Melatonin Signaling Controls the Daily Rhythm in Blood Glucose Levels Independent of Peripheral Clocks

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Abstract

Melatonin is rhythmically secreted by both the pineal gland and retina in a circadian fashion, with its peak synthesis occurring during the night. Once synthesized, melatonin exerts its effects by binding to two specific G-protein coupled receptors—melatonin receptor type 1 (MT1) and melatonin receptor type 2 (MT2). Recent studies suggest the involvement of MT1 and MT2 in the regulation of glucose homeostasis; however the ability of melatonin signaling to impart timing cues on glucose metabolism remains poorly understood. Here we report that the removal of MT1 or MT2 in mice abolishes the daily rhythm in blood glucose levels. Interestingly, removal of melatonin receptors produced small effects on the rhythmic expression patterns of clock genes within skeletal muscle, liver, and adipose tissue. Taken together, our data suggest that the loss of the daily rhythm in blood glucose observed in MT1−/− and MT2−/− mice does not occur as a consequence of ‘disrupted’ clocks within insulin sensitive tissues. Finally our results highlight a diurnal contribution of melatonin receptor signaling in the daily regulation of blood glucose levels.

Introduction

Melatonin is synthesized by both the pineal gland and retina, and functions to drive the temporal variation in a vast array of physiological processes from sleep/wake cycles to reproductive physiology. In mammals, both its synthesis and secretion occurs at night, under the control of the circadian clock located within the suprachiasmatic nucleus (SCN) of the hypothalamus [1]. Once synthesized, melatonin exerts its effects by binding two specific G-protein coupled receptors—melatonin receptor type 1 (MT1) and melatonin receptor type 2 (MT2). Both MT1 and MT2 have been shown to activate several signaling pathways, most notably the Gi/cAMP and Gq/Phospholipase C (PLC)/Ca2+ pathways [2,3].

Several lines of evidence suggest a role for melatonin receptors in the regulation of glucose metabolism. Indeed, both MT1 and MT2 are present within the pancreas where they appear to exert a predominantly inhibitory effect on insulin secretion via receptor mediated attenuation of adenylate cyclase and guanylate cyclase [4–7]. In addition, in vitro studies utilizing a
glucagon producing α-cell line, demonstrate that melatonin produces a direct stimulatory effect on glucagon secretion via a PLC dependent mechanism [8]. This secretory response was blocked in the presence of melatonin receptor antagonists; thus demonstrating that MT₁ and MT₂ within pancreatic islets are coupled to signaling pathways involved in the modulation of both insulin and glucagon secretion.

It has also been postulated that signaling through melatonin receptors is capable of enhancing systemic glucose tolerance via a more direct effect on glucose uptake. In support of this notion, in vitro studies have reported that melatonin administration stimulates glucose uptake in both skeletal muscle and adipose tissue [9,10]. Furthermore a recent study utilizing melatonin receptor knock-out mice, demonstrates that removal of MT₁ leads to the development of insulin resistance and glucose intolerance [11]. In line with data obtained in rodents, recent human genetic studies link polymorphisms in MT₁ and MT₂ to increased risk of developing insulin resistance and diabetes [12–16].

One of the most pronounced rhythmic aspects of physiology within both humans and rodents is the daily regulation of blood glucose levels across the day [17–21]. Studies now confirm the presence of clock genes within many peripheral tissues [22] where they function to regulate important physiological and metabolic outputs [23]. Additional experimental evidence has also demonstrated that neuronal and humoral signals emanating from the SCN synchronize the expression pattern of these genes in peripheral tissues [22,23]. Interestingly, melatonin —via MT₁ receptor signaling—has been implicated in the regulation of clock gene transcription within many peripheral tissues [24–26].

To date, relatively few studies have characterized the effect of melatonin on the temporal organization of glucose metabolism. Removal of endogenous melatonin levels by pinealectomy alters the daily rhythm in blood glucose levels by increasing night-time glucose concentrations [27]; and MT₁−/− mice display higher mean blood glucose levels over a 24 hour period [28]. It remains unclear how melatonin exerts its control on the temporal regulation of blood glucose levels and to what extent this regulation is controlled by the ability of melatonin to synchronize clock genes within insulin sensitive tissues. Therefore we hypothesized that mice lacking melatonin receptors will display altered daily rhythms in blood glucose levels as a consequence of ‘disrupted’ clocks within insulin sensitive tissues. Here we report that removal of MT₁ and MT₂ in mice abolishes daily rhythms in blood glucose levels independently of its effects on peripheral tissue clocks in skeletal muscle, adipose tissue, and the liver. Furthermore, MT₁−/− and MT₂−/− mice exhibit a clear daytime blunting in the amplitude of their blood glucose rhythms- thereby demonstrating that the effects of melatonin receptor deletion are not restricted to the night phase.

**Materials and Methods**

**Sample collection and Blood glucose measurements**

Melatonin proficient (C3H-f+/+; WT) and melatonin proficient mice lacking MT₁ (C3H-f+/+; MT₁−/−) or MT₂ (C3H-f+/+; MT₂−/−) were used in this study [29]. Male mice (3–5 months) maintained in a 12 hour Light: Dark (LD) cycle (lights on at 7am [denoted ZT0] and lights off at 7pm [ZT12]), food and water were available ad libitum. For tissue collection, mice were anesthetized by isoflurane, and then killed by cervical dislocation every 3 hours over a span of 24 hours. Skeletal muscle (quadricep), liver, and white adipose tissue (epididymal fat) were collected with sterile forceps, immediately frozen on dry ice, and stored at -80°C until use. All experimental procedures were performed in accordance with the NIH Guide on Care and Use of Laboratory Animals and were approved by the Morehouse School of Medicine Animal Care and Use Committee.
In a separate time course study, blood glucose levels were measured every 3 hours over a span of 24 hours. Separate animals were used for each time point to avoid inducing stress from repeated sampling. Mice were restrained using a commercially available restraint, and a single droplet of blood obtained by puncturing the tail vein with a sterile lancet. Blood glucose concentrations were then assessed using a One Touch Basic glucometer (Lifescan, Milpitas, CA).

