposure to LL and HFD leads to synergistic disruption of pancreatic islet clock characterized by the impaired amplitude and the phase islet clock transcriptional oscillations. Consequently, whereas exposure to HFD under LD leads to appropriate adaptive beta cell response and preservation of normal glucose homeostasis, HFD under LL results in development of diabetes characterized by loss of circadian rhythms in insulin secretion, impaired beta cell function, and increased rate of beta cell apoptosis.

Light is the strongest entrainment signal for the mammalian circadian system. Thus, inappropriate exposure to nighttime light (typical of shift work and other conditions common to modern lifestyle) is associated with the disruption of circadian rhythms and deleterious consequences for human health [27]. Importantly, accumulating evidence from epidemiological, clinical, as well as animal studies consistently show that disruption of circadian rhythms negatively impacts metabolic health and augments susceptibility for T2DM

[13, 28, 29]. For example, series of studies by Fonken and colleagues have eloquently demonstrated that exposure of mice to dim light (~ 5 lux) at night (designed to recapitulate light pollution levels in urban areas) results in disruption of circadian rhythms in feeding and locomotor activity and coincides with induction of glucose intolerance and exaggerated inflammatory response [30, 31]. Interestingly, dim light at night is also associated with increased body mass and fat accumulation which is attributed to the alter timing of feeding (with no change in total daily caloric intake) [31]. Consistent with these observations, our results also show no difference in total daily caloric intake in rats between LD and LL and clear disruption in behavioral rhythms following exposure to LL. However, rats exposed to LL alone did not display increased body mass or epididymal fat accumulation on either chow or HFD. This discrepancy may be due multiple factors such as species differences, lighting condition as well as duration of treatment. However, previous studies in rats have documented absence of weight gain after exposure to continuous bright light illumination [32]. Nonetheless, absence of increased weight gain in LL groups in our study have excluded a potential confounding factor in evaluating metabolic and islet effects of HFD (which in itself is associated with increased body mass).

LL impairs central clock function by inducing desynchronization of circadian oscillators in the SCN thus resulting in the loss (or dampening) of behavioral and endocrine circadian outputs [33]. This observation is consistent with our results. Also, consistent with previous studies, LL led to dampening of the amplitude of circadian clock oscillators in pancreatic islets [19]. Interestingly, combination of LL and HFD synergized to impair cyclical expression and also altered the phase of circadian clock oscillations in islets

suggestive of an additive deleterious effect of HFD and LL on the islet clock. We have recently shown that feeding activity couples molecular oscillators in the islet to the SCN [34]. Thus behavioral arrhythmicity in feeding and locomotor activity induced by LL likely contributed to impaired islet clock functionality observed in the present study. Moreover, LL-induced loss of endocrine circadian outputs (e.g. melatonin and corticosterone) might have also contributed to loss of islet circadian rhythms, since melatonin and corticosterone has been shown to modulate entrainment of peripheral oscillators (including pancreatic islets) [35, 36].

One of the key observations in our study is that concomitant exposure to LL and HFD recapitulates key features of pancreatic beta cell failure characteristic of T2DM. In particular, LL-HFD islets exhibit loss of circadian insulin secretion, impaired glucose-stimulated insulin release, disrupted islet architecture and increased frequency of beta cell apoptosis. Interestingly, beta cell mass in LL-HFD rats remained comparable to LD-HFD counterparts suggesting that compensatory increase in beta cell proliferation/neogenesis is still able to account for increased beta cell turnover observed in LL-HFD rats in the 10 weeks of the present study. As in humans with evolving T2DM, persistent increase in beta cell apoptosis in LL-HFD will likely result in eventual decline in beta cell mass leading to further loss of metabolic control and exaggeration of diabetic phenotype [37].

These observations raise the question, what mechanism accounts for induction of beta cell failure following exposure to LL-HFD? This is likely attributed to LL-mediated loss of beta cell clock function resulting in compromised beta cell adaptation to oxidative stress

associated with increased metabolic demand placed on the beta cell as a consequence of HFD. The support for this postulate comes from studies in which deletion of key components of the β -cell circadian clock has been shown to diminish glucose-stimulated insulin release, impair mitochondrial function and, importantly, compromise cellular response to oxidative stress (achieved via control of nuclear factor erythroid 2-related factor 2 (Nrf2) [17, 18]. Indeed, the molecular clock in beta cells appears to be essential for orchestrating cellular response to oxidative stress by regulating NRF2 levels, which subsequently regulates a myriad of downstream antioxidant enzymes [17]. This is particularly important for the beta cell which notoriously exhibits low antioxidant potential in the face of significant reliance on mitochondrial metabolism for regulation of insulin release and production [38]. Circadian disruption can thus increase susceptibility to oxidative stress and consequent beta-cell failure, particularly in context of gluco-/lipotoxicity (the known inducers of beta-cell oxidative stress in T2DM) [39]. Subsequently, clock-coordinated response to oxidative stress is critical for beta cells of rats exposed to LL-HFD, since in this group insulin secretion is constitutively elevated throughout the 24 hr cycle driven by loss of circadian variations in fast/feeding cycles and insulin sensitivity. Furthermore, chronic exposure to LL-HFD has been shown to disrupt circadian rhythms in insulin sensitivity and exacerbate insulin resistance in mice [40], an observation consistent with our data. Thus, combination of constitutively elevated beta cell metabolic demand and loss of beta cell clock function makes LL-HFD animals particularly vulnerable to beta cell failure consistent with observed increase in the frequency of beta cell apoptosis and dysfunction.

Loss of melatonin secretion may be another contributory mechanism by which LL

compromises normal adaption to HFD. Melatonin secretion and subsequent receptor signaling is important in the regulation of organisms' circadian rhythms and entrainment of circadian clocks, including beta-cells [36]. Since pineal production and secretion of melatonin is primarily influenced by the duration and intensity of exposure to light [41], exposure to artificial lighting at night results in profound suppression of nighttime melatonin production and secretion [42]. Although, melatonin's involvement in regulation of glucose homeostasis and beta-cell health has been proposed decades ago [43], more recently activation of melatonin receptor signaling has been demonstrated to play an important role in beta cell protection against oxidative stress in human islets [44]. Moreover, in humans, loss of nocturnal melatonin secretion or presence of melatonin receptor genetic variant is associated with higher risk of developing beta cell failure and T2DM [45, 46]. Together these findings emphasize the importance of melatonin signaling in regulation of beta cell function and survival in T2DM.

