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**What are the effects of circadian rhythms on the G-protein coupled receptors involved with  
glucose metabolism?**

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**Senior Honors Project:** Cell and Molecular Biology

## **Abstract**

Circadian rhythms (CR) are 24-hour cycles that regulate physiological processes of the human body, such as sleep and blood glucose levels. They allow organisms to coordinate behavioral, physiological, and molecular processes with the 24-hour light/dark cycle. CRs influence metabolism through transcriptional-translation mechanisms of time-keeping, which are activated by environmental stimuli. It is known that circadian rhythms influence glucose metabolism through a variety of ways including G-protein coupled receptors (GPCRs); however, the extent to which these mechanisms operate are unknown. Disrupted CRs lead to inappropriate GPCR pathway signaling, pancreatic beta-cell failure, and misregulation of gene expression, which results in a predisposition to metabolic disease. Possible drug targets and lifestyle choices may be identified to prevent or treat metabolic diseases due to new research.

## **Objectives**

Glucose metabolism and G-protein coupled receptors (GPCRs) are integral to cell signaling, and the GPCRs that control glucose metabolism are of particular interest. Additionally, the influence of circadian rhythms (CRs) on GPCRs, metabolic function, and disease is becoming more clear. I aim to unite the topics of circadian rhythms and GPCRs that regulate glucose metabolism and to explore the latest breakthroughs in this research field. I will first explain the fundamental understanding of circadian rhythms, GPCRs, and glucose metabolism. Next, I will provide an overview of recent and compelling research that shows an interaction among these topics, with an emphasis on the metabolic effects of disrupted circadian rhythms.

## **Introduction:**

### **Fundamentals of Circadian Rhythms**

The circadian rhythm is a 24-hour cycle that regulates physiological processes of the human body, such as sleep and blood glucose levels (Kalsbeek et al. 2014). Circadian rhythms allow organisms to coordinate behavioral, physiological, and molecular processes within a 24-hour light/dark cycle (Rakshit et al. 2014). The circadian system, referred to as a clock, can be adjusted by environmental stimuli, such as light, temperature, food intake, etc.; however, it also continues without environmental cues (Rakshit et al. 2014). The clock's intrinsic setting and response to periodic environmental stimuli allow organisms to synchronize the time of day and

generation of daily rhythms of behavior, physiology, metabolism, and regulation of gene/protein expression. The circadian clock is composed of a subset of genes and proteins on transcription-translation feedback loops of approximately 24 hours, which is reinforced and slightly altered by the environment. This is achieved through endogenous transcriptional-translational mechanisms of time-keeping that are highly conserved (Rakshit et al. 2014). Highly conserved genes, which tend to indicate essential processes to an organism's survival, control processes that are similar in many different organisms.

The mammalian circadian system is made up of the suprachiasmatic nucleus (SCN), which consists of a pair of small nuclei located in the hypothalamus of the brain above the optic chiasma, as well as peripheral oscillators ("clocks" with rhythmic variations) in many cells throughout the body (Morris et al. 2012; Tsang et al. 2014). The "master clock"/pacemaker is in the SCN where it receives photic (light) information from the retina to synchronize the SCN to the daily light/dark cycle. Photic information impacts how the SCN is coordinated to daily cycles, mostly from LD (light/dark cycle) changes. As the pacemaker, the SCN controls the circadian system throughout the body and synchronizes transcription of circadian oscillators in other cells with LD cycle changes (Rakshit et al. 2014).

The circadian system becomes disadvantageous when lifestyle factors produce misalignment between CRs and the external environment (Li et al. 2012). Some lifestyle factors that cause disruption of normal CRs are night shift work, extended work schedules, and 24-hour light stimulus. Night shift work is when an individual works consistently for 12 hours during the night opposed to usual day work. This activity contradicts the environmental cues associating night with sleep resulting in CR misalignment. Extended work schedules and 24-hour light stimulus results in CR misalignment due to the same contradiction. Artificial lights at night from TVs or city lights lead to a 24-hour light stimulus that also interferes with the environmental cue that darkness signals a time to sleep. The circadian system is essential to an organism's metabolism, and disruption of normal CRs has been linked to increased predisposition to metabolic diseases' especially type 2 diabetes mellitus (T2DM). Due to sleep/wake, fasting/feeding behaviors, organisms experience dramatic fluctuations in energy demands and nutrient supply, causing metabolism to oscillate based on time of day (Bailey et al. 2014). Additionally, metabolic processes oscillate not just due to behavior and environment, but evidence suggests that they are partially influenced by cell autonomous circadian clocks (Bailey

et al. 2014). Importantly, these cells include pancreatic beta cells, which have a prominent role in glucose metabolism.

## **Fundamentals of GPCRs**

GPCRs are targets for a variety of hormones, neurotransmitters, and environmental cues. Additionally, approximately 30% of current pharmaceuticals work through GPCRs, which are the largest class of cell surface receptors. Therefore, GPCR structure and action are very important for biological understanding of diseases associated with GPCR dysfunction and with disease treatment through pharmaceutical drugs. A GPCR is a 7 transmembrane protein with cytoplasmic and extracellular domains. Additionally, there are 5 classes of GPCRs categorized by their sequence and structural similarities: rhodopsin, secretin, glutamate, adhesion and Frizzled/Taste2 (Layden et al. 2010).