### Real Time quantitative RT-PCR (Q-PCR)

RNA was extracted from tissue samples using TRIZOL Reagent (Life Technologies) and reverse transcription was performed on 2 μg of RNA using a High-Capacity RNA-to-cDNA Kit (Life Technologies). Q-PCR was performed using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using iQ SYBR Green Supermix (Bio-Rad Laboratories). The efficiency and specificity of each primer was assessed by generating standard curves to ensure amplification efficiency >90% and performing melt curve analysis to verify the production of a single gene product. Primers used are listed in Table 1 and the PCR program utilized was as follows: 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing-elongation at 60°C for 1 minute. The acquisition of fluorescence data was performed at the end of the elongation step using CFX manager software V 2.1 (Bio-Rad Laboratories). Expression levels of each transcript were normalized by comparison with the amount of 18S rRNA.

### Data analysis

Results are presented as mean ± standard error of the mean (SEM). COSINOR analysis was done using the nonlinear regression model within SigmaPlot V 10.0 (Systat Software, San Jose, CA, USA). This program was used to assess rhythmicity of gene expression and to fit a cosine curve to the gene expression data. The model can be written according to the equation: f(x) = A+B cos [2 π(x)/24] with the f(x) indicating relative expression levels of target genes, x indicating the time of sampling (h), A indicating the mean value of the cosine curve (mesor; midline estimating statistic of rhythm), B indicating the amplitude of the curve (half of the sinusoid) and C indicating the acrophase (h). Transcript levels were calculated relative to the average expression of each dataset throughout 24 hrs to plot temporal expression. One-Way ANOVA was used to compare the amplitude of clock gene expression between genotypes. Where the analysis of variance indicated a significant effect of genotype, a Holm-Sidak post-hoc test was performed. The level of significance for all tests was set at \( p < 0.05 \).

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**Table 1. PCR primers and sequences.**

| Gene | Accession number | Primer sequence 5’ to 3’ | Amplicon size [bp] |
|------|------------------|--------------------------|-------------------|
| Per2 | NM_011066        | Forward: GAAAGCTGTCACCACCATAGA  
Reverse: AACTCGCACTCTTTTCTAGG | 186 |
| Bmal1| NM_007489        | Forward: AACCTTCCCCGACGTCAACAG  
Reverse: AGTCCCTTTTGGCACCTT | 79 |
| Dbp  | NM_016974        | Forward: CCGAGGAACAGAAGGATGA  
Reverse: ATCTGGTTCTCTTCTGAGTTCACCTT | 81 |
| Revbα| NM_145434        | Forward: TGGAGGCTGATTCTTCAAC  
Reverse: CAACTAGAGGATGGCAGCAT | 140 |
| 18S  | K01365.1         | Forward/Reverse: CTCGCTCGCGCTAGTCCCTG GGCGTGCGTACTTAGACAT | 123 |

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Results

Effect of melatonin receptor deletion on daily rhythms in blood glucose

We first examined the effect of melatonin receptor deletion on the daily rhythm in blood glucose levels. As shown in Fig 1, Blood glucose concentrations in WT mice showed significant fluctuations over the light:dark cycle (One-Way ANOVA (post hoc: Holm-Sidak) \( p < 0.001 \), Table 2: COSINOR, \( p < 0.0001 \), with an amplitude (fold above trough) of 29.8 mg/dL and an estimated peak occurring at \( \sim \) ZT 5. Surprisingly, this rhythm was abolished in both MT\(_1\)\(-/-\) and MT\(_2\)\(-/-\) mice (One-Way ANOVA and COSINOR analysis produced \( p > 0.05 \) for MT\(_1\) and MT\(_2\)\(-/-\) mice; Table 2). Interestingly, the loss of blood glucose rhythm in melatonin receptor knock-out mice coincided with differences in daytime glucose levels rather than nocturnal levels. Statistical analysis of glucose concentrations indicated that both MT\(_1\)\(-/-\) and MT\(_2\)\(-/-\) displayed reduced diurnal levels compared to WT mice (One-Way ANOVA (post hoc: Holm-Sidak); Fig 1 (D)).

Daily rhythms in clock and clock-controlled gene expression in skeletal muscle of WT, MT\(_1\)\(-/-\) and MT\(_2\)\(-/-\) mice

Recently, a vast array of metabolic phenotypes arising from tissue specific clock gene mutant mice \([30–37]\) has shed light on the importance of peripheral tissue clocks in the regulation of glucose homeostasis. To begin investigating the influence of melatonin receptor deletion on rhythmic clock gene expression within insulin sensitive tissues, relative mRNA levels were analyzed within skeletal muscle of WT, MT\(_1\)\(-/-\) and MT\(_2\)\(-/-\) mice (Fig 2 and Table 3). In both WT and melatonin receptor knock-out mice, clock genes (Per2, Bmal1 and Reverb \( \alpha \)) and the clock controlled gene Dbp were rhythmically expressed (Table 3, COSINOR, \( p < 0.0001 \)). Bmal1 and Per2 demonstrated a typical antiphasic expression pattern; with an expression peak or trough for Bmal1 occurring at ZT 22 or ZT 11 and Per2 occurring during the day at ZT11 or ZT 22, respectively. As expected, Dbp also exhibited a maximum peak during the mid-light phase. The most notable effects of melatonin receptor deletion on the skeletal muscle clock were on the amplitude of Dbp and Reverb \( \alpha \). MT\(_1\)\(-/-\) mice exhibited a \( \sim 2 \)-fold increase in amplitude for both transcripts, compared to WT and MT\(_2\)\(-/-\) mice (One-Way ANOVA (post-hoc: Holm-Sidak), \( p < 0.001 \)). No differences in the peak phase of Per2, Dbp and Reverb \( \alpha \) were observed among the three genotypes, however with respect to Bmal1, a very subtle phase advance was present in MT\(_1\)\(-/-\) mice with respect to WT and MT\(_2\)\(-/-\) mice (One-Way ANOVA (post-hoc: Holm-Sidak), \( p < 0.001 \); Table 3). Taken together this data suggests that removal of melatonin receptors confers rather small effects on the rhythmic expression pattern of clock genes within skeletal muscle.