In conclusion, lifestyle factors associated with disruption of circadian rhythms and promotion of obesity are becoming commonplace in today's societies. Additionally, there is also increased understanding of a causative interrelationship between circadian disruption and development of obesity and *vice versa* in humans [28]. The results of our study suggest that combination of circadian disruption and diet-induced obesity synergize to promote development of pancreatic islet failure recapitulating many features of islet pathology present in T2DM. This data provides a potential mechanism underlying common epidemiological association between circadian disruption and T2DM.

iii. Bibliography

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CHAPTER 4

i. Introduction

In the manuscript entitled “Behavioral and molecular circadian rhythms in a non-obese rat model of Type 2 Diabetes Mellitus.” by Qian, J. *et al.*, we used the human islet amyloid polypeptide transgenic (HIP) rats: a validated non-obese, beta-cell dysfunction model of T2DM. The expression of circadian rhythms of locomotor activity in the diabetic HIP rats is not significant different from their age-matched wild-type littermates. The molecular clockwork in the SCN and several peripheral tissues as measured by *Per1::LUC* was also normal in the HIP rat. Therefore, we reported that impaired glucose metabolism induced by beta-cell failure did not significantly alter circadian rhythms in behavior or the transcriptional oscillations of *Per1* in the SCN, islets and aorta.

ii. Behavioral and Molecular Circadian Rhythms in a Non-obese Rat Model of Type 2 Diabetes Mellitus

ABSTRACT

It is becoming increasingly clear that circadian regulation of glucose homeostasis and insulin secretion is an important feature of metabolic control in humans. In addition, there are also numerous studies which indicate that metabolic signals also feed back into the circadian system, modulating circadian gene expression and behavior. In recent years, circadian disruption has become increasingly prevalent in modern societies and is likely associated with the increased incidence of Type 2 diabetes (T2DM). T2DM is a metabolic syndrome characterized by glucose intolerance, hyperglycemia and islet pathology. Though it is known that circadian disruption contributes to insulin resistance and beta-cell dysfunction, it is not clear whether the impaired glucose homeostasis disturbs the circadian system in return. To address this question, we examined the expression of circadian rhythms in the human islet amyloid polypeptide transgenic (HIP) rats: a validated non-obese, beta-cell dysfunction model of T2DM. We found that the expression of circadian rhythms of locomotor activity in the 10-month-old diabetic HIP rats is not significantly different from their age-matched wild-type littermates. Furthermore, the molecular clockwork in the SCN and several peripheral tissues as measured by *Per1::LUC* was also normal in the HIP rat. Taken together, this data suggests that glucose intolerance caused by beta-cell dysfunction is not sufficient to disrupt the circadian system.

INTRODUCTION

The circadian system allows organisms to align internal metabolism as well as behavior, physiological and molecular processes with the earth's 24 hour light/dark (LD) cycle. The circadian clock in mammals is expressed within pacemaker neurons of the suprachiasmatic nucleus (SCN) that is entrained by LD cycle and, in turn, maintains proper phase alignment of peripheral tissue clocks present in nearly all cells [1, 2]. The intracellular clock mechanism within the SCN and periphery tissues in mammals is highly-conserved transcriptional and post-translational feedback loops generated by a set of interplaying clock proteins. In the primary feedback loop, CLOCK and BMAL1 are two helix-loop-helix transcription factors that are essential components of the core circadian clock [3], and when dimerized activate transcription of the Period (PER) genes and Cryptochrome (CRY) genes through a circadian E-box regulatory element. Once translated PER and CRY translocate to the nucleus where they function as negative regulators of their own transcription by interacting with CLOCK/BMAL1 complex thereby completing the negative feedback loop [4]. This autoregulatory feedback loop takes ~24 h to complete a cycle and constitutes the main circadian molecular clock.

An unfortunately, common consequence of a modern lifestyle is the disruption of circadian rhythms. Accumulating evidence has revealed that the alteration of circadian rhythms is associated with increased incidence of various pathological conditions, such as type 2 diabetes mellitus (T2DM). Epidemiological studies have suggested a strong association between susceptibility to T2DM and circadian disruption caused by disrupted/deficient sleep patterns [5-8] or working conditions such as rotational shift work

and extended work schedules [9-11]. Interestingly, it has been reported that sleep disturbances are more common among people with diabetes [12]. Adult type 2 diabetics have higher rates of sleep apnea, insomnia, excessive somnolence and hypnotic use than healthy controls [13-16]. Though it is suggested that sleep disturbances among diabetic patients may be due to physical discomfort and psychosocial factors, whether circadian disruption also occurs in diabetics and contributes to the sleep disorders remains unknown.

Metabolism is an important circadian output. Conversely, metabolic signals also feed back into the circadian system, modulating circadian gene expression and behavior. Nutrition and feeding-controlled hormones have profound effects on circadian clock function. For examples, in cell cultures, insulin causes an acute induction of *Per1* mRNA production [17, 18]. Addition of a glucose bolus to the culture medium downregulates *Per1* and *Per2* mRNA levels and induces circadian expression of clock genes [19]. In addition, it is known that misaligned feeding paradigm can shift the phase of circadian clock gene expression in peripheral tissues, such as liver, adipose tissue and pancreas in rodents [20, 21]. Further animal studies have revealed that depletion of endogenous insulin by streptozotocin or inhibition of insulin action by S961 suppresses the feeding-induced circadian phase shift [22, 23]. Together, these results indicate that glucose as well as insulin may contribute to the circadian regulation in animals, which leads us to wonder whether circadian clock will be disturbed when glucose metabolism and regulation is impaired - in particular, when an individual develops T2DM.

Hyperglycemia as well as alterations in the normal cyclic pattern of glucose tolerance are hallmark features of T2DM [24]. Emerging evidence point to pancreatic beta-cell failure

as one of the key pathophysiological events responsible for the development of T2DM. One potential mechanism of the beta-cell failure is increased beta-cell apoptosis induced by cytotoxic oligomers of islet amyloid polypeptide (IAPP) [25-27]. To gain insight into whether the pathophysiology of T2DM alters the circadian clock, we undertook studies in wild-type (WT) Sprague Dawley rats and diabetic Sprague Dawley rats transgenic for human islet amyloid polypeptide (HIP rats). The HIP rat develops diabetes at ~10 months of age with islet pathology closely resembling that in humans with T2DM [28]. It also recapitulates the defects in insulin secretion and insulin action present in humans with T2DM [29]. Importantly, this diabetes rat model doesn't become obese with disease progress. This allows us to rule out the confounding effects of obesity on the circadian system. Thus, the HIP rat provides an excellent model to test our hypothesis that pathophysiology of T2DM disrupts circadian rhythms. To address this hypothesis, we sought to determine whether circadian rhythms in locomotor activity and *Per1* circadian transcriptional oscillations in the SCN and other peripheral tissues are disrupted in the diabetic HIP rats.

METHODS

Animals

Male Sprague Dawley and h-IAPP transgenic rats on the Sprague Dawley background were used in the current study. The hemizygous HIP rats overexpressing human islet amyloid polypeptide in the pancreatic beta-cells spontaneously developed diabetes between 5 and 10 months of age. The generation and phenotypes of the HIP rats has been described in detail previously [30].

In addition, we have cross-bred the hemizygous HIP rats with a previously described homozygous transgenic rats in which the mouse *Period-1* promoter was linked to luciferase reporter (Per1:LUC rats) [21]. The cross-breeding generates HIP rats hemizygous for Per1:LUC, referred to as HIP:Per1:LUC rats (n=16), and wild-type rats hemizygous for Per1:LUC, referred to as WT:Per1:LUC rats (n=15).

The rats were bred and housed individually throughout the study at the University of California Los Angeles animal facility and kept in custom-made environmentally controlled soundproof chambers under standard 12/12 hour LD cycle (lights on at 6:00 hr, lights off at 18:00 hr, all times in Pacific Standard Time). All rats were entrained to 12:12 LD conditions for a minimum of 2 weeks prior to any manipulations. The University of California Los Angeles Institutional Animal Care and Use Committee approved all surgical and experimental procedures described in the manuscript.