In 1981-1994 Gilman and Rodbell established that guanine nucleotide-binding proteins (G proteins) transmit signals into cells following activation of GPCRs (Gilman 1995 and Rodbell 1995). G proteins are heterotrimers (complexes of three protein subunits) of  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits. The G protein is inactive when the  $G\alpha$  subunit is bound to guanine diphosphate (GDP). Upon ligand-binding by a GPCR, the  $G\alpha$  subunit is activated by release of GDP and binding to guanine triphosphate (GTP) (Figure 1). Simultaneously, the  $G\alpha$  subunit dissociates from the  $\beta\gamma$  complex, allowing the  $G\alpha$  subunit and the  $\beta\gamma$  complex to activate downstream effector proteins and propagate the signal to the cell interior (Figure 1C) (Kobilka 2007).

Unique  $G\alpha$  subunits from one of the four major classes of  $\alpha$  subunits (Gs, Gi, Gq, and G12/13) form a complex with each GPCR. Each class also consists of multiple subtypes; for example, human genome sequencing revealed 16 genes for  $\alpha$  subunits with alternative splicing generating 23 different  $\alpha$  subunit isoforms. Additionally, 5  $\beta$  subunits and 12  $\gamma$  subunits have been identified in the human genome, allowing many combinations of  $\beta\gamma$  complexes. Additionally, these signal transduction pathways are regulated by multiple non-G proteins that can influence each step, allowing for modulation of responses (Kobilka 2007). Such pathways are essential in the regulation of glucose metabolism primarily involving insulin and glucagon.

## **Metabolic control by GPCRs**

Glucose metabolism involves feeding and fasting, which control available glucose/carbohydrate sources. Despite increases and decreases of carbohydrates over time, the body attempts to maintain nearly constant glucose levels (Kobilka 2007). There are many glucose-level regulating factors, but insulin and glucagon are the primary hormones. After a meal, blood glucose levels increase. Glut2 (GLUcose Transporter in liver and beta cells) transports glucose into pancreatic beta cells (Figure 2, Step 1) (Kobilka 2007). Glucose is metabolized as it is taken up into beta cells, leading to increased ATP (adenosine triphosphate- an energy molecule of the body) production. This glucose processing increases the ATP:ADP (adenosine triphosphate:adenosine diphosphate) ratio, resulting in cell depolarization due to closing of potassium channels in the cell membrane (Figure 2, Step 2) (Kobilka 2007). Subsequently, membrane calcium channels open and allow calcium to enter the cell (Figure 2, Step 3). The calcium influx causes insulin secretion into the blood by islet beta cells (Figure 2, Step 4) (Kobilka 2007).

High blood glucose levels are lowered due to the actions of insulin and the beta-adrenergic GPCR. Insulin then circulates and acts on a variety of tissues, most importantly are adipose (fat), muscle, and liver. The GPCR that regulates glucose metabolism by binding insulin is the beta-adrenergic receptor (Dehvari et al. 2012). Furthermore, genetic polymorphisms (differences) in the beta-adrenergic receptor (GPCR of glucose metabolism) lead to varying levels of baseline insulin sensitivity. Insulin binds to this receptor and stimulates intracellular signaling pathways that lead to the translocation of glucose transporters (Glut4 in fat and muscle cells) to the cell membrane, increasing glucose uptake (Layden et al. 2010). Glucose is an important energy source in adipose and muscles. Additionally, it can be a stored energy source when converted into fat or glycogen. Hepatocytes (liver cells) produce glucose via breakdown of glycogen or synthesis from non-carbohydrate sources. Insulin receptor binding in hepatocytes leads to increased glycogen synthesis and inhibition of the liver's glucose production. These mechanisms allow blood glucose levels to fall after the meal (Layden et al. 2014).

When blood glucose is low, glucagon is released from pancreatic alpha cells to increase blood glucose levels. Glucagon acts primarily to increase glucose production by liver cells, by either glycogen breakdown or synthesis of glucose via gluconeogenesis, thus increasing blood glucose levels. Insulin and glucagon secretion are homeostatic responses to blood glucose fluctuations that maintain blood glucose levels within a fairly narrow range (Layden et al. 2014).

Glucose is the primary instigator of islet cell secretion of insulin and glucagon, and thus when glucose no longer can stimulate islet cell secretion, like in T2DM, blood glucose levels are no longer regulated within the normal range. Although glucose is the primary regulator of islet cell secretion, current research has identified additional factors that regulate secretion and their respective pathways, particularly GPCR pathways. Such factors could help keep blood glucose levels within the normal range for individuals that have a decreased regulatory action on islet cell secretion. Current research involves defining the role of GPCRs in normal islet physiology and diabetes pathophysiology. The melatonin receptor is a GPCR that has been shown to have an association with predisposition to T2DM. Other possible glucose regulatory factors that are being explored are herbal supplements, like baicalein from a Chinese root (Fu et al. 2014), and chemically synthesized structures, such as imeglimin (Pacini et al. 2015 and PubChem Open Chemistry Database 2008), that have exhibited possible metabolic regulatory effects by improved insulin sensitivity and beta-cell function in clinical results. These factors are possible pharmaceutical targets or dietary supplements that could treat or alleviate symptoms of metabolic dysfunction due to the association of metabolic disease with insulin resistance and beta-cell dysfunction.