Daily rhythms in clock and clock-controlled gene expression within liver and adipose tissue of WT, MT\(_1\)\(-/-\) and MT\(_2\)\(-/-\) mice

Regulation of blood glucose levels occurs as a concerted effort between skeletal muscle, liver, and adipose tissue. Therefore in addition to skeletal muscle, we examined clock gene expression within liver and adipose tissue of melatonin receptor knock-out mice. For both liver and adipose tissue, clock and clock controlled genes were rhythmic in all three genotypes (Tables 4 & 5, COSINOR, \( p < 0.0001 \)). In WT mice, Per2 peaked at \( \sim \) ZT14, Bmal1 peaked at \( \sim \) ZT21, Dbp \( \sim \) ZT 8 and Reverb \( \alpha \) \( \sim \) ZT 5 (Figs 3 & 4, Tables 4 & 5). Removal of either MT\(_1\) or MT\(_2\) receptors produced subtle effects on phase and amplitude of these rhythms in both tissues. In the liver, removal of MT\(_2\) resulted in a significant reduction in the amplitude of Per2 with respect to MT\(_1\)\(-/-\) mice (One-Way ANOVA (post-hoc: Holm-Sidak), \( p < 0.05 \))—this effect was
Fig 1. Loss of melatonin receptors disrupts daily rhythms in blood glucose. Blood glucose levels every 3 hours across the light/dark cycle in WT (A), MT$_{1}^{-/-}$ (B) and MT$_{2}^{-/-}$ (C) mice fed ad libitum are denoted within each respective plot. Results are expressed as mean ± SEM in which the black solid line in (A) represents the periodic sinusoidal function of the blood glucose rhythm as determined by COSINOR analysis (p<0.05, N = 3–4 for each time point and
not present in adipose tissue or skeletal muscle. Similar to the effect observed in skeletal muscle, MT1-deficiency resulted in a significant increase in the amplitude of Reverb α in adipose tissue, with respect to WT and MT2−/− mice (One-Way ANOVA (post-hoc: Holm-Sidak), p < 0.05) - this effect was not present in the liver. Taken together, these results demonstrate that the removal of melatonin receptors induces rather small, tissue-specific effects on the rhythmic expression pattern of clock genes, suggesting that function of the molecular circadian clockwork is not drastically affected by the loss of melatonin receptors. Thus, the effect of melatonin receptor deficiency on daily glucose rhythms appears to be independent of molecular clock function.

Discussion

Within our bodies a single day can be partitioned into two separate metabolic phases - one characterized by activity and feeding, and the other by rest and fasting. In this regard, it is critical that metabolic timing cues be properly distributed to temporally separate distinct metabolic functions. The temporal coordination of glucose metabolism is critical for the maintenance of glucose homeostasis and energy balance [38]. Disturbances in glucose availability and insulin action have been linked to a variety of metabolic disorders such as obesity, metabolic syndrome, and type 2 diabetes. Notably, one of the most pronounced rhythmic aspects of physiology within both humans and rodents is the daily regulation of blood glucose levels across the 24 hr day [17–21].

In the present study we report that melatonin signaling regulates the daily rhythm in blood glucose levels, independent of clock gene expression within insulin sensitive tissues. Melatonin proficient mice containing both melatonin receptors (MT1 & MT2) exhibited a clear rhythm in blood glucose levels marked by a diurnal peak occurring prior to the onset of activity (Fig 1). This observation is in agreement with previous studies in both rodents and humans in which glucose concentrations rise prior to onset of activity [19, 39–40]. Albeit some discrepancies in the precise timing of this rise, with studies in rats and humans depicting a more defined ‘awakening’ rise in blood glucose levels. These inherent fluctuations in glucose concentrations are dependent on the SCN, occur independently of feeding rhythms, and can be attributed to temporal variations in both hepatic glucose production and glucose tolerance [41]. In this way, the SCN is thought to play a role in preparing an individual for the upcoming activity period by increasing plasma glucose concentrations and facilitating tissue glucose uptake.

Interestingly, within both MT1−/− and MT2−/− mice the daily rhythm in blood glucose levels was abolished (Fig 1), thereby suggesting a role for melatonin receptors in the regulation of this rhythm. Our results differ slightly from those of a previous report by Muhlbauer et al. [28] demonstrating that MT1−/− have increased mean blood glucose levels over a 24 hr. period. The discrepancy between our findings and theirs could be due to the age of the mice and the precise

| Table 2. Blood glucose rhythms in WT, MT1−/− & MT2−/− mice. |
|-------------|-------------|-------------|-------------|
| Cosinor Parameter | WT | MT1−/− | MT2−/− |
| Mesor | 123.9±3.7 | — | — | — |
| Amplitude | 29.8±5.2 | <0.0001 | — | 0.38 | — | 0.20 |
| Acrophase(h) | 4.6±0.7 | — | — | — |

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bleeding protocol used in each study. In our study we utilized mice 3–4 months of age while the study by Muhlbauer et al. assessed mice at 1.5 months of age; additionally it is not clear whether this study used a repeated bleeding protocol or single bleed protocol to assess the blood glucose rhythm. As the loss of blood glucose rhythm was observed when either MT1 or MT2 was knocked out, it raises the interesting possibility that the maintenance of this rhythm could involve signaling through the formation of MT1/MT2 heteromers [42]. Recently, a functional role for these heteromers was demonstrated in the retina where deletion of either MT1 or MT2 abolished the daily rhythm in electroretinogram (ERG) responses [43].