Activity measurement and analysis

WT and HIP rats at 10 months of age were housed individually in cages outfitted with

the optical beam infrared sensor system designed to monitor circadian behavioral rhythms (Respironics, Murrysville, PA). Their gross motor activity was recorded as counts per 3 min interval. The rats were first on a 12:12 LD cycle. After 2 weeks of entrainment under this light condition, rats underwent a phase advance of 6 hours, and then 2 weeks later a phase delay of 6 hours. The advancing phase shift was achieved by ending the light period 6 hours earlier. The delaying phase shift was accomplished by extending the light period by 6 hours. Another 2 weeks later, the animals were placed in constant darkness (DD) for at least 2 weeks to assess their circadian patterns of activity. Analysis of locomotor activity rhythms was performed as described previously[31]. Briefly, we examined the period and power of activity rhythms over the last 7 day period within both LD and DD cycle by periodogram analysis combined with the χ^2 test (ClockLab software, Actimetrics, Chicago, IL). Lower power can be a reflection of a decrease in precision, amplitude, or an increase in variability in the behavioral rhythm. The number of counts per day provides an indication of activity levels, and the total activities of each cycle in activitive phase is designated alpha (α) while the remaining activities in the rest phase is rho (ρ). Fragmentation was calculated by determining the number of activity bouts (maximum gap: 21 min) per day. Precision was calculated by determining the difference between the daily onset of activity and lights off under LD conditions or from the best-fit through the daily onsets under DD conditions.

Intraperitoneal glucose tolerance tests

Rats were fasted 6 h before the procedure and then injected intraperitoneally with 50% dextrose at a dose of 1.5 g/kg body weight. Blood samples were obtained at time-points 0, 10,

20, 40, 60 and 90 min. Plasma glucose was measured by the glucose oxidase method (YSI Glucose Analyzer, Yellow Springs, OH). Insulin and glucagon were measured using an ELISA assay (Alpco, Salem NH).

Real-time monitoring of bioluminescence

The methods were similar to those previously described (Nakamura et al., 2010 & Qian et al., 2013). 5 to 6-month-old HIP:Per1:LUC and WT:Per1:LUC rats were anesthetized at about 9:00 Pacific Standard Time. Pancreatic islets were isolated using standard collagenase method. About fifty islets per rat were laid onto the tissue culture membranes (Millipore) in 35mm dishes containing 1.2ml culture medium (Dulbecco's modified Eagle's medium [D5030-10L, Sigma–Aldrich] supplemented with 11mmol/L glucose, 10 mmol/L HEPES [pH 7.2], 2mM glutamine, B27 [2%, GIBCO], 0.1 mM luciferin [beetle luciferin, potassium salt, Promega]). Brains were sectioned in the coronal plane at 4 °C using a motorized vibratome at a thickness of 300 µm. Aorta were prepared by hand with scalpels. Brains and aortas were placed into identical culture conditions. Dishes were sealed and placed into a LumiCycle luminometer (Actimetrics, Inc.), which was kept inside a standard light-resistant tissue culture incubator at 37 °C. Bioluminescence was measured by photomultiplier tube for 1 min at intervals of 10 min and continuously recorded for at least 7 days.

For analysis of rhythm parameters, original data were detrended by subtraction of the 24 h running average from the raw data, and then smoothed with a 3 h running average (Lumicycle Data Analysis, Actimetrics). Peak was defined as the highest point of smoothed data and the 1st peak after 24 h in vitro was used as a phase marker. Period is calculated as

the average time difference between adjacent peaks. Phase is defined as the timing of the peak of the first intact circadian cycle. Amplitude was calculated as the difference between the highest and lowest 12-h means for the first intact circadian cycle. Normalized amplitude was calculated by dividing the original amplitude by the average value of the baseline at first peak and trough.

Immunohistochemistry and Immunofluorescence

WT and HIP rats were euthanized by intravenous sodium pentobarbital 120 mg/kg. The pancreas was then rapidly removed from euthanized rats and fixed in 4% paraformaldehyde overnight at 4 °C. Paraffinembedded pancreatic sections were stained first for hematoxylin/eosin and insulin (guinea-pig anti-insulin, 1:100; Zymed, Carlsbad, CA).

As for WT:Per1:LUC and HIP:Per1:LUC rats, the pancreas islets were isolated and fixed in 4% paraformaldehyde. Paraffinembedded pancreatic islet sections were co-stained by immunofluorescence for insulin (guineapig anti-insulin, 1:100; Zymed) and terminal deoxynucleotidyl transferase biotin-dUTP nick endlabeling (TUNEL method; Roche Diagnostics, Mannheim, Germany) for visualization of beta-cell apoptosis. Slides were viewed using a Leica DM6000 microscope (Leica Microsystems, Bannockburn, IL) and images acquired using OpenLab 5 (PerkinElmer, Waltham, MA).

Western blotting

Proteins (25–50 µg per lane) were separated on a 4%–12% Bis-Tris NuPAGE gel (Invitrogen) and blotted onto a PVDF membrane (FluoroTrans; VWR, 29301856).

Membranes were probed overnight at 4 °C with primary antibodies against phospho-JNK (Cell Signaling Technology), GAPDH (Cell Signaling Technology, 2118S) and cleaved caspase-3 (Cell Signaling Technology, 9661S). Horseradish peroxidase–conjugated secondary antibodies were from Invitrogen. Proteins were visualized by enhanced chemiluminescence (Millipore, WBKLS0500).

RESULTS

Diurnal Metabolic Profiles and Islet Morphology of wild-type and HIP rats

10 month-old WT and diabetic HIP rats were maintained under 12:12 hr LD cycle with ad libitum access to food. WT rats display a robust circadian rhythm in insulin secretion (peak: 1600h, trough: 1000h, $P < 0.05$; Fig. 4-1B), but no oscillation in glycemia (Fig. 4-1A). Plasma glucagon concentration in WT rats did not display a significant diurnal rhythm (Fig. 4-1C). As expected, diabetic HIP rats exhibited an overall higher level of plasma glucose compared to WT rats, which was accompanied by a reduced level of plasma insulin throughout the day (Fig. 4-1A & B). In addition, HIP rats tended to have an even bigger