## **Diabetes**

Diabetes mellitus has been strongly linked to desynchronized circadian rhythms (Rakshit 2014; Li et al. 2012; Kimple et al. 2014; Morris et al. 2012) and is a prevalent disease among humans. Diabetes mellitus results from elevated blood glucose levels due to decreased insulin secretion and/or sensitivity and it is classified as type 1 or type 2. Type 1 diabetes is an autoimmune disease whereby the body's immune system specifically destroys beta cells, which in healthy individuals secrete insulin and are found in the pancreatic islets of the pancreas. Therefore, an insufficient amount of insulin exists, leading to the inability of sufficient glucose uptake in cells. Type 1 diabetes is fatal without supplemental insulin treatment (Layden et al. 2010).

Type 2 diabetes is caused by insulin resistance, i.e. impaired insulin signaling and cellular action of insulin, which is often associated with obesity (Kimple et al. 2014). The body responds to insulin resistance by secreting more insulin to overcome the resistance. In some individuals, however, pancreatic beta cells fail to secrete enough insulin to overcome the resistance, resulting in type 2 diabetes. Additionally, type 2 diabetic patients have elevated

glucagon levels, which acts to worsen hyperglycemia by increasing liver glucose synthesis. About 90 percent of all diabetes is accounted for by type 2 diabetes. Due to the long-term health consequences, such as continual blood glucose monitoring, and increasing appearance of diabetes among youth, knowledge of its pathophysiology and improved treatments are important to current research (Layden et al. 2010).

### **New research/ Influences on CR synchronization**

The circadian system directly controls many physiological functions, particularly regulation of glucose metabolism. The association of circadian rhythm disruption and T2DM (type 2 diabetes mellitus) is best supported by the studies of shift-workers, who show an increased prevalence of T2DM (Kalsbeek 2014, Spiegel et al. 1999, 2001, Buxton et al. 2010, 2012, Scheer et al. 2009, Schmid et al. 2007, 2009, Klingenberg et al. 2013, Benedict et al. 2011, Salgado-Delgado et al. 2010, 2013). These studies also suggest that disrupted or poor sleep for anyone can cause increased T2DM risk. Additionally, sleep loss would have negative effects on the treatment or management of those already diagnosed with diabetes (see below for further discussion). Current research suggests strong links between chronic circadian misalignment and T2DM in humans. Due to the strong links between CR misalignment and T2DM, more research on what influences CR synchronization to the environment and the master clock (SCN) will be examined. This will lead to better understanding of their role in normal and aberrant glucose metabolism. Such influences are human clock genes, peripheral autonomous clocks, and behaviors such as shift work.

### **Human clock genes**

Variation in circadian rhythm genes as well as expression of other genes, correlates with glucose homeostasis (Turek et al. 2005). Clock and Bmal1 polymorphisms (variations) show possible links to T2DM and obesity susceptibility (Marcheva 2010). Notably, other CR genes have been linked to glucose levels, high fasting glucose levels, adiposity, and insulin sensitivity (Kalsbeek 2014). Epidemiological studies on shift work are still the strongest evidence that links T2DM (metabolic disease) and CR disruption. Similar evidence has been found in studies on individuals with deficient or disrupted sleep. The disruption results in hormonal dysfunctions that may lead to metabolic diseases like T2DM and obesity, partly through changes in glucose metabolism



(Kalsbeek 2014, Spiegel et al. 1999, 2001, Buxton et al. 2010, 2012, Scheer et al. 2009, Schmid et al. 2007, 2009, Klingenberg et al. 2013, Benedict et al. 2011, Salgado-Delgado et al. 2010, 2013).

Research from using a genome-wide association study (GWAS) has provided additional evidence of circadian system influence on glucose metabolism and beta cell health. A GWAS is an experimental technique that rapidly scans genetic markers across the complete genomes, of many people to find associations between genetic variations and a particular disease (National Human Genome Research Institute 2014). Using a GWAS approach, the findings can be used for disease detection, treatment, and prevention. In this case GWAS was used to find genetic variations involved in both the circadian system and glucose metabolism (Rakshit et al. 2014).

Genetic variants in key circadian system genes (Cry2 and MTNR1B) were shown to be associated with increased human T2DM predisposition risk (GWAS from Rakshit et al. 2014, Zhang et al. 2010, and Pi et al. 2012). Cry2, a key component of the circadian system was shown to be associated with increased fasting glucose levels and degeneration of beta cell function, which contributes to increased T2DM risk (Zhang et al. 2010). GWAS studies from ethnically diverse groups globally also have extensively demonstrated a link between a variance in the melatonin receptor 2 (melatonin is a key circadian hormone) and increased T2DM risk and degenerative beta cell function (Pi et al. 2012). Variations in CR and other associated genes and their expression influences glucose metabolism.

## **Peripheral autonomous clocks and glucose metabolism:**

### **Pancreatic clock**

Blood glucose levels fluctuate but are regulated to stay within a relatively narrow range in healthy individuals. The highest levels of blood glucose are at the beginning of the person's active/awake phase due to food consumption. Insulin secretion mirrors the rhythm of food intake. Mouse and human pancreatic islet cells have been shown to have an autonomous circadian rhythm (Kalsbeek 2014). This supports the idea of a circadian control over pancreatic function. In fact, insulin release that is stimulated by glucose is dependent on a functional circadian clock, because glucose alone will not induce significant insulin release (Kalsbeek 2014).

