DISORDERS OF CIRCADIAN RHYTHMS

EDITED BY: Arturo Ortega, Mario Caba and Pevet Paul
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frontiers Research Topics

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DISORDERS OF CIRCADIAN RHYTHMS

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EDITORIAL
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Editorial: Disorders of Circadian Rhythms

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Keywords: cancer, circadian rhythm, clock genes, metabolism, neurological diseases, shift work

Editorial on the Research Topic

Disorders of Circadian Rhythms

Circadian rhythms are biological oscillations with a period of about 24 h, which allow the organisms to anticipate changes in the environment. These rhythms are maintained by an innate genetically determined time-keeping system called *"the molecular circadian clockwork,"* of which the suprachiasmatic nucleus (SCN) of the hypothalamus is the master biological clock and is mainly synchronized by light. The circadian system also includes peripheral clocks located in multiple cell types and tissues; these are entrained by both SCN (neural and humoral signaling) as well as other SCN-independent cues (food and temperature), resulting in a synchronized organism.

As has been established, circadian rhythmicity has a profound effect on the physiological and behavioral organization of vertebrates, so disruption of these rhythms is associated with the development of multiple clinical conditions, such as mental and metabolic diseases, cancer, addiction, and pain. In the past years it has become evident that important etiological and therapeutic connections exist between clock-based features of an organism and its pathologies. However, the functional links between disturbances of the circadian rhythms and overall health in animal models and humans are yet to be characterized.

This E-Book comprises *state-of the-art* Reviews, Original Research and Perspective contributions that feature current advancements in the molecular mechanisms and the impact of *gene-environment interactions* of circadian rhythms in diverse pathologies.

A perspective article by Nunez et al. analyzes the serious consequences of nocturnal activity in humans. In addition, the advantages and limitations of some animal models used to study these effects are discussed. Loss of circadian homeostasis is associated with pathogenesis of cancer as can be clearly understood after the critical reading of the review paper by Lin and Farkas, which is a remarkable synthesis of our current knowledge of the potential role of altered circadian rhythms in breast cancer. Discrepancies present among different studies that consider or not the rhythmicity of core clock, as well as the advantages to the use of small molecules for studying the links between circadian rhythms and cancer are also critically reviewed.

Méndez and Muñoz analyze the possible role of NADPH as a circadian and cancer-promoting metabolite. In this context, the authors focus particularly on the relationship between circadian rhythms and metabolic reprogramming (Warburg effect).

Several neurodegenerative diseases are linked with alterations in glutamate transport. Chi-Castañeda and Ortega provide a thorough review on the mechanisms of circadian regulation of glutamate transporters, including transcriptional, translational, post-translational and post-transcriptional regulation, both in neuronal and glial cells.

Light is the main synchronizer of the master clock. This oscillator encodes seasonal changes based on the amount of daylight hours (day length) and adjusts numerous biological processes. Seasonality has been documented in sleep duration, appetite, mood, social activity, among others.

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Garbazza and Benedetti recapitulate the current information on the relationship between biological clock and behavior, particularly mood disorders. The effects of seasonal changes of the light-dark cycle and gene polymorphisms of the core clock machinery on the behavior of patients affected by mood disorders are discussed in depth.

Disruption of circadian rhythms is also associated with reproductive problems. Caba et al. recapitulate on the essential role that the circadian clock plays in reproduction, exploiting the rabbit model that offers an extraordinary opportunity to studying this issue. In addition, they emphasize the translational importance of circadian rhythms in reproduction.

The mammalian retina contains an autonomous circadian clock that regulates diverse biochemical, cellular, and physiological processes within the eye. However, the discovery of a self-sustained oscillator in retinal pigment epithelial (RPE) cells is relatively recent, and their regulatory mechanisms are currently unknown. Therefore, using a human retinal pigment epithelium cell line, Morioka et al. studied the role that histamine signaling plays in these cells. The authors propose that the RPE oscillator is entrained by histamine *via* H_1 receptors. In addition, the authors call the attention to the indiscriminate use of antihistaminic drugs that eventually lead to circadian rhythm disorders.

Peripheral circadian oscillators probably perform an essential role in metabolic homeostasis. Several studies have provided evidence that high sugar and/or high fat diets modify rhythmic expression of clock genes in peripheral tissues. In their article, Blancas-Velazquez et al. examined the impact of a high-energy diet on clock gene expression in different reward-related brain areas. They demonstrate that a high fat/high sugar diet affects *Per2* mRNA expression pattern in areas involved in food reward.

The pineal hormone melatonin is one of the major humoral signals from the SCN and regulates main physiological processes, such as the sleep-wake cycle, glucose, and lipid metabolism. The SCN controls melatonin synthesis and release by multisynaptic projections relaying in the superior cervical ganglia (SCG). In this sense, Mul Fedele et al. assessed the effects of SCG surgical removal on rat metabolism and diurnal rhythms of locomotor activity and feeding. Increased adipose tissue, increased body weight/food intake ratio, decreased glycemia, and increased daytime activity was found in the SCGx rats, suggesting that SCG could be altering metabolism by shifting the feeding pattern.

Circadian timing system interacts with metabolic and thermal mechanisms directly involved in the maintenance of body temperature. Accordingly, Machado et al. report cold-induced metabolic response and core clock gene expression variations in skeletal muscle (CLOCK, PER2, CRY1-2, and REV-ERBα) and brown adipose tissue (DBP and REV-ERBα) fluctuation according to the time of the day of the exposure to low temperature. Furthermore, chronic cold exposure also influences expression of genes associated in thermogenesis and substrate oxidation in a time of day and tissue-specific manner.

van der Spek et al. extensively compare clock and metabolic gene expression rhythms in mesenteric-, perirenal-, epididymal-, and subcutaneous white adipose tissue (WAT) depots. Nevertheless, no clear differences in gene expression rhythms between subcutaneous and different intra-abdominal WAT depots were found. Consequently, different WAT depots are not involved with variations in clock gene rhythmicity.

Last but not least, the review by Caba and Mendoza highlights the role of clock genes in meal anticipation. The authors present conclusive evidences demonstrating that rabbit pups are an excellent natural model to study the molecular and brain mechanism of food-anticipatory circadian behavior.

AUTHOR CONTRIBUTIONS

DC-C and AO have read all the contributions and wrote the Editorial Article.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Alterations in Metabolism and Diurnal Rhythms following Bilateral Surgical Removal of the Superior Cervical Ganglia in Rats

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Mammalian circadian rhythms are controlled by a master pacemaker located in the suprachiasmatic nuclei (SCN), which is synchronized to the environment by photic and nonphotic stimuli. One of the main functions of the SCN is to regulate peripheral oscillators to set temporal variations in the homeostatic control of physiology and metabolism. In this sense, the SCN coordinate the activity/rest and feeding/fasting rhythms setting the timing of food intake, energy expenditure, thermogenesis, and active and basal metabolism. One of the major time cues to the periphery is the nocturnal melatonin, which is synthesized and secreted by the pineal gland. Under SCN control, arylalkylamine *N*-acetyltransferase (AA-NAT)—the main enzyme regulating melatonin synthesis in vertebrates—is activated at night by sympathetic innervation that includes the superior cervical ganglia (SCG). Bilateral surgical removal of the superior cervical ganglia (SCGx) is considered a reliable procedure to completely prevent the nocturnal AA-NAT activation, irreversibly suppressing melatonin rhythmicity. In the present work, we studied the effects of SCGx on rat metabolic parameters and diurnal rhythms of feeding and locomotor activity. We found a significant difference between SCGx and sham-operated rats in metabolic variables such as an increased body weight/food intake ratio, increased adipose tissue, and decreased glycemia with a normal glucose tolerance. An analysis of locomotor activity and feeding rhythms showed an increased daytime (lights on) activity (including food consumption) in the SCGx group. These alterations suggest that superior cervical ganglia-related feedback mechanisms play a role in SCN-periphery phase coordination and that SCGx is a valid model without brain-invasive surgery to explore how sympathetic innervation affects daily (24 h) patterns of activity, food consumption and, ultimately, its role in metabolism homeostasis.

Keywords: superior cervical ganglion, SCGx, circadian rhythm, metabolism, melatonin

INTRODUCTION

The circadian system, a set of biological clocks that regulate almost all physiological and behavioral processes, has evolved to adapt the organism's physiology to cyclic environmental changes (1–4). In mammals, the master clock resides in the suprachiasmatic nuclei (SCN) of the hypothalamus and is mainly synchronized by the light–dark (LD) cycle (5). The circadian system also includes

peripheral clocks, entrained by the SCN *via* neural and humoral cues, such as rhythmically secreted hormones (6–8), and other SCN-independent cues like food (9).