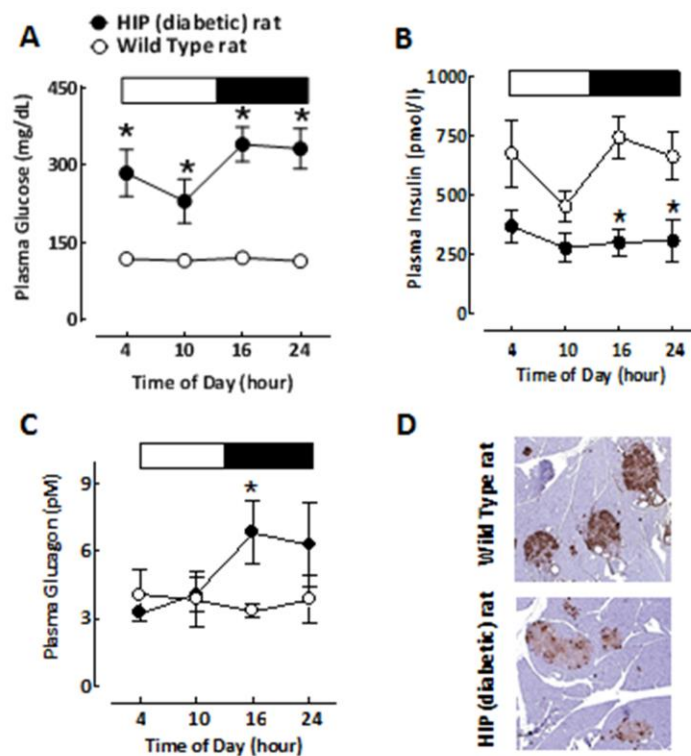


Figure 4-1. The diurnal plasma glucose (A), insulin (B), glucagon (C) concentrations, and pancreatic islet morphology (D) in diabetic HIP rats and wild-type rats at 10 months of age. (A, B, C) Diurnal profiles in plasma glucose, insulin and glucagon secretion measured in rats at 6-hr intervals ($n=4-5$ rats per time point) across the 24-hr day under typical LD cycle. (D) Examples of pancreatic islets stained by immunohistochemistry for insulin (brown) and nuclear stain hematoxylin (blue). Data are expressed as means \pm SE. *HIP vs. wild-type, $P < 0.05$.

day-night difference in plasma glucose concentration (peak: 1600h, trough: 1000h, $P < 0.05$; Fig. 4-1A). This indicated an impaired glycemic control in these rats. Actually, HIP rats displayed a sharp increase in plasma glucagon level in the early night phase (peak: 1600h, trough: 0400h, $P < 0.05$; Fig. 4-1C). In addition, the HIP rats lost their ability to increase plasma insulin level in response to night-time food intake (Fig. 4-1B). Both changes could contribute to the abnormally high level of plasma glucose at night. Morphological examination of the pancreata in HIP rats revealed extensive loss of insulin-positive cells compared with WT rats (Fig. 4-1D), which was consistent with previous findings [30].

Circadian behavioral rhythm are largely not altered in diabetic HIP rats

To better clarify the behavioral phenotypes of the HIP rats, we recorded locomotor activity from 10 month-old HIP and WT rats ($n = 6-7$) of the same genetic background (Sprague Dawley). We designed a behavioral paradigm with four separate conditions to determine the impact of diabetes on daily and circadian rhythm of behavior (Fig. 4-2A). Under both LD and DD conditions, all of the rats were rhythmic. Activity of all WT and HIP rats was primarily nocturnal (Fig. 4-2B). Along with a comparable total activity level (Fig. 4-2E), the HIP rats exhibited no significant difference in power and fragmentation of locomotor behavior rhythm under both LD and DD cycle (Suppl. Table 1). The free-running period was also not altered in the HIP rats (24.44 ± 0.18 vs 24.35 ± 0.18 h for WT vs. HIP, $P > 0.05$, Fig. 4-2C). Most of the circadian parameters that we could measure were not significantly different between WT and HIP rats. To assess the impact of light on circadian behavior, we examined the ability of the HIP rats to re-synchronize to 6 h shifts under LD

conditions. We found that the HIP rats resynchronized to 6-hr advances in the LD cycle at the same speed as their WT littermates (WT: 1 ± 0 days; HIP: 1 ± 0 days, Suppl. Table. 4-1). However, the HIP rats tended to be slower than the WT rats in adjusting to 6-hr delay in the LD cycle (WT: 2.83 ± 0.75 days; HIP: 4.86 ± 2.03 days, $P = 0.07$). Therefore, we didn't find any significant disruptions in the diurnal and circadian rhythms of locomotor activity in the diabetic HIP rats.

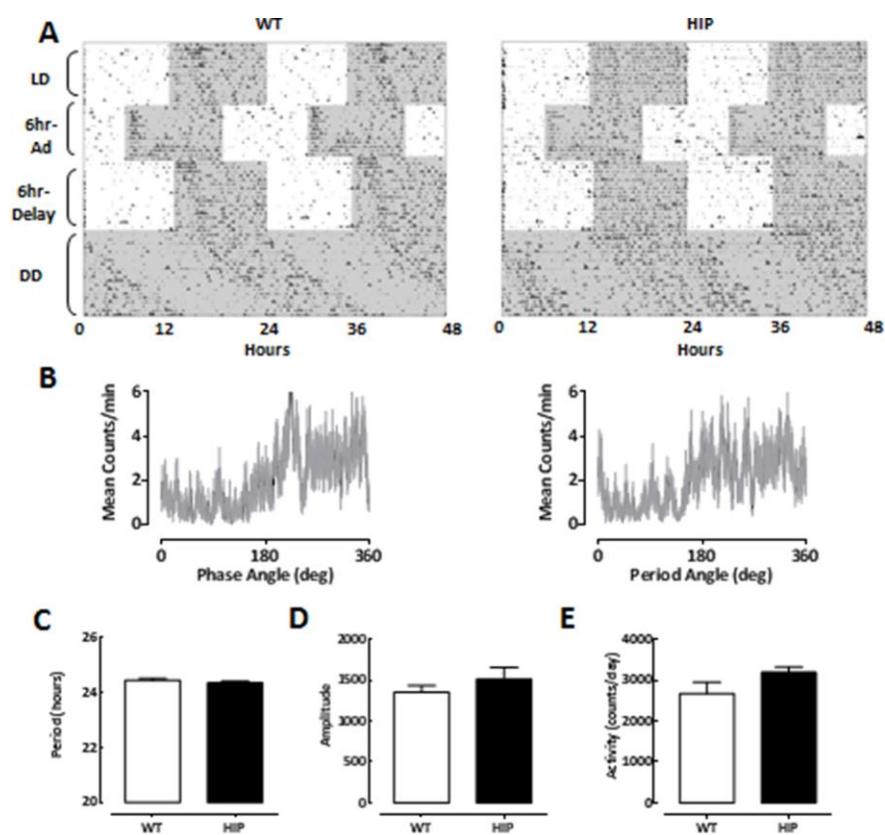


Figure 4-2. Diurnal and circadian rhythms of locomotor activity are not disrupted in the diabetic HIP rats. 10 month-old rats were placed individually in cages with activity monitors, and locomotor activity was recorded under different lighting conditions. Each horizontal row represents an activity record for a 24-hour day that is then double plotted. Successive days are plotted from top to bottom. The grey shading represents darkness. Rats were initially held in LD (12:12), then a 6 hour phase advance was introduced and followed by a 6 hour phase delay. After adjusted back to the LD cycle, rats were released into DD. (A) Panels show examples of the locomotor activity recorded from WT (left) and HIP (right). (B) The average waveform (black line, error bar in grey line) of activity for wild-type (left) and HIP (right) rats measured over 10-days in DD. (C, D) Free running period (C) and power (D) of circadian locomotor activity rhythm under DD condition in HIP (black) and wild-type (white) rats. (E) Average daily activity under DD condition. See Suppl. table 1 for detailed analysis.