Clock disruption changes expression of pancreatic islet genes that control survival, growth, and synaptic vesicle assembly of islet cells. Pancreatic clocks were studied using mice in vivo by three research groups targeting *Bmal1*, specifically in the pancreas or in the beta-cells (Sadacca et al. 2011, Marcheva et al. 2010, and Lee et al. 2013). Sadacca et al. (2014) examined clock gene expression in beta cells by immunohistochemistry and in situ hybridization. Mice lacking pancreas-specific *Bmal1* (KOs) and the controls, functional *Bmal1* littermates, had their islet cells examined for glucose-stimulated insulin secretion, total insulin content, glucose tolerance, and insulin tolerance. Marcheva et al. and Lee et al. did similar experiments. All three research groups showed that conditional removal of the pancreatic clock caused defective beta-cell function and led to type 2 diabetes mellitus. The mice in this experiment presented with impaired glucose tolerance, decreased insulin secretion, and elevated glucose levels. Also, KO and wild type mice possessed similar insulin content in the islets. This suggested that only insulin secretion, and not synthesis, was defective in KO mice (Sadacca et al. 2011 and Marcheva et al. 2010). Additionally, it suggests that *Bmal1* in the pancreas is necessary for normal insulin secretion and glucose metabolism.

Zhao et al. (2012) tested other CR pancreas-specific genes, *Per* and *Cry*, for involvement in glucose homeostasis using KO mice. This resulted in hyperinsulinemia, which is an excess level of insulin in the blood. *Per2* (specific gene from *Per* gene family) KOs showed an increase in glucose-stimulated insulin secretion and a decrease in insulin clearance. *Cry* double KO (simultaneous KO of *Cry1* and *Cry2* genes) mice were also hyperinsulinemic (Zhao et al. 2012).

Marcheva et al. (2010) used *Clock* and *Bmal1* KO mice to further the tests on the pancreas-specific CR genes. Pancreatic islet cells from *Clock* mutant mice or *Bmal1* KO mice show a drastic decrease in glucose-induced insulin secretion. Hypoinsulinemia, abnormally low blood insulin levels, was observed in both *Bmal1* and *Clock* KO mice. Observations were made in whole body circadian gene KO animals, so all tissues were affected. *Clock* mutants and *Bmal1* mutants display reduced glucose tolerance, reduced insulin secretion and defects in size and proliferation of islets. These symptoms also worsened with age (Marcheva et al. 2010). The major effect of disturbed circadian rhythm/clock mechanisms in the pancreatic beta-cells is impaired insulin release which ultimately results in hyperglycemia and defective glucose metabolism.

## **Hypothalamic clock**

Yamamoto et al.'s (1987) research was the first to support that the SCN is involved in the daily rhythm in glucose metabolism (Yamamoto et al. 1987). The experiment showed that SCN lesions (damage) eliminated the daily rhythms in blood glucose and insulin concentrations. Additionally, a pronounced day/night difference in the response to 2-deoxy glucose, a glucose-utilization inhibitor, was revealed.

SCN-lesioned rats no longer exhibited a food intake rhythm. Therefore, the results could have been an indirect effect of the altered food intake rhythm on glucose metabolism, because the glucose and insulin peak (because the hormones fluctuate and peak normally after food intake or in anticipation of food intake) shifts to the daytime when there is restricted feeding during the daytime (Yamamoto et al. 1987). After that experiment, other researchers wanted results from unanswered questions left from the Yamamoto experiment. They tested whether the SCN directly influences glucose metabolism independently from its effect on feeding behavior with several sets of experiments. The experiments consisted of a feeding schedule: 6-meals-a-day with an identical meal every 4 h. This schedule effectively removed the pronounced day/night difference in the rats' food consumption (La Fleur et al. 1999).

The first set of experiments with the feeding schedule showed a clear daily rhythm in glucose and insulin due to meal-induced responses. That is, meal consumption during the dark period resulted in glucose and insulin responses that were larger than responses seen in meal consumption during the light period. Moreover, the daily rhythm in basal blood glucose levels of the rats was very similar to the daily rhythm seen in animals fed *ad libitum*, whereby both showed a daily rise at the time of awakening. *Ad libitum* indicates that food was available at all times, and the animal had free choice with the quantity and frequency of consumption. This clearly supports the role of SCN in directly influencing blood glucose concentrations, independent of the food intake rhythms (La Fleur et al. 1999).

Additional support of the SCN's direct influence is that during fasting rhythmicity was retained in blood glucose concentrations. There was no direct SCN influence on basal blood insulin or glucagon concentrations. This suggests that feeding behavior and/or blood glucose concentrations are more important than direct SCN input to set basal insulin and glucagon secretion (La Fleur et al. 1999).

The hypothalamic clock appears to affect glucose production, glucose uptake, insulin release, and insulin sensitivity. Previous studies (Yamamoto et al. 1987 and La Fleur et al. 1999) did not clarify if or how these central timing mechanisms might be connected to the current increased susceptibility of type 2 diabetes and obesity (Kalsbeek et. al. 2014).

### **The liver clock**

The liver has a significant influence on regulating glucose levels by controlling glucose entry and exit to circulation. Liver-produced glucose has additional interest from researchers due to the strong CR control of glucose metabolism in the liver and the influence of sympathetic and parasympathetic input to the liver. Restricted feeding behavior shifts the rhythmic patterns of liver clock genes. Thus, the liver clock uncouples from the central clock (SCN) with the central clock keeping its original rhythmic patterns of clock gene expression (Puschel 2004).

The liver has over 350 CR transcripts with 10%, including the *Per2* core gene, retaining rhythmicity when a functional hepatocyte clock is absent. Behavioral, hormonal, and autonomic rhythms also have roles in the regulation of liver gene expression rhythms (Kornmann 2007).