One of the major physiological processes controlled by the SCN is metabolism, including metabolic rate and circadian rhythms of food intake (3). Food consumption is normally confined to the wake/active phase, while fasting periods occur during the rest/sleep phase, correlating to the anabolic, and catabolic phases of metabolism, respectively (10). Alterations of the circadian pacemaker can lead to metabolic pathologies, such as obesity or metabolic syndrome (11). For example, shift work, chronic forced circadian desynchronization or mutations of clock genes can affect the pattern of food intake and lead to increased levels of circulating triglycerides, and adipose tissue masses resulting in an augmented body weight (12–15).

Melatonin is a hormone produced by the pineal gland during the dark phase and is considered one of the most important circadian outputs (16). It regulates major physiological processes, including the sleep–wake cycle, and lipid and glucose metabolism (17–22). The SCN interact with the pineal gland through the sympathetic neurons of the superior cervical ganglia (SCG) (23). This interaction modulates the arylalkylamine *N*-acetyltransferase (AA-NAT) activity, the main enzyme responsible for melatonin rhythm generation in vertebrates (24). The elimination of the pineal melatonin rhythm, or a reduction of its amplitude, renders the circadian pacemaker a less self-sustained, often damped, oscillatory system (25). On the other hand, forced circadian desynchronization induced by an LD cycle of 22 h in rats (26) or by shift work in humans (27) disrupts rhythmic melatonin secretion.

The SCG are the uppermost ganglia of the paraventral sympathetic chain and innervate the pineal gland, among others structures (28). Superior cervical ganglionectomy (SCGx) is a reliable model to study the role of sympathetic innervation on neuroendocrine interactions (29–31). Moreover, SCGx has been used to determine the influences of the circadian clock (i.e., the SCN) on neuroendocrine functions. In this sense, SCGx disrupts the circadian system by depressing melatonin secretion and suppressing its rhythm (32, 33), presumably by the inhibition of pineal AA-NAT activity (34). This also results in an abolition of the rhythmic excretion of urinary 6-sulphatoxymelatonin, a melatonin metabolite (35). In addition, the SCG also cover other territories such as other glands, brain areas, and the cardiovascular system, which might also be implied in metabolic regulation (36–41).

Taking into account that the lack of melatonin can produce circadian alterations, and that sympathetic innervation from the SCG covers diverse neuroendocrine effectors, the aim of our work was to study if SCGx can affect rat metabolism and whether this is related to an impairment of the circadian clock.

MATERIALS AND METHODS

Ethics Statement

All animal procedures were approved by the Institutional Animal Care and Use Committee at the School of Medicine, National University of Cuyo, Mendoza, Argentina (Protocol ID 9/2012) and were conducted in accordance with the National Institutes of Health's Guide for Care and Use of Laboratory Animals and the Animal Research: Reporting *In Vivo* Experiments (ARRIVE) Guidelines.

Animals

Young (3 months old) male Wistar rats were raised in our colony and maintained in a 12:12 h LD cycle (with zeitgeber time 12—ZT 12—defined as the time of lights off; light intensity averaging 300 lux at the cage level), in a controlled environment with food and water *ad libitum*.

Locomotor Activity Rhythms

Animals were transferred to individual cages equipped with infrared motion sensors. Locomotor activity was assessed by the interruption of the infrared beam and recorded every 5 min (Archron, Argentina). The locomotor activity rhythm analysis was performed using the "el Temps" program (http://www.el-temps.com). Locomotor activity onset was defined as the 10-min bin that contained at least 50% of the maximum activity/bin followed by another bin of at least another 50% of the maximum activity bin within 40 min. Entrainment to the LD cycle was confirmed by periodogram analysis (χ^2 test). Phase angle was measured as the difference (in minutes) between activity onset and lights off. Total daytime activity was assessed by the area under the curve (AUC) of the waveform of each animal. Activity was expressed as a percentage of the total activity or relative activity by comparing post-surgery activity to the activity counts of the 3 weeks previous to the surgery (pre-surgery) as the post-/pre-ratio.

Surgery

Bilateral superior cervical ganglionectomy (SCGx) was performed as described by Savastano et al. (31). Briefly, under ketamine (50 mg/kg of body weight)/xylazine (5 mg/kg of body weight) anesthesia, the ventral neck region was shaved and disinfected. The salivary glands were exposed through a 2.5 cm vertical incision and retracted to uncover the underlying muscles. The carotid bifurcations were identified through the carotid triangles and the SCG were removed after sectioning the sympathetic trunks, the external carotid nerves, and the internal carotid nerves. For sham-operated animals, the same procedure was performed but the ganglia were not removed.

Animal Weight and Food Intake Measurements

Body weight and food consumption were monitored weekly at ZT10. After a 3-week pre-surgery baseline, animals were subjected to bilateral SCGx or a sham procedure ($n = 9$ per group), and body weight and food intake were measured for another 10 weeks. Food efficiency (FE) was analyzed by the body weight/food intake ratio.

The food intake rhythm was analyzed in both groups at week 11. Daytime (i.e., during lights on) and nighttime (during lights off) food intakes were measured daily at the end of the light and dark phases for 10 days ($n = 5$ per group). Daytime and nighttime

feedings were expressed as a percentage of total food consumed per day.

Glycemia and Glucose Tolerance Test (GTT)

At week 10, glycemia was measured at ZT10 using PTS PanelsTM test strips for CardioChekTM Brand Analyzer (Hannover, Germany) ($n = 9$ per group).

At week 13, a GTT was performed after 18 h fast ($n = 5$ per group). Glycemia was measured as mentioned above before and 15, 30, 60, and 120 min after glucose administration (orogastric, 3 g/kg of body weight from a 30% solution of D-glucose), at ZT10. The AUC of glycemia vs. time was calculated above each individual baseline (basal glycemia).

Fat Weight Measurements

At the end of week 13, animals were decapitated under anesthesia, and epididymal, retroperitoneal, mesenteric, and inguinal adipose tissues were collected and weighed ($n = 5$ per group). Fat weight was expressed as relative to body weight.

Statistical Analysis

Data were expressed as mean ± SEM and analyzed using PRISM5 (GraphPad Software Inc., La Jolla, CA, USA). Statistical difference between means was determined by Student's *t*-test. For the grouped statistical analysis, two-way ANOVA or repeated measures two-way ANOVA was used with Bonferroni as post-test. $p < 0.05$ was considered significant and $p < 0.01$ highly significant.

RESULTS

Global Metabolism Is Affected by Bilateral Superior Cervical Ganglionectomy

To study the effect of SCGx on rat metabolism, animals were subjected to ganglionectomy or a sham procedure at the middle of week 3 ($n = 9$ per group). Body weight and food consumption were measured, and FE (body weight/food intake ratio) was calculated. Rats subjected to SCGx did not exhibit differences in body weight (**Figure 1A**) but had significant lower food intake when compared with sham animals (**Figure 1B**), throughout the 10 weeks after surgery. An FE analysis (42) showed metabolic differences between the two groups. FE was higher in ganglionectomized animals, revealing that these rats gained more body mass per gram of consumed food than controls (**Figure 1C**).

Ganglionectomy Increases Daytime Locomotor Activity

Rats subjected to SCGx or sham surgeries ($n = 9$ per group) were placed individually in cages with infrared sensors to study their activity distribution during the day. An activity rhythm analysis demonstrated that entrainment to the LD cycle and activity phase angle were not affected by ganglionectomy (**Table 1**; **Figure 2A**). Moreover, SCGx animals did not show differences in the levels of total activity as post-/pre-surgery ratio (**Table 1**; **Figure 2B**; SCGx group: 1.08 ± 0.083; sham-operated group: 0.99 ± 0.042; data expressed as mean of post-/pre-surgery ± SEM). However, locomotor activity of ganglionectomized animals during the lights-on phase increased after surgery and remained higher throughout the 10-week post-surgery interval (**Figure 2C**). Moreover, the relation between the AUC of daytime activity after and before surgery was significantly higher in the SCGx animals (**Table 1**; **Figure 2D**; SCGx group: 5.492 ± 0.4126; sham group: 1.992 ± 0.2212; data expressed as mean of post-/pre-surgery ± SEM). This increase