Phenotypic characterizations

To further evaluate whether diabetic symptom such as hyperglycemia and formation of IAPP aggregates will affect molecular clockwork in both SCN and peripheral tissues, we cross-bred the Per1:LUC rats with the HIP rats and generated two kinds of mutant rats. Both of the lines had one copy of Per1:LUC but one line overexpressed h-IAPP in the pancreatic beta-cell, while the other did not have any copies of h-IAPP and could be used as a control. It has been previously reported [30] that the HIP rats are prediabetic at 5-month of age, exhibiting a deficit in insulin secretion and a less mean body weight. The body weight of 5-6 month old HIP:Per1:LUC rats was slightly but significantly less than that of WT:Per1:LUC rats (518.38 ± 17.94 vs. 590 ± 18.81 g for WT vs. HIP, $P < 0.05$; Suppl Fig. 4-1). In addition, 5-6 month old HIP:Per1:LUC rats also had impaired glucose tolerance. The ipGTT showed that after the glucose injection (1.5g/kg at 0min), the plasma glucose concentration dropped at a much slower rate in the HIP:Per1:LUC rats than their WT counterparts (Fig. 4-3B). Insulin secretion in response to hyperglycemia was also deficient in the HIP:Per1:LUC rats (Fig. 4-3C).

As expected, morphological examination of pancreatic islets in HIP:Per1:LUC rats by 6 months of age revealed enlarged islets as a result of adaptation to hyperglycemia. Due to the accumulation of amyloid peptides, the proportion of the islet occupied by beta-cell (stained by insulin) was decreased in HIP:Per1:LUC rats compared with their WT littermates (Fig. 4-3D). We also observed a greater rate of beta-cell apoptosis in the HIP:Per1:LUC rats using TUNEL staining (Fig. 4-3E).

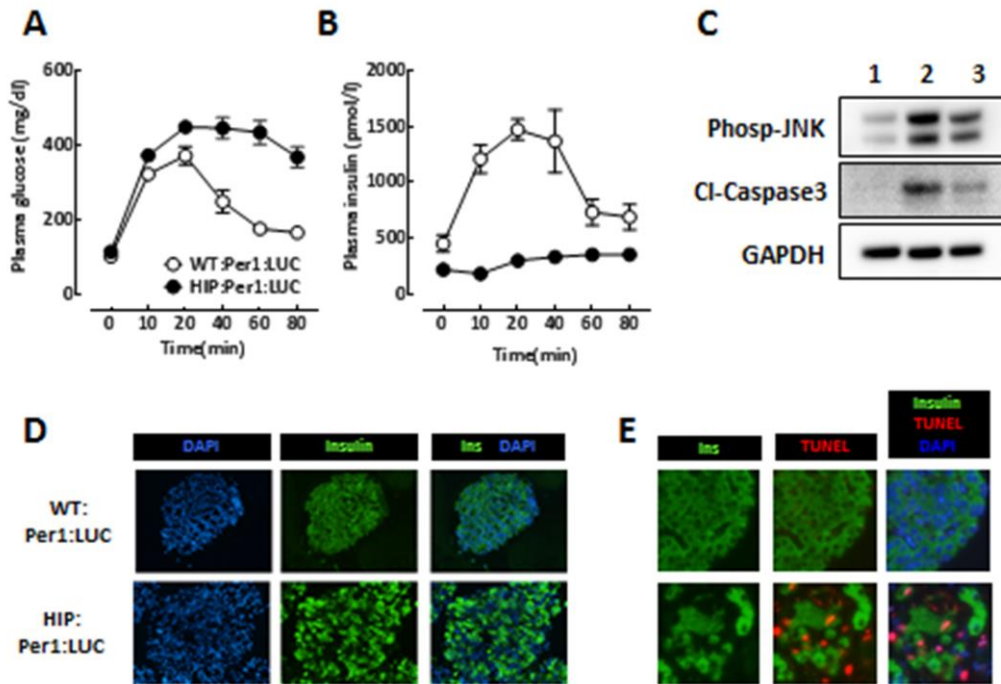


Figure 4-3. Characterization of HIP:Per1:LUC rats. (A, B) Serial changes in plasma glucose (A) and insulin level (B) measured at various times during an intraperitoneal glucose tolerance test in HIP:Per1:LUC (black dots) and WT:Per1:LUC (white dots) rats. (C) Examples of western blots measuring marker of cellular stress phosph-JNK (top), marker of cell apoptosis cleaved-caspase 3 (middle), and loading control GAPDH (bottom) in pancreatic islets from WT:Per1:LUC (column 1) and HIP:Per1:LUC (column 2&3) (D) Representative examples of pancreatic islets stained by immunofluorescence for insulin (green), marker of cell apoptosis TUNEL (red), and nuclear stain DAPI (blue) in WT:Per1:LUC (top panels) and HIP:Per1:LUC rats (bottom panels). All rats are 5-6 mo of age. Data are expressed as means \pm SE.

It is known that under diabetic conditions, oxidative stress and endoplasmic reticulum stress are induced in the pancreatic beta-cells, leading to the activation of the JNK pathway which plays a major role in apoptosis[32]. To confirm that there is an increased rate of apoptosis, we measured by western blot analysis the level of phosphor-JNK, a marker for activated JNK pathway, and cleaved-caspase 3, another biomarker of apoptosis (Fig. 4-3F). The result showed a higher level of phosphor-JNK and cleaved-caspase 3 in the pancreatic islets from the HIP:Per1:LUC rat.

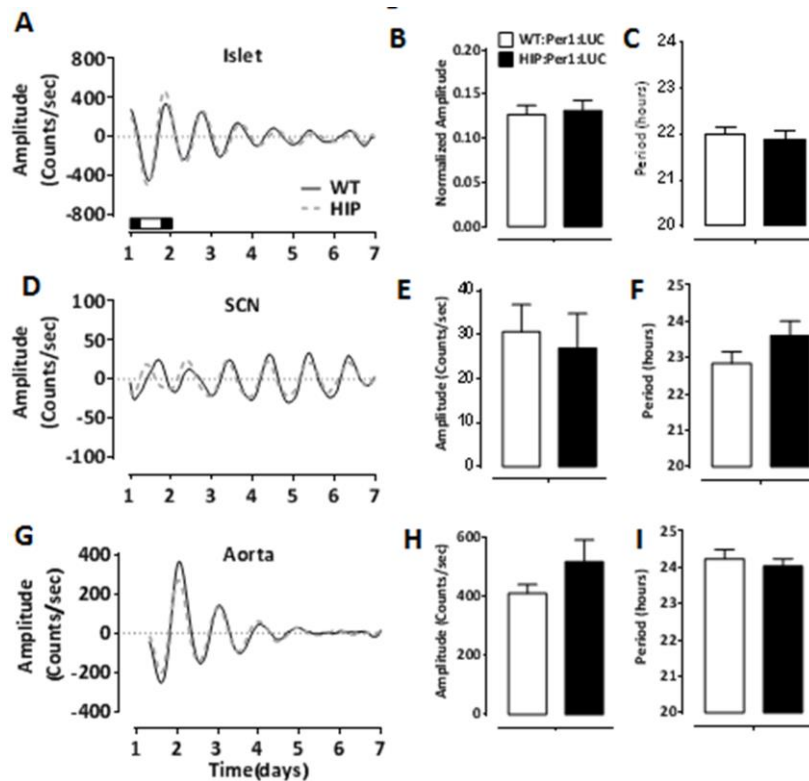


Figure 4-4. *PER1:LUC* rhythms in the suprachiasmatic nucleus (SCN) and peripheral tissues of HIP rats. (A, D, G) Example bioluminescence waveforms from the pancreatic islets (A), SCN (D), and aorta (G) of HIP (grey) and WT (black) rats. (B) Normalized *Per1:LUC* amplitude in the pancreatic islets of HIP (black) and WT (white) rats. See METHODS for procedure of normalization. (E, H) *Per1:LUC* amplitude in the SCN (E) and aorta (H) of HIP and WT rats. (C, F, I) Average period of *Per1:LUC* amplitude in the pancreatic islet (C), SCN (E), and aorta (H) of HIP and WT rats. All rats are 6 mo of age ($n=6-7$ per group). Data are expressed as means \pm SE. *HIP:*Per1:LUC* vs. WT:*Per1:LUC*, $P < 0.05$.