Glucose homeostasis is known to involve the concerted effort of the hypothalamic clock in the SCN as well as peripheral clocks within insulin sensitive tissues such as skeletal muscle, the liver, and adipose tissue [44–47]. To date, a number of metabolic phenotypes have been produced by tissue specific clock gene knock-out mice and serve to highlight the diverse role of the circadian clock in regulating whole body glucose metabolism. Muscle specific Bmal1 Knockout mice

![Image](https://example.com/image.png)

**Fig 2.** Expression levels of clock and clock controlled genes within skeletal muscle of WT, MT1−/−, and MT2−/− mice. For each respective plot: the black circle (WT), red circle (MT1−/−) and blue circle (MT2−/−) indicate the expression levels of target genes calculated relative to the average expression of each dataset throughout the 24hrs. Results are represented as mean ± SEM. The black (WT), red (MT1−/−) and blue (MT2−/−) lines represent the periodic sinusoidal functions determined by COSINOR analysis (p<0.05, N = 4–6 for each time point and genotype). The bar graphs corresponding to each plot depict the amplitude of each oscillating transcript as calculated by COSINOR analysis and normalized to the value of the WT amplitude. **p<0.001, One-Way ANOVA (post hoc: Holm-Sidak) WT vs. MT1−/−; ***p<0.001, One-Way ANOVA (post hoc: Holm-Sidak) MT1−/− vs. MT2−/−.**

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are insulin resistant and have reduced protein levels of GLUT4 [30], while liver specific deletion of Bmal1 leads to a stark increase in glucose tolerance [31]. Interestingly, adipose specific Bmal1-/- mice become obese [34], whereas deletion within the pancreas results in defective β-cell function [32,33,35].

Given the loss of the blood glucose rhythm in MT1-/- and MT2-/- mice, it was rather surprising that we observed only marginal effects on rhythmic clock gene expression within insulin sensitive tissues of MT1-/- and MT2-/- mice. Indeed, previous studies have established a role for melatonin signaling in the regulation of clock gene expression [24–26]. In our analysis, the most evident effects observed by melatonin receptor deletion, were tissue specific increases in the amplitude of clock controlled transcripts within MT1-/- mice. Within skeletal muscle, removal of MT1 resulted in a nearly 2 fold increase in the amplitudes of Reverb and Dbp.

### Table 3. COSINOR analysis of clock controlled genes within skeletal muscle of WT, MT1-/- and MT2-/- mice.

| Gene | Cosinor Parameter | WT | MT1-/- | MT2-/- |
|------|------------------|----|--------|--------|
|      | Output | Regression p-value | Output | Regression p-value | Output | Regression p-value |
| Per2 | Mesor | 1.58±0.10 | <0.0001 | 1.58±0.12 | <0.0001 | 1.52±0.08 | <0.0001 |
|      | Amplitude | 1.47±0.14 | 1.52±0.17 | 1.40±0.12 |
|      | Acrophase(h) | 11.87±0.37 | 10.83±0.43 | 12.51±0.31 |
| Bmal1 | Mesor | 1.70±0.22 | <0.0001 | 1.57±0.12 | <0.0001 | 1.60±0.11 | <0.0001 |
|      | Amplitude | 1.79±0.31 | 1.55±0.17 | 1.66±0.16 |
|      | Acrophase(h) | 21.43±0.66 | 0.70±0.44 | 22.20±0.38 |
| Dbp  | Mesor | 1.35±0.12 | <0.0001 | 1.93±0.19 | <0.0001 | 1.46±0.09 | <0.0001 |
|      | Amplitude | 1.14±0.17 | 2.10±0.28 | 1.28±0.12 |
|      | Acrophase(h) | 8.50±0.58 | 7.07±0.49 | 9.21±0.38 |
| Reverb | Mesor | 1.28±0.08 | <0.0001 | 1.71±0.11 | <0.0001 | 1.24±0.11 | <0.0001 |
|      | Amplitude | 1.07±0.12 | 2.00±0.15 | 1.17±0.16 |
|      | Acrophase(h) | 4.28±0.41 | 4.23±0.29 | 4.52±0.50 |

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### Table 4. COSINOR analysis of clock genes and clock controlled genes within the liver of WT, MT1-/- and MT2-/- mice.

| Gene | Cosinor Parameter | WT | MT1-/- | MT2-/- |
|------|------------------|----|--------|--------|
|      | Output | Regression p-value | Output | Regression p-value | Output | Regression p-value |
| Per2 | Mesor | 1.39±0.09 | <0.0001 | 1.48±0.16 | <0.0001 | 1.25±0.05 | <0.0001 |
|      | Amplitude | 1.22±0.12 | 1.46±0.22 | 0.95±0.07 |
|      | Acrophase(h) | 13.75±0.38 | 11.59±0.59 | 13.58±0.29 |
| Bmal1 | Mesor | 1.92±0.12 | <0.0001 | 1.74±0.08 | <0.0001 | 1.91±0.09 | <0.0001 |
|      | Amplitude | 2.28±0.17 | 1.81±0.12 | 2.21±0.13 |
|      | Acrophase(h) | 21.90±0.29 | 22.54±0.24 | 21.15±0.22 |
| Dbp  | Mesor | 3.86±0.48 | <0.0001 | 3.51±0.49 | <0.0001 | 2.83±0.33 | <0.0001 |
|      | Amplitude | 5.49±0.66 | 5.62±0.71 | 4.22±0.46 |
|      | Acrophase(h) | 8.44±0.47 | 8.48±0.45 | 8.65±0.41 |
| Reverb | Mesor | 3.48±0.42 | <0.0001 | 2.87±0.42 | <0.0001 | 3.40±0.49 | <0.0001 |
|      | Amplitude | 4.79±0.59 | 4.29±0.60 | 5.10±0.63 |
|      | Acrophase(h) | 6.18±0.47 | 5.48±0.53 | 6.77±0.46 |

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and within adipose tissue a 1.5 fold increase in the amplitude of Reverb α (Fig 4; Table 5). Our findings within adipose tissue, are consistent with a recent study in which pinealectomy increased the amplitude of Reverb α at its peak [48]. As we observed no coincident changes on Reverb α amplitude in MT2⁻/⁻ mice, this could suggest a receptor specific role for MT1 signaling in dampening the daily amplitude of Reverb α within adipose tissue and skeletal muscle. Interestingly, strong amplitude increases in Dbp and Reverb α have been reported within the pancreas of MT2⁻/⁻ mice, with no effect in MT1⁻/⁻ mice [28]- further substantiating tissue specific roles on the modulation of clock gene expression by MT1 and MT2.