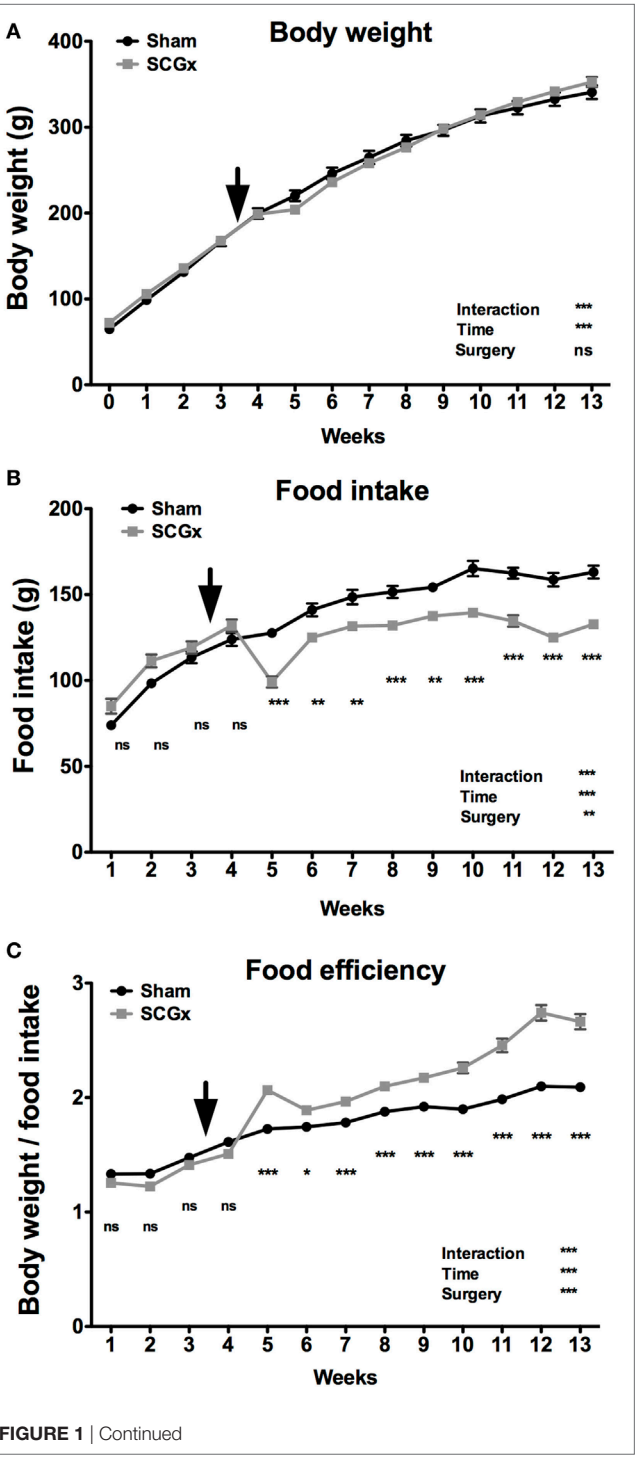

FIGURE 1 | Continued

FIGURE 1 | Bilateral superior cervical ganglionectomy affects metabolic variables. Rats subjected to SCGx at week 3.5 did not exhibit differences in body weight [**(A)**; repeated measures two-way ANOVA: $p = 0.0002$, $F = 3.229$ for interaction, $p < 0.0001$, $F = 1,588$ for time, $p > 0.05$, $F = 0.008$ for surgery; $n = 9$ per group], but had significant lower food intake throughout the 10 weeks after surgery [**(B)**; repeated measures two-way ANOVA: $p < 0.0001$, $F = 35.51$ for interaction, $p < 0.0001$, $F = 222.8$ for time, $p = 0.0015$, $F = 14.92$ for surgery, followed by Bonferroni post-tests: ***$p < 0.001$, **$p < 0.01$; $n = 9$ per group]. A food efficiency (body weight/food intake ratio) analysis demonstrated metabolic differences between the two groups with higher levels in ganglionectomized animals [**(C)**; repeated measures two-way ANOVA: $p < 0.0001$, $F = 42.75$ for interaction, $p < 0.0001$, $F = 374.7$ for time, $p < 0.0001$, $F = 76.49$ for surgery, followed by Bonferroni post-tests: ***$p < 0.001$, *$p < 0.05$; $n = 9$ per group]. The rats used in this work were still growing from young-to-adulthood and therefore increasing their body mass and food consumption over time. Arrows indicate the day of surgery. Repeated measures two-way ANOVA results are depicted at the bottom right of each figure. Values are given as mean ± SEM.

TABLE 1 | Effects of SCGx on the diurnal rhythm of locomotor activity.

	Sham	SCGx	*p*-Value
Period (min)	1,441 ± 0.645	1,442 ± 1.323	0.522
Phase angle (min)	6.50 ± 1.190	7.00 ± 1.080	0.766
Total activity (post-/pre-surgery)	0.99 ± 0.042	1.08 ± 0.083	0.351
Daytime activity (post-/pre-surgery)	1.99 ± 0.221	5.49 ± 0.412	<0.0001
Nighttime activity (post-/pre-surgery)	1.01 ± 0.003	0.91 ± 0.005	<0.0001

occurs at the expense of a reduced nighttime activity (**Table 1**, SCGx group: 0.91 ± 0.005; sham-operated group: 1.01 ± 0.003; data expressed as mean of post-/pre-surgery ± SEM).

Ganglionectomy Increases Food Intake during Daytime

We next studied the daily pattern of food consumption, which can be affected by circadian alterations (13). Ganglionectomized animals had a lower level of food intake per day (**Figure 3A**; 19.06 ± 0.5960 g for SCGx group; 22.80 ± 0.8027 g for sham group, $n = 5$ per group).

As it was observed with the activity rhythm, a food intake rhythm analysis revealed increased food consumption during daytime (**Figure 3B**; 16.68 ± 0.9030 g for SCGx group; 6.160 ± 0.2015 g for sham group), and a slightly but significantly lower feeding activity during the night (**Figure 3C**; 83.48 ± 0.8864 g for SCGx group; 93.63 ± 0.7122 g for sham group).

SCGx Animals Exhibit Lower Basal Levels of Blood Glucose but Higher Adipose Tissue

Six weeks after surgery, a glycemia analysis at ZT10 showed lower levels of blood glucose in SCGx rats (**Figure 4A**; 48.89 ± 4.464 mg/dl for SCGx group; 78.50 ± 4.392 mg/dl for sham group; $n = 9$ per group).

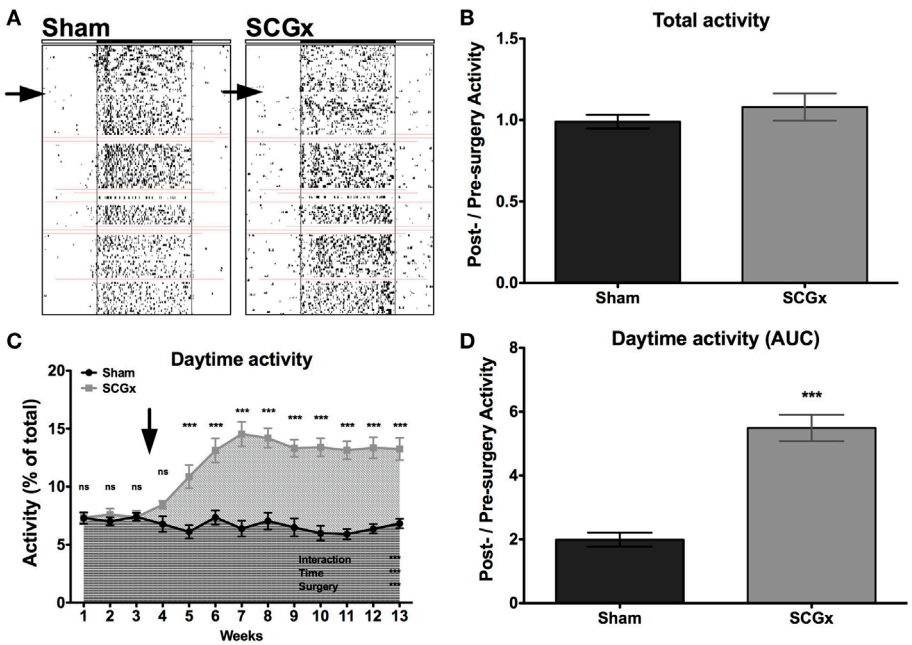

FIGURE 2 | Ganglionectomy affects locomotor activity rhythm. **(A)** Representative actograms for animals subjected to SCGx or sham procedure ($n = 9$ per group). Red lines indicate the moments that the system did not record activity. **(B)** A locomotor activity analysis showed no differences in the levels of total activity, as post-surgery/previous-to surgery ratio (SCGx group, 1.08 ± 0.083; sham group, 0.99 ± 0.042; values are given as mean ± SEM; *t*-test: $p < 0.353$; $n = 9$ per group), but the activity of SCGx animals during daytime (i.e., during lights on) increased after surgery and remained higher throughout the 10-week post-surgery interval [**(C)**; repeated measures two-way ANOVA: $p < 0.0001$, $F = 16.55$ for interaction, $p < 0.0001$, $F = 11.50$ for time, $p < 0.0001$, $F = 43.69$ for surgery, followed by Bonferroni post-tests: ***$p < 0.001$; $n = 9$ per group]. This increased daytime activity is evidenced in the area under the curve (AUC) from post-surgery/pre-surgery ratio, that was significantly higher in the SCGx animals when compared with the sham group [**(D)**; SCGx group: 5.492 ± 0.4126; sham group: 1.992 ± 0.2212; values are given as mean ± SEM; *t*-test: ***$p < 0.0001$, $t = 7.475$; $n = 9$ per group]. Repeated measures two-way ANOVA results are expressed at the bottom right of the figure. Asterisks above the curve indicate significant *p*-values of the Bonferroni post-test. The arrows correspond to the day of surgery.