Overall, these data show that our HIP:*Per1:LUC* rats demonstrate a similar diabetic phenotype as the HIP rats. At the age of 6 month, they already have glucose intolerance and an increased rate of beta-cell apoptosis.

PER1:LUC rhythm in the SCN and peripheral tissues of HIP rats

The diabetic HIP rats did not show disrupted behavior rhythms. Tracking of cell bioluminescence with a clock gene luciferase fusion construct was used for assessment of circadian oscillators *ex vivo*. Therefore, to explore the properties of the molecular clock in

various tissues from the diabetic rats, we recorded PER1-driven bioluminescence rhythms of SCN, aorta and pancreatic islets (Fig. 4-4) from the HIP:Per1:LUC rats at the age of 6 month.

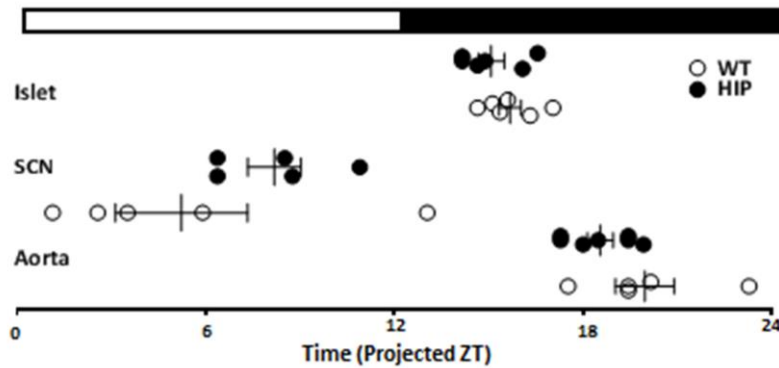


Figure 5. Phase of *PER1:LUC* rhythms in the suprachiasmatic nucleus (SCN) and peripheral tissues of HIP rats. Average timing of *PER1:LUC* 1st peak in the pancreatic islets (top), SCN (middle), and aorta (bottom) of HIP (black) and WT (white) rats. Individual samples are presented as smaller dots. The x-axis represents ZT (Zeitgeber) time of the LD cycle which the animals were under before euthanized. Data are expressed as means \pm SE. *HIP:Per1:LUC vs. WT:Per1:LUC, $P < 0.05$.

The period of Per1:LUC in the SCN, aorta and pancreatic islets from HIP:Per1:LUC rats was not significant different from that of WT:Per1:LUC rats (SCN: 23.61 ± 0.40 vs. 22.84 ± 0.32 h, aorta: 24.24 ± 0.25 vs. 24.03 ± 0.20 h, islets: 21.98 ± 0.17 vs. 21.86 ± 0.23 h for WT vs. HIP, Fig. 4-4C, F&I). As for the amplitude of Per1:LUC rhythm, no significant difference was observed in the SCN and aorta (SCN: 41.94 ± 10.66 vs. 78.37 ± 21.23 counts/min, aorta: 411.29 ± 29.60 vs. 518.47 ± 73.45 counts/min for WT vs. HIP, Fig. 4-4B, E&H). However, the absolute amplitude of Per1:LUC rhythm in the pancreatic islets was significantly larger in the HIP:Per1:LUC rats than in their WT littermates (data not shown). This is possibly due to the increased size of the islets as an adaptation to hyperglycemia. Therefore, here we employed a normalization method to analyze the amplitude of Per1:LUC rhythm in pancreatic islets. The normalized amplitude did not show a significant different between HIP

and WT rats (0.127 ± 0.010 vs. 0.132 ± 0.012 for WT vs. HIP, Fig. 4-4C). Then we examined the timing of the PER1:LUC peaks, which would reflect the phasing of gene expression in the various tissues. There was no difference between the genotypes in the timing of peaks from all three tissues. (Fig. 4-5)

DISCUSSION

Sleep disturbances due to circadian disruption are associated with impaired glucose homeostasis and consequent increased susceptibility to T2DM [33-35]. Sleep disturbances in the forms of obstructive sleep apnea, altered sleep architecture and excessive daytime sleepiness are also more prevalent among T2DM patients than the general population [12, 36, 37]. This raises the possibility that T2DM is not only the consequence of the sleep disturbance but also the cause of it. Several reasons have been postulated to explain the high prevalence of sleep disturbances in T2DM including psychosocial factors, physical complications (pain and nocturia), obesity, and fluctuations in metabolic control [12, 36, 38]. Importantly, the circadian system is an important regulator of the temporal pattern of sleep. As accumulating evidence has suggested that metabolic flux can affect the circadian system [39], we wanted to determine whether the impaired glucose metabolism in T2DM would exert any effects on the circadian system. Here we report that impaired glucose metabolism induced by beta-cell failure did not significantly alter circadian rhythms in behavior or the transcriptional oscillations of *Per1* in the SCN, islets and aorta.

To explore potential circadian deficits induced by T2DM, we used a human IAPP transgenic rat model (HIP rat) that closely recapitulates the metabolic defects and beta-cell failure in patients with T2DM [29, 40]. Importantly, HIP rats remain lean even as the disease progresses [30]. Since obesity is strongly associated with T2DM [41, 42] and has a known disruptive effect on circadian rhythms [43, 44], this model allows us to test the effects of hyperglycemia and hypoinsulinemia on the circadian system independently of obesity. In addition, as proteotoxicity due to accumulation of toxic human IAPP oligomers in the

pancreatic islets is one of the mechanisms resulting in beta-cell failure, we can also assess whether there is any impact of proteotoxicity on pancreatic islet circadian clock using the HIP rat model. First, we confirmed that there is a dramatic change in the diurnal metabolic profiles in the diabetic HIP rats. Under normal conditions, plasma insulin level is higher in the dark phase in nocturnal animals. This diurnal oscillation of plasma insulin is independent of food intake, but can be augmented by their nocturnal feeding behavior [45, 46]. There is also a diurnal rhythm in plasma glucose in mammals. This rhythm is mainly driven by food intake, and is responsive to insulin rhythm [45, 47]. Somewhat surprisingly, we did not see a clear diurnal rhythm in plasma glucose in the WT rats, a finding perhaps related to their age (10 months). Still compared to WT animals, we observed that the age-matched diabetic HIP rats were severely hyperglycemia. The plasma glucose levels of these HIP rats were particularly higher in the dark phase. Consistent with previous studies [30], the diabetic HIP rats had a reduced overall insulin level due to a significant beta-cell loss. They also lost the diurnal rhythm in plasma insulin. Glucagon concentrations in the WT rats did not display a significant diurnal rhythm [48]. As insulin restrains glucagon secretion [49], the increased plasma glucagon level in the dark phase observed in the diabetic HIP rats could be attributed to the diminished insulin release.