Acute insulin injections cause the *Per2* and *Rev-Erb $\alpha$*  rhythms in the liver to have a phase-advance, which is a jump in CR cycles based on the 24 hour time of cycles (Kuriyama et al. 2004). Other experiments also showed a phase advance of the hepatic clock when insulin signaling might have been affected, but the oscillations of liver clock genes are still maintained in diabetic mice (diabetes was induced using a drug called streptozotocin). The same results hold for glucocorticoids. Adrenal hormones are necessary for the majority of the liver transcriptome. The hepatic clock gene expression pattern shifts due to acute glucocorticoid injections. The shift is prevented by deletion of the glucocorticoid receptor (GR) in the liver (Balsalobre 2000). Glucocorticoid signaling is vital to maintaining fasting glucose maintenance by stimulating gluconeogenesis in the liver. Additionally, it has been shown that abnormal activation of the GR contributes to diabetic hyperglycemia.

However, GR's mediation role in the circadian regulation of hepatic metabolism is unknown, because a GR-KO mice with restricted feeding does not prevent the phase shift of liver clock genes. The role of autonomic innervation is also unknown; however, denervation of both hepatic sympathetic and parasympathetic nerves results in an elimination of the daily glucose rhythm. The denervation has a minor effect on liver clock gene rhythms (Cailotto et al. 2005).

Thus, autonomic innervation is not a requirement for the maintenance of hepatic clock gene rhythms. Also, the GR-KO shows that clock gene rhythms are not adequate themselves to drive the blood glucose rhythms (Kalsbeek et al. 2014).

All the hepatic tissue-specific clock KOs mentioned above show more precise roles for core clock genes involved in hepatic glucose metabolism. Mice with liver-specific *Bmal1* disruption have an increased glucose tolerance in addition to normal insulin production and normal body fat content. In contrast, liver-specific *Cry* KO was found to inhibit gluconeogenesis (body creates glucose from fat or muscle break down) induced by glucagon. Additionally, when expression of *Cry* protein was increased in diabetic KO mice's liver it resulted in improved glucose tolerance (Zhang et al. 2010). GR and CRY1 complex in hepatocytes and then inhibit transcription of phosphoenolpyruvate kinase, an enzyme involved in gluconeogenesis (Lamia et al. 2011).

In conclusion, a liver-specific KO of the molecular clock mechanism seems to result in impaired glycogenesis and consequently, a decreased hepatic glucose production and increased glucose tolerance (Kalsbeek et al. 2014). A misalignment of the liver clock from the central clock (SCN) or the environment impairs glucose homeostasis and produces characteristics of T2DM.

### **Muscle clock**

A major location of insulin-stimulated glucose disposal is skeletal muscle. Because of this, muscle insulin resistance has been considered one of the earliest factors in the pathogenesis of metabolic syndrome. Due to insulin binding and inducing glucose uptake in muscles, researchers tested to see if muscles showed evidence of autonomous CRs like pancreatic cells.

Dyar et al. (2014) used muscle-specific *Bmal1* KO mice to examine skeletal muscle. Skeletal muscles use lipid metabolism (stored energy) at night, due to resting, and glucose metabolism during the day when there is more activity and feeding. At this light/dark transition, the metabolism switch is expected to be impaired by reduced pyruvate dehydrogenase (PDH- an enzyme in glycolysis and thus glucose metabolism) activity and the affiliated block in insulin-stimulated glucose uptake (Dyar et al. 2014). Meaning a regulator needs to overcome blocks from lipid metabolism to allow the switch in metabolism. The experiment found that the functional muscle clock is the regulator that is primed to choose a certain fuel source depending

on the time of day. The experiment showed that inactivation of muscle-specific *Bmal1*(KO) and consequent muscle clock disruption lead to a condition of reduced metabolic flexibility, characterized by an altered glucose metabolism and muscle insulin resistance (Dyar et al. 2014). The results of which suggested that preparation for the transition from the rest/fasting phase to the active/feeding phase is the muscle clock's major role, when the predominant fuel for skeletal muscle becomes glucose (Dyar et al. 2014).

Overall, Dyer's study found that disruption of the intrinsic muscle clock causes muscle insulin resistance and impaired glucose metabolism in muscles and that the muscle autonomous clock's major role was to transition between lipid and glucose metabolism (Dyar et al. 2014).

### **Major findings and environmental factors**

Glucose metabolism shows dramatic oscillations depending on the time of day. Increased physical activity raises non-insulin-mediated glucose utilization (Rose & Richter 2005, Alberts et al. 2006, Calvo et al. 2008), whereas food consumption leads to glucose disposal via insulin-mediated uptake by the cell (Woerle et al. 2003). Glucose metabolism rhythms are not only influenced by behavior fluctuations, but they can continue without them due to other influences. Rhythms persist even when rats are fasted (La Fleur et al. 1999), and circulating glucose levels increase before waking in humans and rodents (Bolli et al. 1984). Destruction of SCN and genetic manipulation of circadian clock components disrupt glucose homeostasis (La Fleur et al. 1999, La Fleur et al. 2001).

Doi et al. (2010) presented experimental support that CRs are involved in mediation of oscillations which are reduced in expression/activity of glycogen synthase and hepatic glycogen levels in *Clock* gene mutant mice. When fasting, glucose homeostasis is maintained by the liver breaking down fats, carbohydrates, and proteins to make glucose, thus raising blood glucose levels (gluconeogenesis). The body activates gluconeogenesis through the  $\beta$ -adrenergic receptors and the receptor's second messenger, cAMP. A study by Zhang et al. (2010) showed that the core clock component, *CRY*, controls gluconeogenesis, depending on the time of day, by activating this pathway and signaling through  $\beta$ -adrenergic receptors (Zhang et al. 2010).