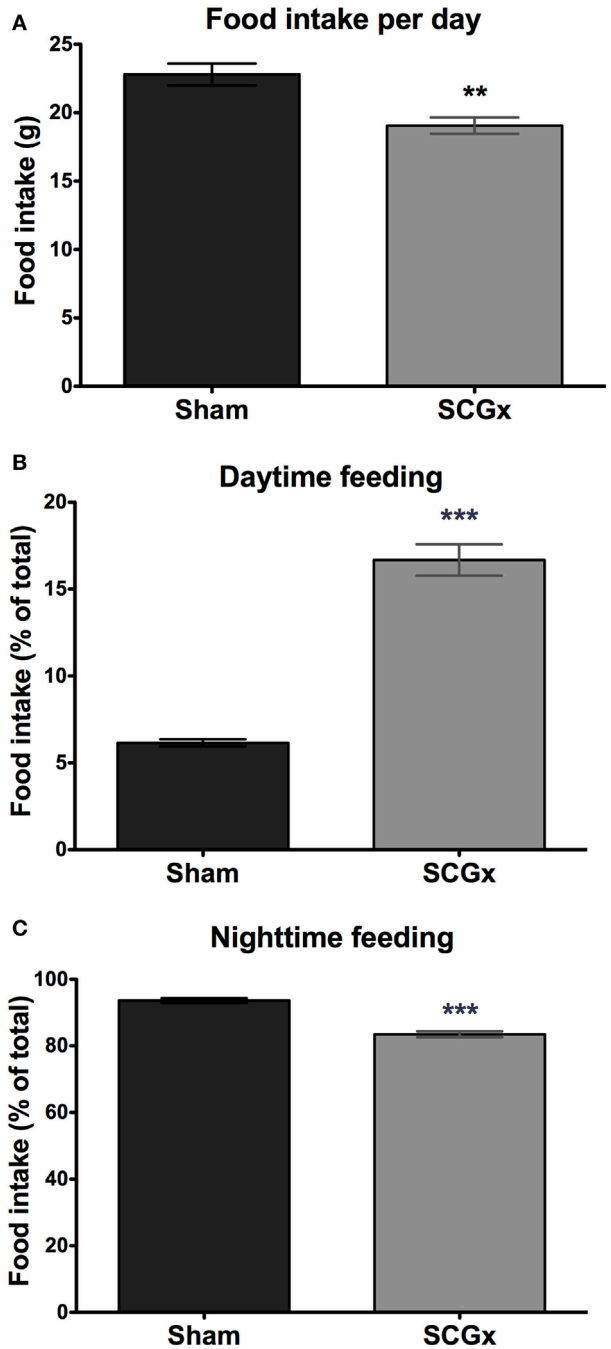

FIGURE 3 | Daytime feeding is increased in SCGx animals. Ganglionectomized animals had a lower level of food intake [**(A)**; SCGx group: 19.06 ± 0.5960 g; sham group: 22.80 ± 0.8027 g; values are given as: mean ± SEM; *t*-test: $^{**}p = 0.0057$, $t = 3.738$; $n = 5$ per group]. Feeding rhythm was also affected: daytime (i.e., during lights on) food consumption was higher in SCGx rats [**(B)**; SCGx group: 16.68 ± 0.9030 g; sham group: 6.160 ± 0.2015 g; *t*-test: $^{***}p < 0.0001$, $t = 11.37$; $n = 5$ per group], and lower during the nighttime (i.e., during lights off) [**(C)**; SCGx group: 83.48 ± 0.8864 g; sham group: 93.63 ± 0.7122 g; *t*-test: $^{***}p = 0.0001$, $t = 8.926$; $n = 5$ per group], compared with sham animals. Values are given as mean ± SEM.

At week 13, a GTT was performed ($n = 5$ per group). Surprisingly, there were no differences in glycemia kinetics (**Figure 4B**) or in the AUC of the GTT (**Figure 4C**; 935 ± 57.04 mg/dl for SCGx; 1,008 ± 65.66 mg/dl for sham) between ganglionectomized and sham animals.

Finally, to better understand the increased body mass in SCGx animals, we studied the fraction of the body weight that is represented by adipose tissue. For this, we measured the levels of mesenteric, epididymal, retroperitoneal, and total fat at the end of week 13 (**Figure 5**), and found adipose tissue significantly increased in SCGx when compared with sham animals (epididymal fat: SCGx group, 0.0186 ± 0.0005; sham group, 0.0162 ± 0.0004; retroperitoneal fat: SCGx group, 0.0154 ± 0.0002; sham group, 0.0130 ± 0.0007; mesenteric fat: SCGx group, 0.002 ± 0.0003; sham group, 0.002 ± 0.0003; total fat: SCGx group, 0.0362 ± 0.0007; sham group, 0.0318 ± 0.0011; $n = 5$ per group).

DISCUSSION

The impact of the superior cervical ganglionectomy (SCGx) on hormone secretion, and blood glucose and insulin release has been reported before (40, 43–46) but its role on body weight homeostasis remains to be fully established. In this work, we assessed the impact of SCGx on rat metabolism and diurnal rhythms. Rats subjected to SCGx showed: (1) increased FE (i.e., gained more weight per gram of food consumed); (2) increased activity during the lights-on phase of the photoperiod; (3) increased feeding during daytime; (4) reduced glucose levels, without changes in glucose tolerance, at ZT10; and (5) increased adipose tissue mass.

The SCG provide sympathetic innervation to diverse areas including the hypothalamus, the pineal gland, cephalic blood vessels, the choroid plexus, the eye, the myocardium, the salivary and thyroid glands, and the carotid body (12, 40, 41). Removal of the superior cervical ganglia can cause loss of vasoconstriction control of brain and pituitary blood vessels (47), changes in cerebrospinal fluid production from the choroid plexus (48), and other central effects in response to partial sympathetic denervation (49). Moreover, abolition of the peripheral sympathetic innervation of the brain by SCGx is associated with several neuroendocrine changes in mammals, which include the disruption of water balance (37), and the alteration of normal photoperiodic control of reproduction (50, 51).

As previously mentioned, the mammalian circadian system is held in synchrony by the SCN through endocrine and autonomic outputs (52, 53). One of the mayor endocrine cues is the pineal hormone melatonin. Its synthesis and release is driven by the SCN through a multisynaptic pathway relaying in the SCG (54, 55). This interaction determines the rhythmic production of the hormone, whose day–night profile is modulated by daylength (23), encoding photoperiodic changes in the metabolic state (56).

Previous evidences have shown that SCGx decreases the secretion of melatonin and suppresses its rhythm (32, 33). The relationship between melatonin and the circadian control of metabolism has been demonstrated before. Pinealectomy and