To test whether behavior rhythms were affected by T2DM, we compared the diurnal and circadian rhythm of locomotor activity in 10 month-old diabetic HIP rats with age-matched WT animals. We did not see any significant difference in a variety of circadian parameters of locomotor activity (Fig. 4-2 and Suppl. Table 4-1). Our results indicated that the dramatically altered diurnal plasma glucose and insulin levels are not sufficient to change the circadian

behavior. These results are contrary to what has been observed in leptin-resistant/deficient T2DM mice model. Both db/db mice and ob/ob mice exhibited reduced power in activity rhythm and an increased percentage of activity during light phase [50, 51]. Since it has been reported that high fat diet attenuates circadian rhythm of locomotor activity in mice [44], we presume that the different observations in our study are due to the effect of obesity in these mouse models. In fact, the development of T2DM in these mice is largely caused by obesity-induced insulin resistance[52], while the HIP rats maintain a slightly lower body weight than WT rats after 6 month of age[30]. There was no evidence for entrainment deficits in the diabetic HIP rats as well. The diabetic HIP rats did not take more days to adjust to 6h phase advance or delay in the LD cycle. It has been reported that retinopathy, one type of complication of T2DM, has the potential to impair photoentrainment in mice [53]. Of course, it is possible that older HIP rats would start to develop retinopathy and exhibit deficits in photic regulation of the circadian system.

Interacting molecular feedback loops driving rhythmic transcription and translation of key clock genes such as *Period* [54] are at the core of the oscillatory mechanism responsible for driving circadian oscillations. The rhythmic expression of clock genes is not only present in the master clock, the SCN, but also in most periphery tissues. These molecular rhythms persist in isolated tissue cultures and allow us to measure circadian oscillations in *Per1*-driven bioluminescence [55, 56]. Thus, to look for possible deficits in this molecular clockwork, we generated the HIP:Per1:LUC rats by cross-breeding the Per1:LUC rats with the HIP rats. We confirmed that the HIP:Per1:LUC rats had a similar T2DM phenotype as that of the HIP rats. They had impaired glucose tolerance and a greater extent of beta-cell

apoptosis at 6 months of age (Fig. 4-3B &C). These transgenic rats allowed us to measure *ex vivo* *Per1*-bioluminescence rhythm in different tissues. Consistent with the unchanged circadian behavior, *Per1*-bioluminescence rhythms in the SCN of the HIP:Per1:LUC rats were not significantly different from the WT:Per1:LUC rats. This suggested that the autonomous clock gene oscillations in the SCN are quite resistant to the changed metabolic flux in T2DM. The pancreatic islets in the 6 month-old HIP:Per1:LUC rats was much larger than those in the WT:Per1:LUC due to adaptation to hyperglycemia. As tissue size will affect the total amount of photons emitted and add to the amplitude of the bioluminescence rhythm, here we normalized the amplitudes of pancreatic islets by the baseline of the raw bioluminescence readout. We did not observe any disruptions in *Per1*-bioluminescence rhythm in the pancreatic islets of the HIP:Per1:LUC rats, indicating hyperglycemia and accumulation of IAPP are not sufficient to disrupt the islet clock. Cardiovascular complications is the most common cause of death in people with T2DM, so we also looked at the *Per1*-bioluminescence rhythms in the aorta and did not observe any disturbance in the HIP:Per1:LUC rats, either. These results do not rule out the possibility that there might be mild selective alterations in some clock gene expression. For example, one previous study looked at mRNA expression of clock genes in the liver and adipose tissues of non-obese type 2 diabetic Goto-kakizaki rats [57]. Though most of the mRNA expressions of the key clock genes they examined were not altered, a mild change in *Dbp* mRNA expression pattern in adipose tissues and a mild attenuation in rhythmic mRNA expression of *Per2* in liver were observed.

In conclusion, we find that the diurnal and circadian rhythms in locomotor activity are

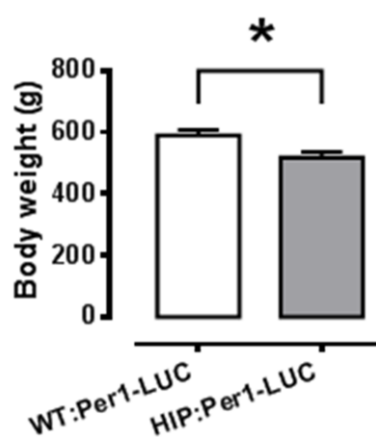
not impaired in the diabetic HIP rats in the spite of dramatically altered diurnal plasma glucose and insulin patterns. Using the *Per1*-driven bioluminescence report system, we demonstrated that the molecular clockworks largely remain intact in the SCN, islets and aorta. Our results suggest that pathological complications of T2DM, such as hyperglycemia, hypoinsulinemia and islet pathology, do not significantly disrupt the circadian system. Therefore, the high prevalence of sleep disorders observed among patients with T2DM could be attributed to other factors, such as psychosocial factors, physical discomforts or obesity-induced circadian disruption. Further studies are needed to delineate the exact causes responsible for the sleep disturbances in T2DM which will be important for the development of a targeted therapy to prevent further exacerbation of the disease.

iii. Supplemental Data

	WT	HIP
	LD	
Power, SEM	1855.35 ± 257.27	2018.07 ± 286.21
Average activity, counts/day	2099.86 ± 551.32	2195.09 ± 494.36
Fragmentation, bouts/day	10.5 ± 1.53	9.73 ± 1.22
	6h-Advance	
Average days to shift	1 ± 0	1 ± 0
	6h-Delay	
Average days to shift	2.83 ± 0.75	4.86 ± 2.03
	DD	
Power, SEM	1351.82 ± 203.35	1512.82 ± 380.27
Period	24.44 ± 0.18	24.35 ± 0.18
Average activity, rev/day	2672.68 ± 689.18	3.188.51 ± 340.61
α/p ratio	2.38 ± 0.75	2.45 ± 0.65
Fragmentation, bouts/day	11.22 ± 0.81	10.77 ± 0.81
Precision, min	45.52 ± 5.21	37.81 ± 5.24

* P-val < 0.05

*Suppl. Table 4-1. Key circadian parameters in 10 month-old wild-type and HIP rats. Rhythms in locomotor activity were examined using periodogram analysis. Comparisons were made between littermate WT and mutant mice with a Student's t-test with * indicating a significant difference at P<0.05.*



*Suppl. Fig. 4-1 Average body weight of 6 month-old HIP:Per1:LUC and WT:Per1:LUC rats. Data are expressed as means ±SE. *HIP:Per1:LUC vs. WT:Per1:LUC, P < 0.05.*

iv. Bibliography

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CHAPTER 5

i. Summary and Discussion

The presence of circadian clock in pancreatic islet has been previously shown by several means. First, rtPCR analysis in islet collected at different time points throughout the day displayed robust circadian oscillation of clock gene expression [1, 2]. In addition, bioluminescent imaging of islets isolated from *Per2:LUC* mice demonstrated autonomous clock expression in pancreatic islets *in vitro* [1]. Consistent with these studies, we utilized *Per1:LUC* transgenic rats (in which mouse *Per1* promoter is linked to a luciferase reporter) to report well defined in phase high amplitude circadian cycles of *Per1*-driven luciferase expression in individual rat islets with ~24 h period (Chapter 2). Interestingly, we found that the islet clock is particularly sensitive to low glucose level. When islets were cultured at low glucose concentrations (<5 mM), they showed substantial lower amplitude, longer period, and altered phase of *Per1* bioluminescence oscillations compared to islet cultured at normal to high glucose concentration (>11mM). As components of the circadian clock can sense alterations in the cell's metabolism as well as regulate cellular metabolism [3], this observation suggests potentially important inter-relationships between cellular, metabolic, and circadian cycles in pancreatic islets [4].