In all tissues observed, increases in the amplitude of Reverb α were not accompanied by a coincident decreases in the expression level of Bmal1 [49]. In the present study we demonstrate that daily variations in blood glucose levels do not directly depend on rhythmic cycling of clock genes within insulin sensitive tissues, as evidenced by the fact that the disruption of this rhythm in melatonin receptor knock-out mice occurred independently of clock disruption. The identification of numerous tissue specific circadian transcripts within peripheral tissues highlights that circadian regulation extends far beyond core clock components [50,51]. Furthermore it has now become increasingly evident that the timing of the biological clock is subject to a plethora of post-transcriptional and post-translational regulatory mechanisms[52]. Therefore we cannot rule out the possibility that melatonin signaling could indirectly affect blood glucose rhythms by altering the functioning of the clock at the post transcriptional or post-translational level. Along these lines recent studies have begun to suggest a potential role for melatonin in proteosomal degradation [53,54]. It has been shown that the increase in blood glucose concentrations prior to the onset of activity occurs as a result of increased glucose production by the liver rather than decreased glucose utilization by insulin sensitive tissues[41]. Therefore the clear daytime blunting in the amplitude of the blood glucose rhythm within melatonin receptor knock-out mice likely reflects alterations in hepatic glucose production. Indeed previous studies have suggested that melatonin is capable of activating a hypothalamic-liver communication which may contribute to circadian adjustments of gluconeogenesis by suppressing gluconeogenesis [55,56]. Furthermore pinealectomized rats display glucose intolerance and a desynchronized pattern of

Table 5. COSINOR analysis of clock genes and clock controlled genes within adipose tissue of WT, MT1⁻/⁻ and MT2⁻/⁻ mice.

| Gene   | Cosinor Parameter | WT Output | Regression p-value | MT1⁻/⁻ Output | Regression p-value | MT2⁻/⁻ Output | Regression p-value |
|--------|-------------------|-----------|--------------------|----------------|--------------------|----------------|--------------------|
| Per2   | Mesor             | 1.11±0.06 | <0.0001            | 1.16±0.07      | <0.0001            | 1.17±0.09      | <0.0001            |
|        | Amplitude         | 0.49±0.10 |                    | 0.69±0.10      |                    | 0.76±0.13      |                    |
|        | Acrophase(h)      | 12.84±0.68|                    | 13.89±0.56     |                    | 12.56±0.64     |                    |
| Bmal1  | Mesor             | 1.34±0.10 | <0.0001            | 1.16±0.05      | <0.0001            | 1.19±0.06      | <0.0001            |
|        | Amplitude         | 0.98±0.14 |                    | 0.79±0.07      |                    | 0.77±0.09      |                    |
|        | Acrophase(h)      | 20.58±0.58|                    | 22.56±0.33     |                    | 22.11±0.45     |                    |
| Dbp    | Mesor             | 1.30±0.18 | <0.0001            | 1.27±0.12      | <0.0001            | 1.14±0.09      | <0.0001            |
|        | Amplitude         | 1.32±0.24 |                    | 1.03±0.16      |                    | 0.96±0.14      |                    |
|        | Acrophase(h)      | 7.79±0.76 |                    | 10.55±0.60     |                    | 10.20±0.52     |                    |
| Reverb | Mesor             | 1.34±0.14 | <0.0001            | 1.54±0.14      | <0.0001            | 1.35±0.10      | <0.0001            |
|        | Amplitude         | 1.14±0.19 |                    | 1.72±0.19      |                    | 1.12±0.14      |                    |
|        | Acrophase(h)      | 4.81±0.67 |                    | 6.23±0.49      |                    | 5.88±0.57      |                    |

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(Fig 2; Table 3); and within adipose tissue a 1.5 fold increase in the amplitude of Reverb α (Fig 4; Table 5). Our findings within adipose tissue, are consistent with a recent study in which pinealectomy increased the amplitude of Reverb α at its peak [48]. As we observed no coincident changes on Reverb α amplitude in MT2⁻/⁻ mice, this could suggest a receptor specific role for MT1 signaling in dampening the daily amplitude of Reverb α within adipose tissue and skeletal muscle. Interestingly, strong amplitude increases in Dbp and Reverb α have been reported within the pancreas of MT2⁻/⁻ mice, with no effect in MT1⁻/⁻ mice [28]- further substantiating tissue specific roles on the modulation of clock gene expression by MT1 and MT2. In all tissues observed, increases in the amplitude of Reverb α were not accompanied by a coincident decreases in the expression level of Bmal1, implying that additional modulatory effects may be in place to regulate the amplitude of Bmal1 [49].
 gluconeogenesis, marked by increased nighttime glucose levels[27]. All these notions are consistent with a role for nocturnal secretion of melatonin lowering night-time glucose concentrations. Here we demonstrate that global disruption of melatonin receptors does not affect nocturnal glucose levels, and in fact appears to have a more pronounced effect during the day. This could suggest a more indirect effect of melatonin on the temporal regulation of gluconeogenesis. Future studies would need to be done to further elucidate indirect and direct effects of melatonin receptor signaling on gluconeogenesis.

The loss in blood glucose rhythms observed in melatonin receptor knock-out mice does not appear to have resulted from differences in the food intake rhythm (personal communication with Charlotte von Gall). These results are consistent with pinealectomy studies demonstrating that removal of melatonin has no apparent effects on the rhythm of food intake[57]. It is possible that the loss of the blood glucose peak could result from increased insulin concentrations.

Fig 3. Expression levels of clock and clock controlled genes within liver of WT, MT1<sup>−/−</sup>, and MT2<sup>−/−</sup> mice. For each respective plot: the black circle (WT), red circle (MT<sub>1</sub><sup>−/−</sup>) and blue circle (MT<sub>2</sub><sup>−/−</sup>) indicate the expression levels of target genes calculated relative to the average expression of each dataset throughout the 24hrs. Results are represented as mean ± SEM. The black (WT), red (MT<sub>1</sub><sup>−/−</sup>) and blue (MT<sub>2</sub><sup>−/−</sup>) lines represent the periodic sinusoidal functions determined by COSINOR analysis (p<0.05, N = 4–6 for each time point and genotype). The bar graphs corresponding to each plot depict the amplitude of each oscillating transcript as calculated by COSINOR analysis and normalized to the value of the WT amplitude. *p<0.05, One-Way ANOVA (post-hoc: Holm-Sidak) MT<sub>1</sub><sup>−/−</sup> vs. MT<sub>2</sub><sup>−/−</sup>.