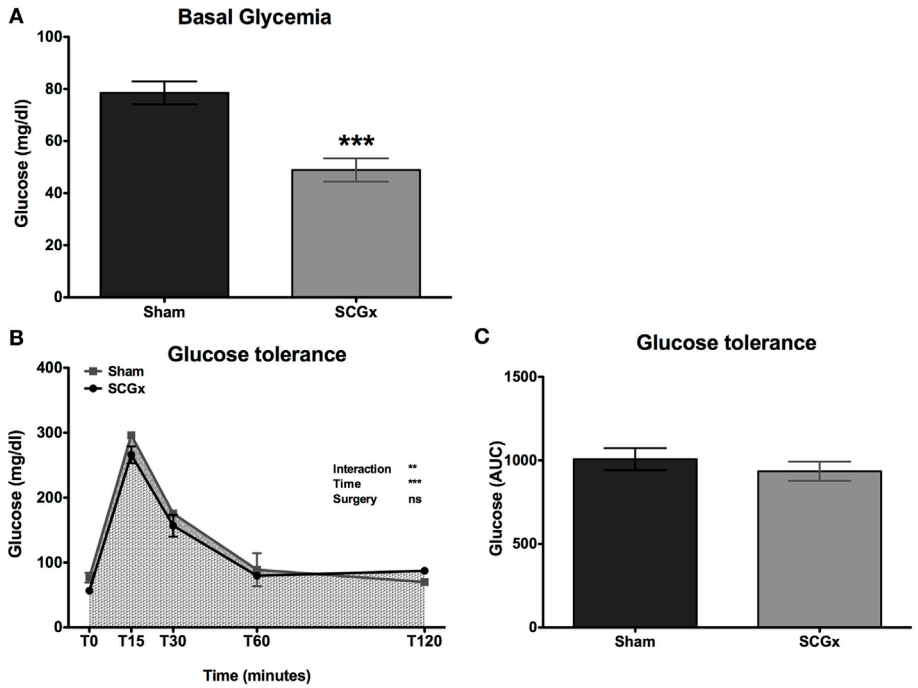

FIGURE 4 | SCGx animals exhibit lower basal levels of blood glucose, with normal glucose tolerance. Basal glucose levels at ZT10 were measured at week 10. We found lower levels in SCGx rats when compared with the sham ones [**(A)**; SCGx group: 48.89 ± 4.464 mg/dl; sham group: 78.50 ± 4.392 mg/dl; *t*-test: ***$p = 0.0003$, $t = 4.706$; $n = 9$ per group]. At week 13, a glucose tolerance test (GTT) was performed ($n = 5$ per group). Glycemia was measured before and 15, 30, 60, and 120 min after glucose administration. There were no differences in glycemia kinetics **(B)** or in the area under the curve of the GTT [**(C)**; SCGx group: 935 ± 57.04 mg/dl; sham group: 1,008 ± 65.66 mg/dl; *t*-test: $p = 0.214$, $t = 0.834$; $n = 5$ per group] between ganglionectomized and sham animals. Values are given as: mean ± SEM. Repeated measures two-way ANOVA results are shown at the right of the figure.

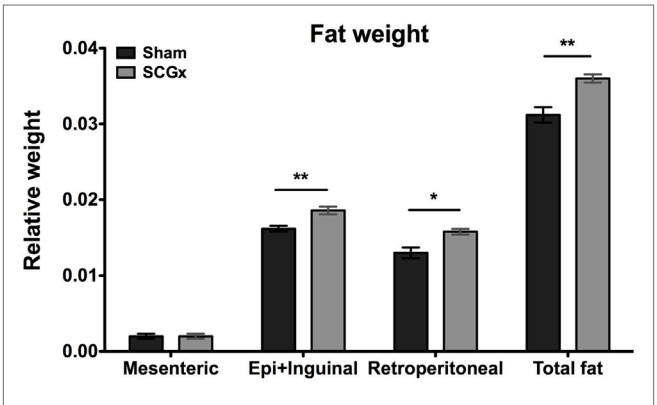

FIGURE 5 | Ganglionectomized rats exhibit higher levels of adipose tissue. Epididymal + inguinal (Epi + Inguinal), retroperitoneal, and mesenteric fat were collected at the end of week 13, and their weights were relativized to body weight for each animal. Fat tissue was significantly increased in SCGx compared with sham animals (for epididymal + inguinal fat, SCGx group: 0.0186 ± 0.0005; sham group: 0.0162 ± 0.0004; *t*-test: **$p = 0.0053$, $t = 3.795$; for retroperitoneal fat, SCGx group: 0.0154 ± 0.0002; sham group: 0.0130 ± 0.0007071; *t*-test: *$p = 0.0125$, $t = 3.207$, for mesenteric fat, SCGx group: 0.002 ± 0.0003; sham group: 0.002 ± 0.0003; *t*-test: $p = 1$, $t = 0$; and for total fat, as the collective weight of epididymal + inguinal, retroperitoneal, and mesenteric fat, SCGx group: 0.0362 ± 0.0007; sham group: 0.0318 ± 0.0011; *t*-test: **$p = 0.0032$, $t = 4.14$; values are given as: mean ± SEM; $n = 5$ per group).

melatonin administration or replacement (57, 58) significantly changes body weight, as well as glucose levels and its utilization in different tissues (59). In our model, we found decreased levels of glucose at ZT10, but a GTT showed no differences between SCGx and sham-operated animals. In contrast, pineal ablation in rats was shown to increase glucose levels (57).

Furthermore, leptin secretion is strongly associated with glucose and lipid metabolism, and has been shown to be modulated by melatonin (60). Moreover, the administration of melatonin in experiments conducted in rats and rabbits induced a reduction in body weight, serum lipids, adiposity, blood glucose, and insulin levels associated with the intake of a high-fat diet, suggesting a protective role of melatonin (20, 61, 62).

Taking into account our results, SCGx mimics the effect of pinealectomy on the neuroendocrine system only in some aspects, affecting several areas that include, but are not restricted to, the pineal gland. Although we cannot state that all SCGx-induced changes presented here are exerted *via* a suppressed pineal function, it is tempting to speculate that the diurnal timing of locomotion and feeding might be related to the lack of melatonin feedback to the circadian clock.

The importance of timed feeding and circadian physiology of metabolism has been extensively studied (63, 64). In this sense, an increased fat anabolism during daytime (i.e., the rest phase) due to food consumption at this time, may explain the lower levels of

blood glucose, and lead to increased adiposity in the SCGx group. Previous studies showed that animals fed during the light phase exhibit an increased body weight and food consumption, alterations in leptin, insulin, corticosterone, glucose, and free fatty acid levels in plasma, fat accumulation, liver steatosis, and metabolic syndrome (65–69). These alterations arise from a completely reversed clock-gene expression in the liver, kidney, heart, and pancreas, without affecting SCN function (9).

On the other hand, SCGx rats exhibit significantly augmented serum corticosterone and adreno-corticotropin hormone levels, and a suppression of their rhythm (35, 70). Glucocorticoids (GCs) can stimulate the *de novo* synthesis of lipids (71). It has been reported that rats exposed to long-term treatment with GCs show a slower body weight gain, reduced food intake, and increased epididymal fat mass (72). Some of the effects reported here might be related to alterations in GC turnover that, in turn, could lead to the increase in FE and lipid accumulation. Indeed, the role of the sympathetic neuro-adipose connections in the regulation of lipolysis and body weight has been studied before (73). Sympathetic denervation leads to an increase in adipose tissue, while nerve stimulation results in fatty acid release, and sympathetic or ganglionic blockade inhibits the mobilization of lipids (74–76). Leptin production is also under the control of the sympathetic system (77), with participation of the SCG (78).

Regarding light synchronization, it has been demonstrated that pinealectomy accelerates the re-entrainment of rats to the new LD schedule (79–82). Moreover, in rodents, melatonin administration synchronizes free-running rhythm and accelerates re-entrainment after phase shifts of the LD cycles (83–85), and reinforces entrainment to shortened 22 h LD cycles in both SCGx and pinealectomized rats (86). We studied the effect of SCGx on the entrainment to the LD cycle and found no significant differences on period, phase angle, or total locomotor activity between SCGx and sham-operated animals. However, SCGx rats showed significant differences in activity during daytime (lights on). In addition, food intake analysis evidenced augmented food consumption during daytime, which may correlate with the activity bouts under the light phase.

Also, it was previously observed that bilateral removal of the SCG delays the synchronization of feeding rhythms with a newly imposed diurnal lighting regimen, but, again, the response to pinealectomy was different (87). In fact, the elimination of pineal rhythmicity cannot account for all of the effects of SCGx on photic entrainment of feeding and locomotor activity rhythms. It can be suggested that SCGx alters the sympathetic innervation of hypothalamic structures implicated in the neural control of feeding, affecting the diurnal rhythm of food intake.

Rhythms in metabolism are orchestrated by the SCN and other inputs from different areas of the hypothalamus, like the mediobasal region, which plays a significant role in metabolic homeostasis (88–93). Other areas, like the dorsomedial hypothalamus, have an important role as a component of the SCN-independent food-entrainable oscillator (94–97). The circadian regulation of body weight depends on the integration of multiple signals of several hypothalamic areas, including the SCN, the arcuate nucleus, the ventromedial hypothalamic nucleus, and the paraventricular nucleus, that control appetite and food intake, deposition of fat, and energy expenditure (11, 53, 98). Melatonin not only couples circadian cues to many body functions but might also be a key player in the regulation of basal metabolic rate (99), independently of other SCG-innervated territories, such as the hypothalamus. In this sense, the results shown in this work provide evidence suggesting that SCGx may be affecting metabolism by changing the feeding pattern (i.e., increasing feeding during daytime), acting over peripheral clocks without affecting the SCN.

In conclusion, these findings provide insights into the metabolic and diurnal rhythms of ganglionectomized rats. SCGx is not only a good model to study the circadian clock influence on neuroendocrine functions, but a reliable approach to investigate the relationship between the circadian system and metabolism, as well as the role of the SCG innervation in the synchronization of the master circadian clock with the peripheral clocks, especially the ones that drive metabolic variables.