Light is the strongest entrainment signal for the master clock, SCN. Peripheral oscillators are synchronized by a variety of signals including direct neuronal control by the SCN, neuroendocrine hormonal signals, as well as indirect signals such alterations in body temperature, sleep-wake cycle and feeding-fasting cycle [5-7]. Sudden change of light-dark cycle shifted the phase of islet clock (Chapter 2). However, feeding has been suggested as the

dominant Zeitgeber for digestive organs, such as liver [8, 9]. As change of photoperiod also changed the feeding cycle, we used a time-restricted feeding protocol to study the separated effect of feeding and light cycle. Our 6-hr daytime restricted feeding paradigm quickly shifted the phase of islet *Per1:LUC* bioluminescence rhythm within 3 days, suggesting that peripheral oscillators in the islet may be coupled to the SCN via modulation of feeding rhythmicity. Possible mechanism for this feeding-induced entrainment in islets is still not clear. A number of potential candidates mediating feeding-induced entrainment have been considered which include, but are not limited to 1) feeding-generated metabolites (e.g. glucose, lipids, NADPH) [10, 11], 2) feeding-induced hormonal factors (e.g. GLP-1, GIP, insulin), [12-14] and 3) feeding-induced regulation of body temperature [8, 15].

To study the molecular mechanisms underlying the relationship between beta-cell clock and beta-cell health, a number of global or beta-cell specific circadian mutant have been generated. These studies have clearly shown that deletion of key components of the beta-cell circadian clock compromise the regulation of glucose homeostasis via impaired beta-cell function [16-18]. In real life, environmental factors (e.g. rotational shift work, exposure to light at night) induced circadian disruption are more common. In Chapter 2, the constant light paradigm was used to mimic the real life situation of exposure light at night. We found that circadian disruption due to continuous exposure to constant light (LL) and resultant loss of circadian rhythms in locomotor activity, feeding and hormonal secretion appears to significantly compromise islet clock integrity. Specifically, LL induced circadian disruption results in impaired amplitude, phase and inter-islet synchrony of clock gene oscillations in rat pancreatic islets. These islets display impaired glucose stimulated insulin secretion, which is

consistent with findings in humans that report induction of glucose intolerance and diminished insulin response to glucose after circadian misalignment [19-21].

The initiation of hyperglycemia in T2DM occurs as a result of the failure to maintain adequate pancreatic beta-cell insulin secretion (i.e. beta-cell failure) to compensate for a progressive decline in insulin action/sensitivity (insulin resistance) [22]. Induction of insulin resistance in T2DM is largely attributed to induction of obesity [23]. Obesity coupled to insulin resistance increases the functional demand per beta cell. Under normal conditions, beta-cell can adapt to this by increasing insulin secretion or expanding beta-cell mass [24, 25]. However, when the metabolic demand overrides the limited capacity of beta-cell compensation, this adaptation might break down and lead to beta-cell failure, especially when beta-cells are already vulnerable due to genetic susceptibility and environmental perturbation. In Chapter 2, we have already demonstrated that environmental conditions that affect circadian rhythms can impair the pancreatic clock and insulin secretion function. Next, in Chapter 3, we investigated whether circadian disruption induced beta cell dysfunction accelerates development of T2DM by compromising normal beta cell functional and morphological adaptation to HFD-induced obesity mediated in part through loss of beta cell clock function.

Our results showed that while HFD did not alter pancreatic islet clock, concomitant exposure to LL and HFD-induced obesity leads to synergistic disruption of pancreatic islet clock function characterized by the impaired amplitude and the phase islet clock transcriptional oscillations. Consequently, whereas HFD-induced obesity under standard LD cycle resulted in preservation of normal glucose homeostasis, HFD-induced obesity under

disrupted LL cycle led to development of diabetes characterized by loss of circadian rhythms in insulin secretion, compromised beta cell function, and induction of beta cell apoptosis. These results indicated that impaired circadian clock can constrain the capacity of beta-cell to functionally and morphologically adapt to obese-induced insulin resistance, ultimately resulting in beta-cell dysfunction and death.

One possible mechanism contributing to the failed adaptation is accumulating oxidative stress. It has been proposed that at least three stages of the glucose-stimulated insulin secretion (GSIS) mechanism (increased glycolytic flux, decreased ADP concentration, and increased intracellular Ca^{2+} concentration) could lead to a dramatic increase in the development of oxidative stress in the beta cells [26]. Oxidative stress leads to damage in organelles, particularly the mitochondria [27]. Beta cell function is highly dependent on ATP production from mitochondria. And because of their inherently low expression of antioxidant enzymes, beta cells are vulnerable to excess ROS [28]. In addition, beta cell circadian clock has been recently shown to orchestrate cellular response to oxidative stress [29], loss of antioxidant cellular defenses due to circadian disruption can make beta cell more vulnerable to oxidative damage. By this reasoning, ROS generated by prolonged beta-cell compensation caused by HFD is not able to be efficiently cleared out in circadian disrupted beta cell. The resultant excessive oxidative stress can eventually lead to beta-cell failure. On the other hand, beta-cell circadian clock appears to be also involved in regulation of key transcription factors regulating beta-cell growth, proliferation as well as maturation [1]. Therefore, circadian disruption induced by LL might also impair the beta-cell capacity to adapt to HFD by interfering with beta-cell hypertrophy and hyperplasia [30].

Deficits in circadian rhythm and sleep are often observed in people suffering from neurodegenerative and neurodevelopmental diseases, such as Parkinson disease, Huntington disease and Autism [31]. The deficits will in return exacerbate the diseases, thus forming a vicious cycle. Sleep disturbances are also very common among T2DM patients [32], though whether circadian disorders underlie the sleep problems remains unknown. As a lot of peripheral oscillators respond to metabolic signals, it is plausible to suspect that the impaired glucose metabolism in T2DM will affect the circadian system. In Chapter 4, we utilized a non-obese rat model of T2DM to address this question. Our results indicated that beta-cell loss and the resultant hyperglycemia do not affect the circadian rhythm of locomotor activity and the islet circadian clock. This result is consistent with our previous *in-vitro* data that *Per1*-driven bioluminescence rhythm is not altered when cultured at high glucose concentration (25mM, Chapter 2). Though it has been reported that addition of glucose can reset the molecular clock of rat-1 fibroblast in culture [10], the effects of chronic exposure to high glucose on circadian clock could be different from those of glucose shock which works more like an entrainment cue.

In the strong association between circadian disruption and T2DM, our studies emphasized the detrimental impact of circadian disruption on the progression of T2DM. Therefore, intervention aimed at improving circadian rhythm holds potential to the development of novel therapeutic and preventative strategies of T2DM.

ii. Bibliography

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