Recently, soleus (leg muscle) and diaphragm muscles showed decreased glucose uptake and oxidation in skeletal myocyte-specific *Bmal1* null mice (CR muscle-specific KO), accompanied by pyruvate dehydrogenase dysregulation (Dyar et al. 2014). This study suggests

that skeletal muscle CR clocks significantly impact glucose utilization and thus affect glucose metabolism. Another model of CR clock dysregulation used melatonin receptor null mice, which showed altered CRs of blood insulin levels and insulin transcripts (Muhlbauer et al. 2009).

Glucagon is also important in maintaining blood glucose homeostasis. Like insulin, glucagon displays diurnal release patterns (Gagliardino et al. 1978, Tasaka et al. 1980). Ruiter et al. (2003) revealed CR and feeding behaviors control 24-hr blood glucagon concentrations. Melatonin (an important synchronizer of circadian rhythms) affects expression of pancreatic glucagon and peripheral glucagon action (Bahr et al. 2011). All of the above experiments together show a contribution of cell/tissue autonomous clocks to glucose metabolism rhythms.

CRs regulate glucose metabolism, but metabolism also influences CRs. In this way disruption and misalignment between different tissue clocks or CR clocks and the external environment disrupts homeostasis of glucose metabolism and leads to diseases. Factors that can produce circadian misalignment are feeding, ethanol consumption, obesity, and diabetes. Abnormal CR clock function during these conditions may contribute to the cause of metabolic disease and other diseases (Bailey 2014).

### **Shift work and Sleep Deprivation**

Shiftwork causes un-syncing light sensitive central pacemaker (SCN) and peripheral clocks (Kalsbeek 2014). Studies have examined sleep/circadian disruptions on glucose metabolism in humans with controlled laboratory settings to eliminate variables, such as timing of meals. The first clear demonstration that sleep loss could negatively impact human beta-cell function and/or insulin sensitivity was done by Spiegel et al. (1999). They reported that 5 days of 4 hours of sleep deprivation per night led to impaired glucose tolerance after the first meal and to an intravenous glucose tolerance test (IVGTT). Beta-cell function was examined during the IVGTT and shown to have acute insulin release and insulin sensitivity. Another recent study also demonstrated glucose intolerance and a decrease in insulin sensitivity after one week of sleep restriction (Buxton et al. 2010).

In another effort to qualify the effects of shift work, Scheer et al. (2009) employed a longer study and used an 11-day forced dyssynchrony consisting of repeating 28 hour days to induce circadian misalignment in healthy adults. This experiment resulted in circadian misalignment (eating and sleeping 12 hours out of phase from habitual times), increased blood

glucose and insulin, decreased leptin (hormone that regulates fat storage and fullness), and increased blood pressure. Due to circadian misalignment, some subjects exhibited pre-diabetic symptoms.

Subsequent studies exposed subjects to circadian misalignment to better understand the specific effects of circadian misalignment on glucose metabolism. During these studies healthy adults ate, slept, and functioned 12 hours out of phase with their typical daily living schedules (shift-work model). Living with 1–3 weeks of circadian misalignment was reported to cause postprandial (after a meal) hyperglycemia and glucose intolerance (Scheer et al. 2009). Some individuals (~40%) even showed notable glucose intolerance that is classified as “prediabetic.” More significantly, 3 weeks of circadian misalignment and simultaneous sleep restriction resulted in beta-cell failure. However, these effects normalized 9 days after normal circadian alignment (Scheer et al. 2009). Similar results indicating beta-cell failure were seen in a 3-week nocturnal lifestyle experiment (Qin et al. 2003).

Later researchers aimed to test sleep deprivation as a CR misalignment factor in contrast to the studies previously done on shift work. Additionally, a combination of sleep deprivation and circadian disruption with the “28 hour days” led to a decreased metabolic rate and increased postprandial (after a meal) glucose, thus increasing insulin resistance in healthy adults (Buxton et al. 2012). Studies utilizing acute sleep restriction (three consecutive nights of 4□hrs of sleep/night) results in individuals with a reduction in insulin sensitivity (Klingenberg et al. 2013).

Schmid et al. (2007 and 2009) observed a significant decrease in circulating glucagon levels following one night of total sleep deprivation, defined as 24 hours of constant wakefulness, or one night of mild sleep restriction (4.5 hrs of sleep) in healthy young men when compared with glucagon levels in healthy men allowed to sleep for 7□ hrs. Sleep deprivation showed a significant increase in morning blood glucose, ghrelin (fullness hormone) as well as both nighttime and daytime cortisol levels compared to the control group allowed to sleep 8 hrs with a normal 24 hr sleep-wake cycle (Benedict et al. 2011). This strong evidence supports disturbed sleep association with a predisposition to negative metabolic effects and abnormal regulation of glucose and energy metabolism.

Salgado-Delgado et al. (2010) used the “night-work” model in rats to examine timing of feeding in conjunction with shiftwork as CR misalignment factor. Rats that had forced activity



and/or food consumption (night-work) during the normal rest/sleep period were observed to have increased adiposity, body weight, and glucose intolerance when compared to rats with forced activity during the normal active/awake period. Moreover, this experiment disturbed the normal daily rhythms in both clock and metabolic genes and caused hepatic steatosis (buildup of fat in liver AKA fatty liver) (Salgado-Delgado et al. 2013). These results support that activity and/or light during the normal rest/sleep phase causes dyssynchrony among intracellular metabolic pathways and different organ systems. Consequently, dyssynchrony or circadian misalignment may be part of the reason that night work and shift work have a strong association with obesity and metabolic diseases.