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after melatonin receptor deletion. Although there have been reports of a circadian rhythm in insulin secretion[17], studies demonstrating that fasted rats do not show an insulin rhythm [58], suggest that the food intake rhythm drives the rhythm in insulin secretion. Therefore it is unlikely that major differences in the insulin secretory response occur independently of food intake within melatonin receptor knock-out mice.

Taken together our data demonstrates that melatonin receptors are critical for the temporal regulation of glucose homeostasis. Given that no coincident disturbances were found in the rhythmic expression of clock genes within insulin sensitive tissues, it could suggest that this regulation occurs at the level of the central nervous system-possibly the SCN- by a mechanism which involves coupling of nutrient sensing by the brain to glucose production by the liver. Alternatively, an additional explanation could be that melatonin signaling acts directly on the
expression/activity of genes involved in glucose metabolism. Peripheral regulation alone, for example melatonin binding to receptors within the pancreas, skeletal muscle or liver is unlikely as the effects of melatonin receptor deletion on blood glucose levels are not restricted to the dark phase (time window when melatonin is secreted). Future studies will be needed to further elucidate the role of melatonin receptor signaling in the regulation of the daily regulation of blood glucose levels.

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Author Contributions

Conceived and designed the experiments: SO KB GT. Performed the experiments: SO KB SCA. Analyzed the data: SO KB GT. Wrote the paper: SO KB GT.

References

1. Dubocovich ML, Delagrange P, Krause DN, Sugden D, Cardinali DP, Olcese J. International Union of Basic and Clinical Pharmacology. LXXV. Nomenclature, classification, and pharmacology of G protein-coupled melatonin receptors. Pharmacol Rev 2010 Sep; 62(3):343–80. doi:10.1124/pr.110.002832 PMID: 20605968

2. Tosini G, Owino S, Guillaume JL, Jockers R. Understanding melatonin receptor pharmacology: latest insights from mouse models, and their relevance to human disease. Bioessays 2014 Aug; 36(8):778–87. doi: 10.1002/bies.201400017 PMID: 24903552

3. Brydon L, Roka F, Petit L, de CP, Tissot M, Barrett P, et al. Dual signaling of human Mel1a melatonin receptors via G(2), G(3), and G(q/11) proteins. Mol Endocrinol 1999 Dec; 13(12):2025–38. PMID: 10596579

4. Kemp DM, Ubeda M, Habener JF. Identification and functional characterization of melatonin Mel 1a receptors in pancreatic beta cells: potential role in incretin-mediated cell function by sensitization of cAMP signaling. Mol Cell Endocrinol 2002 Jun 14; 191(2):157–66. PMID: 12062899

5. Peschke E, Muhlbauer E, Musshoff U, Csernus VJ, Chankiewitz E, Peschke D. Receptor (MT(1)) mediated influence of melatonin on cAMP concentration and insulin secretion of rat insulinoma cells INS-1. J Pineal Res 2002 Sep; 33(2):63–71. PMID: 12153439

6. Picinato MC, Haber EP, Cipolla-Neto J, Curi R, de Oliveira Carvalho CR, Carpinelli AR. Melatonin inhibits insulin secretion and decreases PKA levels without interfering with glucose metabolism in rat pancreatic islets. J Pineal Res 2002 Oct; 33(3):156–60. PMID: 12220330

7. Stumpf I, Muhlbauer E, Peschke E. Involvement of the cGMP pathway in mediating the insulin-inhibitory effect of melatonin in pancreatic beta-cells. J Pineal Res 2008 Oct; 45(3):318–27. doi: 10.1111/j.1600-079x.2008.00593.x PMID: 18363673

8. Bahr I, Muhlbauer E, Albrecht E, Peschke E. Evidence of the receptor-mediated influence of melatonin on pancreatic glucagon secretion via the Gaalphaq protein-coupled and PI3K signaling pathways. J Pineal Res 2012 Nov; 53(4):390–8. doi: 10.1111/j.1600-079x.2012.01009.x PMID: 22672634

9. Ha E, Yim SV, Chung JH, Yoon KS, Kang I, Cho YH, et al. Melatonin stimulates glucose transport via insulin receptor substrate-1-phosphatidylinositol 3-kinase pathway in C2C12 murine skeletal muscle cells. J Pineal Res 2006 Aug; 41(1):67–72. PMID: 16842543

10. Lima FB, Matsushita DH, Hell NS, Dolnikoff MS, Okamoto MM, Cipolla NJ. The regulation of insulin action in isolated adipocytes. Role of the periodicity of food intake, time of day and melatonin. Braz J Med Biol Res 1994 Apr; 27(4):995–1000. PMID: 8087099

11. Contreras-Alcantara S, Baba K, Tosini G. Removal of melatonin receptor type 1 induces insulin resistance in the mouse. Obesity (Silver Spring) 2010 Sep; 18(9):1861–3.