ETHICS STATEMENT

All animal procedures were approved by the Institutional Animal Care and Use Committee at the School of Medicine, National University of Cuyo, Mendoza, Argentina (Protocol ID 9/2012) and were conducted in accordance with the National Institutes of Health's Guide for Care and Use of Laboratory Animals and the Animal Research: Reporting *In Vivo* Experiments (ARRIVE) Guidelines.

AUTHOR CONTRIBUTIONS

MG and SP performed experiments for the paper; MF, MG, and SP analyzed data; MF, DG, EM, and SP wrote the manuscript; DG and EM provided reagents and funding for the study.

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The Cost of Activity during the Rest Phase: Animal Models and Theoretical Perspectives

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For humans, activity during the night is correlated with multiple pathologies that may reflect a lack of harmony among components of the circadian system; however, it remains difficult to identify causal links between nocturnal activity and different pathologies based on the data available from epidemiological studies. Animal models that use forced activity or timed sleep deprivation provide evidence of circadian disruptions that may be at the core of the health risks faced by human night and shift workers. One valuable insight from that work is the importance of changes in the distribution of food intake as a cause of metabolic imbalances associated with activity during the natural rest phase. Limitations of those models stem from the use of only nocturnal laboratory rodents and the fact that they do not replicate situations in which humans engage in work with high cognitive demands or engage voluntarily in nocturnal activity (i.e., human eveningness). Temporal niche switches by rodents have been observed in the wild and interpreted as adaptive responses to energetic challenges, but possible negative outcomes, similar to those associated with human eveningness, have not been systematically studied. Species in which a proportion of animals shows a switch from a day-active to a night-active (e.g., grass rats) when given access to running wheels provide a unique opportunity to model human eveningness in a diurnal rodent. In particular, the mosaic of phases of brain oscillators in night-active grass rats may provide clues about the circadian challenges faced by humans who show voluntary nocturnal wakefulness.

Keywords: shift work, circadian rhythms, temporal niche, eveningness, grass rats

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HUMAN NIGHT WORK

Our contemporary global society has created demands that require many of us to be active during the natural rest phase of our species, the night. This is exemplified by increasing number of individuals who work nights and thus are awake and engaged with the environment at least for part of the time normally dominated by human sleep. There is a rich, albeit mostly correlational, literature linking night and shift work to a multitude of pathologies including higher risk of cancer (1), metabolic syndrome (2), hypertension (3), cognitive deficits (4), and female infertility (5), among other health and behavioral problems. Many of these negative outcomes may stem from a lack of harmony among different components of the circadian system (6).

The circadian system consists of a principal oscillator located in the hypothalamic suprachiasmatic nucleus (SCN) (7, 8) that entrains to the light–dark cycle *via* direct retinal projections from melanopsin-containing retinal ganglion cells (9). Outputs of the SCN serve to synchronize a multitude of extra-SCN oscillators in the brain and in the peripheral organs (10). Night or shift

work is associated with exposure to environmental influences that challenge the temporal regulation of many behavioral and physiological functions. When undisturbed, the resulting daily rhythms are kept at optimal phase relations among themselves and synchronized (entrained) to the 24-h day–night cycle by the circadian system. Some of the challenges faced by the circadian system of night workers include exposure to nocturnal light, activity during the natural sleep period, and food consumption during the rest phase of the cycle. It is very likely that many of the health and behavioral problems of night or shift workers stem from circadian disruptions resulting from external (light) and internal (metabolic signals; release of neurotransmitters) stimuli that interfere with, or override, circadian signals emanating from the SCN (6, 11). However, it is difficult to draw conclusions about causality from epidemiological studies with humans. A number of animal models have been developed to circumvent those limitations.

ANIMAL MODELS OF NIGHT WORK: INSIGHTS AND CHALLENGES

Animal models of shift work have used almost exclusively laboratory rodents (see Ref. (12) for a review) that are forced to be active during their rest phase by placing them in rotating wheels for several hours every day (13–15) or alternatively, by keeping them awake during the rest phase using gentle stimulation whenever the animal gives signs of falling sleep (16). Forced activity for 8 h/day during the normal rest phase of laboratory rats, sustained for five consecutive days per week, results in increased abdominal fat accumulation and the display of several indicators of metabolic syndrome, including impaired glucose tolerance (13). These animals also shift their food intake to the light period (13, 14) and show reduced general activity as well as a reduced activity-rhythm amplitude on days off from the forced activity regime (17). Interestingly, providing food only during the normal active (dark) phase prevents many of the effects of forced activity during the light phase (15), and at least in studies using rats, restricting feeding to the light phase in otherwise undisturbed animals mimics the effects of the forced activity manipulation (15). A different study that used a forced activity paradigm similar to that of experiments reporting increases in body weight and adiposity, surprisingly found a reduced body weight in the shift-working rats (18). Differences in housing conditions or stress level of the animals could be responsible for the different outcomes, but of note is the observation that in these shift-work animals that lost weight, the amount and distribution of activity on days off did not differ from those of control animals not exposed to the forced activity regime. Thus, both changes in energy expenditure and the emergence of day-time feeding appear to contribute to the metabolic effects of forced activity during the normal rest phase of nocturnal laboratory rats.

There is ample evidence that sleep deprivation *per se* can negatively affect metabolism and energy balance (19, 20). Experiments in which chronic timed sleep restriction, with opportunity for sleep recovery within the 24-h period, are most relevant as animal models of shift work. Several studies using mice have reported metabolic deficits including abnormal glucose and lipid metabolism when the animals are deprived of sleep during the first 6 h of the night for two blocks of 5 days separated by 2 days of *ad lib* sleep (16, 21). Restricting feeding to the night prevented these metabolic effects (16); however, different from what was reported for rats, restricting feeding to the light phase without sleep restriction did not result in metabolic anomalies (16). Also different from most of the forced activity work with rats, sleep restriction did not affect body weight in mice (16).

Although studies using forced timed activity or timed sleep restriction provide causal links between the human experience of night shift work and circadian, metabolic, and energy disruptions reported for these workers, they have some clear limitations. First, the use of nocturnal laboratory rodents poses questions about how generalizable the findings are to diurnal species such as ours. Also, even within nocturnal rodents, the limited data hint at possible differences between laboratory rats and mice (12), some of which likely stem from differences in body size and how that affects responses to metabolic challenges. Using a wider range of species, including day-active (DA) ones, would add significantly to the value of animal models of human shift work that use forced activity or timed sleep restriction.

HUMAN EVENINGNESS

Although humans are clearly diurnal, many of us become active during our normal rest phase, the night. This change in phase preference is not limited to those engaged in shift or night work. For example, many young adults shift their activity phase and display what is known as "eveningness," which involves being active during a large proportion of the night. There is compelling evidence that in humans, voluntary shifts to a nocturnal activity profile result in substantial negative outcomes, including eating disorders (22), diabetes, and metabolic syndrome (23). Further, eating at the inappropriate phase has been linked to obesity in humans and animals (24, 25). These problems have clear negative impacts on the human capital of society. The animal models that simulate human shift work using forced activity or timed sleep deprivation (see above) are not ideal to study the consequences of the apparent voluntary temporal niche switch of human eveningness. For example, the expression of Fos protein in the brain of grass rats (*Arvicanthis niloticus*) is remarkably different if the animals are forcibly kept awake at night compared to when they show unconstrained night wakefulness (26, 27) (more about this animal model below). Interestingly, shifts in the balance between day/night activity, like those seen when eveningness emerges in teenagers, have been reported for other mammalian species, both in the field and in the laboratory (28–30) and thus represent potential models for understanding the causal links between human voluntary nocturnal activity and the negative outcomes associated with it.

MAMMALIAN TEMPORAL NICHE SWITCHES

Mice, which are strictly nocturnal in standard laboratory conditions, can switch to diurnality when observed under more natural conditions for extended periods of time (31). An influential

hypothesis to account for these switches by mice postulates that they occur in response to energy challenges (32). Specifically, this perspective suggests that situations in which animals experience negative energy balance favor the display of a diurnal phenotype (33). Laboratory work testing this hypothesis has used a "work for food" paradigm in which mice get food only if they run in a wheel, thus emulating the foraging demands of the wild (34). The workload to obtain a particular amount of food is manipulated to resemble environments with different densities of resources. Under those conditions, increasing the workload induces a phase advance of the activity (or work) rhythm, such that normally nocturnal mice show predominantly diurnal activity (32). Reduced ambient temperature, while kept with *ad lib* food availability, also induces a shift to diurnal activity, and enhanced workload and low ambient temperature challenges have additive effects with respect to this temporal niche switching in mice (35). The change in the phase preference for the display of activity when mice experience a negative energy balance is not accompanied by a shift in the phase of the SCN oscillator, but peripheral oscillators in the liver and adrenal gland show a phase that more closely resembles that of diurnal mammals (35).