### **Timing of feeding**

Another source of circadian misalignment during shift work is the timing of food consumption. Bray et al. (2013) found evidence suggesting that food consumption causes dyssynchrony amongst the peripheral clocks. Peripheral unlinking is associated with metabolic homeostasis disruptions that lead to increased adiposity, dyslipidemia (abnormal amount of lipids), and decreased glucose tolerance. This metabolic profile is often seen in individuals with night-eating syndrome (Stunkard and Allison 2003, Arble et al. 2009, Bray et al. 2013).

Furthermore, a caloric-dense, high-fat meal consumed at the end of the awake/active phase causes an increase in adiposity, decreased glucose tolerance and increased blood insulin and leptin in mice compared to the exact same meals consumed at the beginning of the active phase (Bray et al. 2010). In human subjects that were forced to ingest the majority of their calories right before overnight sleep had metabolic abnormalities, including dissociation between plasma insulin and glucose levels (Qin et al. 2003). These experiments show that food consumption at inappropriate times can lead to circadian misalignment associated imbalances in metabolism.

### **Alcohol consumption**

Similar to timing of feeding, alcohol influences glucose metabolism as it is a carbohydrate source. Additionally, the liver processes alcohol, so alcohol consumption would suppose involvement of several tissues of glucose metabolism and tissue-specific autonomous clocks. Gut (Swanson et al. 2011, Forsyth et al. 2013, Summa et al. 2013), liver (Filiano et al. 2013, Zhou et

al. 2014), and other peripheral tissue CRs can be altered by chronic alcohol consumption, which contributes to tissue injury and circadian misalignment. Additionally, ethanol consumption induced liver clock gene expression to advance a phase, but not an advance in the SCN (Filiano et al. 2013 and Zhou et al. 2014). This is misalignment between the master clock (SCN) and a peripheral clock (in this case-the liver), leading to metabolic issues. Furthermore, the studies showed altered diurnal rhythms in several genes involved in lipid, carbohydrate, and energy metabolism (Filiano et al. 2013), later confirmed by Zhou et al. (2014). These studies support the idea that alcohol consumption has a significant influence on misaligning CRs from the environment as well as tissue-specific clocks leading to metabolic issues.

### **New solutions:**

#### **Baicalein Protects against Type 2 Diabetes via Promoting Islet $\beta$ -Cell Function in Obese Diabetic Mice**

Insufficient mass and dysfunction of beta-cells that leads to a decrease in beta-cells insulin secretion are characteristics of diabetes. Fu et al. (2014) used two models, one of middle-aged obese mice, the other of middle-aged obese T2DM mice to test the possible antidiabetic properties of baicalein. Baicalein, a chemical compound isolated from the herbal Chinese root *Scutellaria baicalensis*, was used in both models. The models induced obesity through a high-fat diet. The later mouse model induced T2DM using low dose streptozotocin injections. The first model of obese mice supplemented with baicalein showed an improvement in glucose tolerance and glucose-stimulated insulin secretion (GSIS) in middle-aged obese mice induced by a high-fat diet (Fu et al. 2014).

The second model of T2DM mice showed that baicalein supplements improved hyperglycemia, glucose tolerance, and blood insulin levels significantly, which are associated with an improvement in survival and mass of beta-cells (Fu et al. 2014). These results show that baicalein may directly control pancreatic beta-cell function and mass, thus acting as an antidiabetic agent. This compound can be used to treat T2DM in both issues of hyperglycemia and beta-cell function, where most medications only focus on one.

#### **Melatonin receptors in diabetes: a potential new therapeutical target?**

Melatonin is made in the pineal gland and secreted in a circadian fashion, and it mediates CRs and affects physiological functions. MT1 and MT2 are GPCRs in mammals that control the actions of melatonin. Researchers used in vivo and in vitro studies of rats as well as GWAS that showed a connection between melatonin and diabetes. The results showed that melatonin has a prominent role in controlling glucose metabolism and diabetes pathogenesis (She et al. 2014).

Studies were done on human genetic variants of MTNR1A (encodes MT1) and MTNR1B (encodes MT2) using GWAS as well as rat KO of the receptor genes. Results suggested MTNR1B variants directly inhibited beta-cell function and thus insulin secretion. This receptor then has a main role in increasing insulin secretion and beta-cell function. The studies showed a possible mechanism of T2DM caused by defective melatonin signaling and associated MT2 and T2DM predisposition at the protein level. With the melatonin receptor influencing aberrant glucose metabolism and T2DM, the receptor offers a potential drug target to alleviate diabetes (She et al. 2014). If a molecule was able to bind to the melatonin receptor, and thus increase insulin secretion and beta-cell function, then it could be a treatment for individuals with T2DM.