12. Lyssenko V, Nagorny CL, Erdos MR, Wierup N, Jonsson A, Spigel P, et al. Common variant in MTNR1B associated with increased risk of type 2 diabetes and impaired early insulin secretion. Nat Genet 2009 Jan; 41(1):82–8. doi: 10.1038/ng.288 PMID: 19060908

13. Bouatia-Naji N, Bonnefond A, Cavalcanti-Proenca C, Sparso T, Holmkvist J, Marchand M, et al. A variant near MTNR1B is associated with increased fasting plasma glucose levels and type 2 diabetes risk. Nat Genet 2009 Jan; 41(1):89–94. doi: 10.1038/ng.277 PMID: 19060909
14. Bonnefond A, Clement N, Fawcett K, Yengo L, Vaillant E, Guillaume JL, et al. Rare MTNR1B variants impairing melatonin receptor 1B function contribute to type 2 diabetes. Nat Genet 2012 Mar; 44(3):297–301. doi: 10.1038/ng.1053 PMID: 22286214

15. Li C, Shi Y, You L, Wang L, Chen ZJ. Melatonin receptor 1A gene polymorphism associated with polycystic ovary syndrome. Gynecol Obstet Invest 2011; 72(2):130–4. doi: 10.1159/000323542 PMID: 21474908

16. Bouatia-Naji N, Bonnefond A, Cavalcanti-Proenca C, Sparso T, Holmkvist J, Marchand M, et al. A variant near MTNR1B is associated with increased fasting plasma glucose levels and type 2 diabetes risk. Nat Genet 2009 Jan; 41(1):89–94. doi: 10.1038/ng.277 PMID: 19060909

17. Jolin T, Montes A. Daily rhythm of plasma glucose and insulin levels in rats. Horm Res 1973; 4(3):153–6. PMID:4748471

18. Peret J, Macaire I, Chanez M. Schedule of protein ingestion, nitrogen and energy utilization and circadian rhythm of hepatic glycogen, plasma corticosterone and insulin in rats. J Nutr 1973 Jun; 103(6):866–74. PMID: 4705272

19. La Fleur SE, Kalsbeek A, Wortel J, Bujs RM. A suprachiasmatic nucleus generated rhythm in basal glucose concentrations. J Neuroendocrinol 1999 Aug; 11(8):643–52. PMID: 10447803

20. Bolli GB, De FP, De CS, Perriello G, Ventura MM, Calcinaro F, et al. Demonstration of a dawn phenomenon in normal human volunteers. Diabetes 1984 Dec; 33(12):1150–3. PMID: 6389230

21. Van CE. Diurnal and ultradian rhythms in human endocrine function: a minireview. Horm Res 1990; 34(2):45–53. PMID: 1965834

22. Reppert SM, Weaver DR. Coordination of circadian timing in mammals. Nature 2002 Aug 29; 418(6901):935–41. PMID: 12198538

23. Saini C, Suter DM, Liani A, Gos P, Schibler U. The mammalian circadian timing system: synchronization of peripheral clocks. Cold Spring Harb Symp Quant Biol 2011; 76:39–47. doi: 10.1101/sqb.2011.76.010918 PMID: 22179985

24. Johnston JD, Tournier BB, Andersson H, Masson-Pevet M, Lincoln GA, Hazlerigg DG. Multiple effects of melatonin on rhythmic clock gene expression in the mammalian pars tuberalis. Endocrinology 2006 Feb; 147(2):959–65. PMID: 16269454

25. von GC, Weaver DR, Moek J, Jilg A, Stehle JH, Korf HW. Melatonin plays a crucial role in the regulation of rhythmic clock gene expression in the mouse pars tuberalis. Ann N Y Acad Sci 2005 Apr; 1040:508–11. PMID: 15891103

26. von GC, Garabette ML, Kell CA, Frenzel S, Dehghani F, Schumm-Draeger PM, et al. Rhythmic gene expression in pituitary depends on heterologous sensitization by the neurohormone melatonin. Nat Neurosci 2002 Mar; 5(3):39–47. doi: 10.1038/nn09253 PMID: 11836530

27. La Fleur SE, Kalsbeek A, Wortel J, van dV, Bujs RM. Role for the pineal and melatonin in glucose homeostasis: pinealectomy increases night-time glucose concentrations. J Neuroendocrinol 2001 Dec; 13(12):1025–32. PMID: 11726988

28. Muhlbaier E, Gross E, Labuca K, Wolgast S, Peschke E. Loss of melatonin signalling and its impact on circadian rhythms in mouse organs regulating blood glucose. Eur J Pharmacol 2009 Mar 15; 606(1–3):61–71. doi: 10.1016/j.ejphar.2009.01.029 PMID: 19374484

29. Baba K, Pozdeyev N, Mazzoni F, Contreras-Alcantara S, Liu C, Kasamatsu M, et al. Melatonin modulates visual function and cell viability in the mouse retina via the MT1 melatonin receptor. Proc Natl Acad Sci U S A 2009 Sep 1; 106(35):15043–8. doi: 10.1073/pnas.0904400106 PMID: 19706469

30. Dyar KA, Ciciliot S, Wright LE, Bienso RS, Tagliazucchi GM, Patel VR, et al. Muscle insulin sensitivity and glucose metabolism are controlled by the intrinsic muscle clock. Mol Metab 2014 Feb; 3(1):29–41. doi: 10.1016/j.molmet.2013.10.005 PMID: 24567902

31. Lamia KA, Storch KF, Weitz CJ. Physiological significance of a peripheral tissue circadian clock. Proc Natl Acad Sci U S A 2008 Sep 30; 105(39):15172–7. doi: 10.1073/pnas.0806717105 PMID: 18778586

32. Lee J, Moulik M, Fang Z, Saha P, Zou F, Xu Y, et al. Bmal1 and beta-cell clock are required for adaptation to circadian disruption, and their loss of function leads to oxidative stress-induced beta-cell failure in mice. Mol Cell Biol 2013 Jun; 33(11):2327–38. doi: 10.1128/MCB.01421-12 PMID: 23547261

33. Marcheva B, Ramsey KM, Buhr ED, Kobayashi Y, Su H, Ko CH, et al. Disruption of the clock components CLOCK and BMAL1 leads to hypoinsulinemia and diabetes. Nature 2010 Jul 29; 466(7306):627–31. doi: 10.1038/nature09253 PMID: 20562852

34. Paschos GK, Ibrahim S, Song WL, Kunieda T, Grant G, Reyes TM, et al. Obesity in mice with adipocyte-specific deletion of clock component Arntl. Nat Med 2012 Dec; 18(12):1766–77. doi: 10.1038/nm.2979 PMID: 23142819
35. Sadacca LA, Lamia KA, deLemos AS, Blum B, Weitz CJ. An intrinsic circadian clock of the pancreas is required for normal insulin release and glucose homeostasis in mice. Diabetologia 2011 Jan; 54 (1):120–4. doi: 10.1007/s00125-010-1920-8 PMID: 20890745