The thermoenergetic hypothesis advanced by Hut and coworkers (30, 32) suggests that diurnality emerges in rodents to reduce energy needs, since days are warmer than nights. While this hypothesis promotes the adaptive value (32) of the temporal niche switch (31), there may also be costs, as activity during the natural rest phase of the mice was accompanied by changes in synchrony of internal rhythms. Specifically, peripheral oscillators shifted their phase angles with respect to the SCN and likely with respect to the melatonin rhythm, which remains nocturnal in other models of temporal niche switching (29, 36).

TEMPORAL NICHE SWITCHES IN DIURNAL SPECIES: THE GRASS RAT AS A MODEL

There are examples of species that are diurnal in the field, but that switch to a nocturnal activity profile in the laboratory (37). Based on the thermoenergetic hypothesis, these observations suggests that these animals may be exposed to energetic challenges in the wild that favor diurnality and that do not exist in the laboratory. More interesting from the perspective of developing a model for human eveningness are species, e.g., Nile grass rats (28) and *Octodon degus* (29), sometimes referred to as dual-phasing animals (29), which are diurnal in the field, and also under standard laboratory conditions, but can show either diurnal or nocturnal phenotype when given access to running wheels (28).

For over 20 years, our group has been developing the grass rat as a diurnal mammalian animal model to study the circadian system (38). To go with their diurnal life style, grass rats feature an abundance of retinal cones (39) and an optic tectum that, relative to body size, is four times the volume of that of laboratory rats (40). Although the phase of the SCN oscillator with respect to the light–dark cycle is similar to that of nocturnal rodents (41), brain and peripheral extra-SCN oscillators, monitored using the pattern of expression of clock gene products, are 180° out of phase in reference to those of nocturnal rodents (41, 42). Interestingly, and pertinent to the discussion of human eveningness, with access to running wheels, some grass rats switch to a predominantly nocturnal display of wheel-running activity. There is evidence of a "compromise" in night-active (NA) grass rats between diurnal tendencies and the display of activity during the normal rest phase of the species. Thus, NA grass rats keep several features of their diurnal profile including the display of frequent sleep episodes and low body temperatures late in the night (28, 43) like those seen in DA animals. This interval of sleep is followed by a pre-dawn peak of activity common to both chronotypes (28). Moreover, even though the NA animals sleep more during the day to recover from the sleep debt created by their nocturnal activity, their day-time sleep is fragmented with relatively short sleep-bout lengths (43).

The retention of some diurnal tendencies in NA grass rats may be due to the diverse responses of extra-SCN brain oscillators to the switch to nocturnal activity. Perhaps not surprisingly, the adoption of a NA profile does not affect the phase of clock gene [PERIOD 1 and 2 (PER1/2)] rhythms in the SCN (41) or the nocturnal production of melatonin (36). However, most extra-SCN brain regions that express rhythms in PER 1/2 display a complete reversal of the time of peak expression when grass rats become NA, thus making the circadian profile of NA grass rats similar to that of nocturnal rodents (41). But, the reversal is not universal, again revealing features that are retained by NA grass rats from their antecedent diurnal profile. Outside the hypothalamus, the central amygdala shows a very similar pattern in NA and DA grass rats that contrasts with what is seen in the rest of the extrahypothalamic brain (41). In the extra-SCN hypothalamus (**Figure 1**), the paraventricular nucleus (PVN) shows a phase reversal in NA grass rats (36), but the ventral subparaventricular zone remains fixed and similar in phase to that of DA animals (41). Most relevant for understanding the sleep fragmentation of NA grass rats during the day is the response of the hypothalamic histaminergic nuclei [i.e., the dorsal and ventral tuberomammillary nuclei (dTMN and vTMN), respectively] to the switch to nocturnality in these animals. Like the PVN, the oscillator of the vTMN of NA grass rats shows a phase reversal, but in sharp contrast that of the dTMN, it retains the phase typical of DA animals (44). These

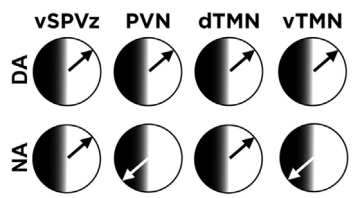

FIGURE 1 | Mosaic of phases of the PERIOD 1 rhythm in hypothalamic extra-suprachiasmatic nucleus (SCN) regions: The phase of the rhythm is similar between day- and night-active (DA and NA, respectively) grass rats for the ventral subparaventricular zone (vZPVz) and the dorsal tuberomammillary nucleus (dTMN). In contrast and similar to most extra-SCN oscillators outside the hypothalamus, the rhythm is 180° out of phase between DA and NA grass rats in the paraventricular nucleus (PVN) and in the ventral tuberomammillary nucleus (vTMN). See text for references.

results suggest that the fragmented recovery sleep of NA grass rats results from a mismatch between rhythms in components of the histaminergic arousal system of the tuberomammillary nuclei (45) and the rest/activity cycle. The work with NA and DA grass rats suggests that although temporal phenotypes are flexible and may change in service of energy homeostasis, those changes are not likely to be complete. The compromises between diurnal and nocturnal features of NA grass rats most likely reflect an internal circadian desynchrony that may be an additional cost paid by humans who voluntarily adopt a nocturnal profile. It would be instructive to determine if temporal niche switches in the wild (31) are also associated with similar circadian costs to accompany risks due to exposure to different competitors and/or predators for whom they lack preparation (32).

FUTURE CONSIDERATIONS

Available models using constrained (forced activity and timed sleep deprivation) or unconstrained (wheel running availability) activity during the natural rest phase of a species do not incorporate the effect of the type of engagement with the environment that goes on during the new active phase. In particular, these models do not replicate instances of human nocturnal activity with significant attentional and cognitive demands, e.g., nurses in hospitals or technicians working at nuclear plants. Experiments in which nocturnal laboratory rats are trained and tested during the day on tasks that demand enhanced attentional performance provide evidence of clear circadian effects that include a shift to a predominantly diurnal chronotype with salient anticipatory activity that persists for days after the training is discontinued (46, 47). Activities with low cognitive demands, such as spatial learning or training using operant tasks with low attentional requirements, do not substantially affect circadian activity; neither do daily handling or restriction of water availability to the light phase. (46, 47). The circadian effects of high cognitive-demand tasks are likely mediated by cholinergic inputs to the SCN (11), which may affect the nucleus in ways different from other forms of temporal niche switches. Determining the effects on metabolism and energy balance of different activities during the rest phase, with varied cognitive demands, would add significantly to the value of animal models of human night work or eveningness. Also important to consider when assessing the cost of constrained or unconstrained switches in temporal niche is the influence of circadian phase on cognitive competence. Thus, just like cognitive effort can affect the temporal distribution of activity, time of day can affect the acquisition (46) or retention of learned tasks (46, 48). How cognitive functions may be affected by changes in the preferred phase for the display of activity in different animal models represents an important area to explore with respect to the cost of activity during the night, both in night work and in human eveningness.

ETHICS STATEMENT

The work from our group discussed here was carried out in accordance with the recommendations of the Michigan State University All University Committee on Animal Use and Care, and the National Institute of Health guide for the Care and Use of Laboratory Animals. All protocols were approved by the Michigan State University All University Committee on Animal Use and Care.

AUTHOR CONTRIBUTIONS

The ideas presented here stem from discussions among the three authors, AN wrote the first draft and LS and LY edited and expanded the initial version.

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MINI REVIEW
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Circadian and Metabolic Perspectives in the Role Played by NADPH in Cancer

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Physiological activity in healthy conditions requires a coordinated interaction between the molecular circadian clock and the network of biochemical pathways. An important metabolic parameter in the interface between these two entities is the redox state. Among the redox coenzymes that regulate the fluxes of enzymatic reactions is the $NADP^+$/NADPH pair. Indeed, the main biosynthetic pathways need NADPH to serve as an electron donor for cellular anabolic transformations. The existence of a metabolic circadian clock is well established, and it was first identified in mammalian red blood cells. The metabolic circadian clock is independent of transcriptional activity and is sustained by the enzymatic complex peroxiredoxin/thioredoxin/NADPH. This complex shows 24-h redox fluctuations metabolizing H_2O_2 in various tissues and species (fungi, insects, and mammals). Although this NADPH-sensitive metabolic clock is autonomous in erythrocytes that lack a nucleus, it functions in concert with the transcriptional circadian clock in other cell types to accomplish the task of timing cellular physiology. During carcinogenesis, circadian alterations influence cell cycle onset and promote tumoral growth. These alterations also deregulate cellular energetics through a process known as aerobic glycolysis, or the Warburg effect. The Warburg effect is a typical response of cancer cells in which the metabolism turns into glycolysis even in the presence of functional mitochondria. This alteration has been interpreted as a cellular strategy to increase biomass during cancer, and one of its main factors is the availability of NADPH. This minireview explores the potential role of NADPH as a circadian and cancer-promoting metabolite.