### **Imeglimin Increases Glucose-Dependent Insulin Secretion and Improves Beta-cell Function in Patients with Type 2 Diabetes.**

Researchers aimed to test imeglimin to see if there was an effect in the glucose-stimulated insulin secretion (GSIS) of human patients (Pacini et al. 2015). Imeglimin was proposed due to its unique mechanism of regulating the bioenergetics of mitochondria. The clinical study was randomized and double-blind with 33 T2DM patients using placebo controls. Patients were never given drugs or withdrawn from their metformin (current T2DM medication) for 2 weeks then placed on 1500mg imeglimin twice a day or a placebo for one week duration (Pacini et al. 2015). At the end of the week, the glucose-stimulated insulin secretion (GSIS) was measured in the patients. For the week, Imeglimin raised insulin secretion in response to glucose, increased glucose sensitivity in beta-cells, and had no effect on glucagon secretion. The study showed that patients with T2DM showed increased  $\beta$ -cell function and thus insulin secretion possibly contributing to the decrease in glucose (Pacini et al. 2015).

### **Conclusions:**

CRs are 24-hour cycles that coordinate behavioral, physiological and molecular processes, with the 24-hour light/dark cycle. Lifestyle factors, such as abnormal sleeping and eating patterns, cause misalignment between clocks and between CR clocks and the environment. Furthermore, CRs are essential to metabolism, and CR disruption is linked to increased predisposition to metabolic diseases; especially (T2DM).

Dramatic fluctuations in energy demands and nutrient supply cause glucose metabolism to oscillate based on time of day, behavior, and environment, and are partially influenced by cell autonomous circadian clocks. Insulin regulates glucose metabolism through interaction with the beta-adrenergic receptor, the main GPCR in glucose metabolism.

Studies on peripheral CR clock removal, simulating CR misalignment, led to some combination of the following results characteristic of defective beta-cell function and T2DM: impaired glucose tolerance, decreased insulin secretion, and elevated glucose levels.

Misalignment factors include timing of feeding, ethanol consumption, sleep deprivation, and shift work. Disruption and misalignment between different tissue clocks or CR clocks and the external environment disrupts homeostasis of glucose metabolism and can lead to diseases, especially T2DM.

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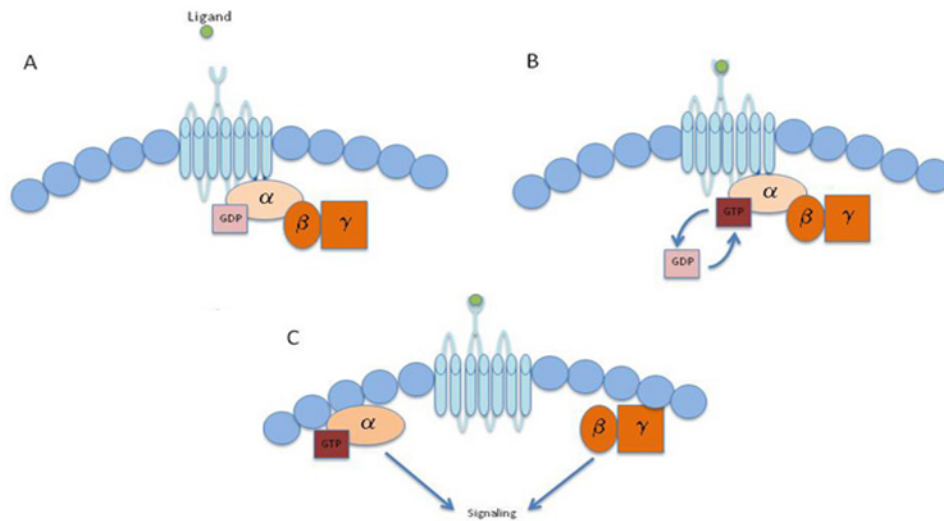
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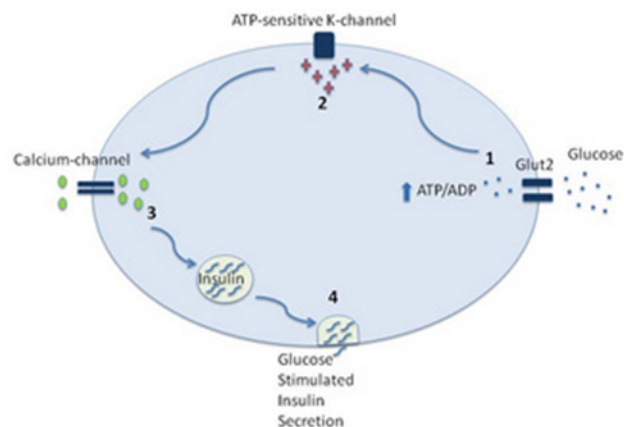
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**Figure 1** GPCR structure and mechanism (Kobilka 2007) G proteins are heterotrimers of  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits. The G protein is inactive (A) when the  $G\alpha$  subunit binds guanine diphosphate (GDP), and upon ligand-binding the  $G\alpha$  subunit releases GDP and binds to guanine triphosphate (GTP) to activate the subunit (B). Simultaneously, the  $G\alpha$  subunit dissociates from the  $\beta\gamma$  complex, allowing the  $G\alpha$  subunit and the  $\beta\gamma$  complex to each activate effector proteins and continue the signal transduction pathway (C).



**Figure 2** Beta cell glucose uptake (Kobilka 2007) Glut2 transports glucose into pancreatic beta cells (Step 1). Glucose is metabolized as it is taken up into beta cells, leading to increased ATP

production, which increases the ATP/ADP ratio, resulting in depolarization due to closing of potassium channels in the cell membrane (Step 2). The depolarization causes membrane calcium channels to open and allow calcium to enter the cell (Step 3). The calcium accumulation causes insulin secretion into the blood by islet beta cells (Step 4).