36. Zhang EE, Liu Y, Dentin R, Pongsawakul PY, Liu AC, Hirota T, et al. Cryptochrome mediates circadian regulation of cAMP signaling and hepatic gluconeogenesis. Nat Med 2010 Oct; 16(10):1152–6. doi: 10.1038/nm.2214 PMID: 20852621

37. Zhao Y, Zhang Y, Zhou M, Wang S, Hua Z, Zhang J. Loss of mPer2 increases plasma insulin levels by enhanced glucose-stimulated insulin secretion and impaired insulin clearance in mice. FEBS Lett 2012 May 7; 586(9):1306–11. doi: 10.1016/j.febslet.2012.03.034 PMID: 22504074

38. Bass J. Circadian topology of metabolism. Nature 2012 Nov 15; 491(7424):348–56. doi: 10.1038/ nature11704 PMID: 23151577

39. Pauly JE, Scheving LE. Circadian rhythms in blood glucose and the effect of different lighting schedules, hypophysectomy, adrenal medullectomy and starvation. Am J Anat 1967 May; 120(3):627–36. PMID: 6037327

40. Bolli GB, De FP, De CS, Perriello G, Ventura MM, Calcinaro F, et al. Demonstration of a dawn phenomenon in normal human volunteers. Diabetes 1984 Dec; 33(12):1150–3. PMID: 6389230

41. La Fleur SE, Kalsbeek A, Wortel J, Fekkes ML, Buijs RM. A daily rhythm in glucose tolerance: a role for the suprachiasmatic nucleus. Diabetes 2001 Jun; 50(6):1237–43. PMID: 11375322

42. Ayoub MA, Levoye A, Delagrange P, Jockers R. Preferential formation of MT1/MT2 melatonin receptor heterodimers with distinct ligand interaction properties compared with MT2 homodimers. Mol Pharmacol 2004 Aug; 66(2):312–21. PMID: 15266022

43. Baba K, Benleulmi-Chaachoua A, Joume AS, Kamal M, Guillaume JL, Dussaud B, et al. Heteromeric MT1/MT2 melatonin receptors modulate photoreceptor function. Sci Signal 2013 Oct 8; 6(296):ra89. doi: 10.1126/scisignal.2304302 PMID: 24106342

44. Kalsbeek A, la FS, Fliers E. Circadian control of glucose metabolism. Mol Metab 2014 Jul; 3(4):372–83. doi: 10.1016/j.molmet.2014.03.002 PMID: 24944897

45. Kalsbeek A, Yi CX, La Fleur SE, Fliers E. The hypothalamic clock and its control of glucose homeostasis. Trends Endocrinol Metab 2010 Jul; 21(7):402–10. doi: 10.1016/j.tem.2010.02.005 PMID: 20303779

46. Lamia KA, Storch KF, Weitz CJ. Physiological significance of a peripheral tissue circadian clock. Proc Natl Acad Sci U S A 2008 Sep 30; 105(39):15172–7. doi: 10.1073/pnas.0806717105 PMID: 18779586

47. Cailotto C, La Fleur SE, Van HC, Wortel J, Kalsbeek A, Feenstra M, et al. The suprachiasmatic nucleus controls the daily variation of plasma glucose via the autonomic output to the liver: are the clock genes involved? Eur J Neurosci 2005 Nov; 22(10):2531–40. PMID: 16307595

48. de Farias TS, de Oliveira AC, Andreotti S, do Amaral FG, Chimin P, de Proenca AR, et al. Pinealectomy interferes with the circadian clock genes expression in white adipose tissue. J Pineal Res 2015 Apr; 58 (3):251–61. doi: 10.1111/jpi.12211 PMID: 25626464

49. McCarthy JJ, Andrews JI, McDearmon EL, Campbell KS, Barber BK, Miller BH, et al. Identification of the circadian transcriptome in adult mouse skeletal muscle. Physiol Genomics 2007 Sep 19; 31(1):86–95. PMID: 17550994

50. Zvonicek S, Pitsyn AA, Conrad SA, Scott LK, Floyd ZE, Kilroy G, et al. Characterization of peripheral circadian clocks in adipose tissues. Diabetes 2006 Apr; 55(4):962–70. PMID: 16567517

51. Cermakian N, Sassone-Corsi P. Multilevel regulation of the circadian clock. Nat Rev Mol Cell Biol 2000 Oct 1; 1(1):58–67. PMID: 11413490

52. Park EJ, Woo SM, Min KJ, Kwon TK. Transcriptional and post-translational regulation of Bim controls apoptosis in melatonin-treated human renal cancer Caki cells. J Pineal Res 2014 Jan; 56(1):97–106. doi: 10.1111/jpi.12102 PMID: 24117987

53. Friend J, Reiter RJ. Melatonin as a proteasome inhibitor. Is there any clinical evidence? Life Sci 2014 Oct 12; 115(1–2):8–14. doi: 10.1016/j.lfs.2014.08.024 PMID: 25219883

54. Faria JA, Kinote A, Ignacio-Souza LM, de Araujo TM, Razolli DS, Doneda DL, et al. Melatonin acts through MT1/MT2 receptors to activate hypothalamic Akt and suppress hepatic gluconeogenesis in rats. Am J Physiol Endocrinol Metab 2013 Jul 15; 305(2):E230–E242. doi: 10.1152/ajpendo.00094.2013 PMID: 23895212

55. Nogueira TC, Lellis-Santos C, Jesus DS, Taneda M, Rodrigues SC, Amaral FG, et al. Absence of melatonin induces night-time hepatic insulin resistance and increased gluconeogenesis due to stimulation
57. Takahashi K, Inoue K, Takahashi Y. No effect of pinealectomy on the parallel shift in circadian rhythms of adrenocortical activity and food intake in blinded rats. Endocrinol Jpn 1976 Oct; 23(5):417–21. PMID: 1009894

58. La Fleur SE. Daily rhythms in glucose metabolism: suprachiasmatic nucleus output to peripheral tissue. J Neuroendocrinol 2003 Mar; 15(3):315–22. PMID: 12588521