Keywords: circadian, redox, NADPH, cancer, metabolism, Warburg effect

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METABOLIC AND TRANSCRIPTIONAL CLOCKS: REDOX HOMEOSTASIS AND CIRCADIAN RHYTHMS

The notion of divergent evolution in various timing systems in several model organisms is well established. For instance, cyanophytes, fungi, insects, and mammals show a set of clock genes that fluctuate daily but without a relationship between their DNA sequences. However, it was rapidly accepted that clock genes control circadian physiology through a network of positive and negative transcriptional loops [(1) and reference within]. Briefly, the positive elements in the primary feedback loop include CLOCK and BMAL1. CLOCK and BMAL1 heterodimerize and initiate transcription of target genes containing E-box *cis*-regulatory enhancer sequences, including PER and CRY. Negative feedback is achieved by PER:CRY heterodimers that translocate back to the nucleus to

repress their own transcription by acting on the CLOCK:BMAL1 complex. Another regulatory loop is induced by CLOCK:BMAL1 heterodimers activating the transcription of retinoic acid-related orphan nuclear receptors, such as *Rev-erbα* and *Rorα*.

Nevertheless, many reports in the past 20 years question the robustness of the transcriptional circadian network as a unique form to sustain the biological measurement of time. It is known now that circadian oscillators are a complex system of transcriptional, posttranscriptional (phosphorylation, sumoylation, and acetylation), and metabolic integrated networks. Moreover, clock gene transcription is sensitive to the metabolic environment, which closely depends on the redox state. Interestingly, both nucleated and anucleated cells display self-sustained redox cycles that influence cellular physiology (2). Such is the case of suprachiasmatic neurons, whose excitability is guided by redox oscillation in a transcriptionally independent manner (2). In this regard, the existence of non-transcriptional rhythms was conclusively demonstrated by Dr. Akhilesh B. Reddy's laboratory while studying human red blood cells (without nuclei in their mature form) (3). They reported the presence of a metabolic circadian oscillator based on the redox cycle of peroxiredoxin enzymes (4). Peroxiredoxins belong to a family of antioxidant enzymes whose main function is the catabolic degradation of hydrogen peroxide by controlling its levels and the associated signaling events (5). Peroxiredoxins are localized in several subcellular organelles. The catalytic mechanisms of peroxiredoxins involve the oxidation of a "reactive" cysteine residue in the active site to sulfenic acid (Cys-SOH), which then forms a disulfide bond with another non-catalytic cysteine residue. In some isoforms, such as 2-Cys peroxiredoxins, there is further oxidation to sulfonic and sulfonic acid forms. Eventually, the thioredoxin system reduces the disulfide bond using NADPH as a cofactor. These redox transformations have rapid turnover, resulting in low levels of intracellular hydrogen peroxide.

O'Neill and Reddy (3) reported circadian fluctuations of peroxiredoxin redox forms in erythrocytes, accompanied by daily variations of NADPH and NADH, and oxidized hemoglobin. Similar NADPH-dependent peroxiredoxin oscillatory systems have been detected in various organisms, including archaeal bacteria. Interestingly, such circadian rhythms are independent of canonical clock genes (6). At present, chronobiologists sustain that the metabolic/redox clock evolved following the Great Oxidation Event, when the Earth's atmosphere became rich in oxygen. According to further evolutionary research, timing systems incorporated diverse circadian genes to reach the current timekeeping mechanism, which exhibits a dual modulation between the transcriptional circadian clock and the redox clock. The redox clock is represented by the NADPH-dependent peroxiredoxin oscillator and by the metabolic reactions mentioned in the first section of this review.

NADPH AS AN ANABOLIC COENZYME

Redox reactions involve a transfer of electrons (even naked or protonated electrons) between molecules. Thus, redox regulation of metabolism is carried out by conjugated redox pairs, with some molecules acting as donors (reducers) and others as acceptors (oxidizers). Thiols, such as glutathione (GSH, reduced form; GSSG, oxidized form), and coenzymes, such as flavin and nicotinamide adenine dinucleotides ($FADH_2/FAD^+$, $FMNH_2/FMN^+$, $NADH/NAD^+$, and $NADPH/NADP^+$), are key participants in metabolic regulation, as they modulate proteins that contain active sulfhydryl groups (enzymes, receptors, cytoskeletal proteins, among others), and the enzymatic activity of various dehydrogenases (7) and NAD^+-dependent enzymes (8). Redox couples in an oxidation–reduction reaction are characterized by a standard redox potential (E'_o, units in volts). E'_o is a measure of the affinity of a redox pair for electrons; negative values mean suitable electron donors, whereas positive values indicate adequate electron acceptors. The E'_o for redox coenzymes and glutathione is in the range of −0.32 V for $NADH/NAD^+$ and $NADPH/NADP^+$, −0.23 V for GSH/GSSG, and −0.22 V for $FADH_2/FAD^+$ and $FMNH_2/FMN^+$. The difference between the redox potentials of two redox pairs is a measure of the driving force for the net electron transfer and is related to the change in Gibbs free energy (ΔG). For example, ΔG under physiological conditions for the electron transfer from NAD(P)H to O_2 is −52.6 kcal/mol (9). Under physiological conditions, the actual oxidation-reduction potential depends on the levels and ratio of the concentrations of the individual members of the redox couple, as well as on the prevalent pH. Each redox pair shows a defined ratio between its elements (reduced/oxidized) according to their subcellular compartments. The complete set of redox pairs makes up the global cellular redox state, a parameter that dictates the unique pattern of electron flux for any cell system.

In particular, the concentrations of $NADP^+$ and NADPH within the cell are lower than those of NAD^+ and NADH (submillimolar range), and under normal metabolic conditions the NADP pool is predominantly in its reduced form. NADPH primarily acts as an electron donor in anabolic or synthetic reactions (10). To accomplish this task, the pool is maintained in its reduced form (the $NADPH/NADP^+$ ratio is kept high) (11). NADPH plays several biological roles [(12) and references within]. It is a coenzyme for glutathione reductase and transferase reactions, and it reactivates thioredoxin reductase and catalase as part of the antioxidant defense system. In addition, NADPH acts as an electron donor for the reductive formation of lipid molecules (cholesterol and fatty acids) and nucleic acids. It is a cofactor for O_2^- generation during NADPH oxidase activity and a protector of mitochondrial DNA integrity. NADPH also acts as a nuclear modulator of gene expression by promoting redox signaling within the nucleus. Finally, it has been shown that NADPH, as a product of the pentose phosphate pathway (PPP), is able to modulate circadian rhythms by extending or shortening the 24-h fluctuations in human cells, mouse tissues, and fruit flies (13).

CIRCADIAN DISRUPTION AND CANCER

Misalignment of the circadian clock with the environment seems to lead to various health alterations such as metabolic diseases and some types of cancer (14). In humans, epidemiological evidence of the adverse association between shift work and disrupted sleep/wake schedules in healthy conditions supports that knowledge (15). In animal models, disruption of circadian cycles by exposure

to light at night dramatically accelerates tumorigenesis and tumor growth (16, 17). In fact, entrainment by restricted feeding inhibits tumor growth in mice with pancreatic adenocarcinoma, with no alteration of the arrhythmic clock gene expression in the tumor in contrast to the synchronization effect in the liver, irrespective to calorie intake. However, genes involved in cell cycle and metabolism were upregulated or downregulated, depending on the circadian time (18). This evidence supports the fact that, aside from the transcriptional regulation of circadian time, other levels of regulation are implicated in the alteration of healthy homeostasis. The fact that resynchronization by restricted feeding delays tumor development highlights the role of metabolism and redox status in tumor growth and progression (19).

Recent studies have shown that peroxiredoxins contribute significantly to the promotion and progression of cancer. Members of the peroxiredoxin family are seemingly overexpressed in several tumor tissues (20–22), and they promote cell proliferation and tumorigenesis through epithelial–mesenchymal transition (23, 24). Hyperoxidation of peroxiredoxins by hydrogen peroxide induces their inactivation, and the sulfiredoxin reductive action reactivates them. In fact, a hyperoxidized form of peroxiredoxin III and sulfiredoxin is in antiphase circadian oscillation in healthy cells (25). Overexpression of peroxiredoxins with a decrease in sulfiredoxin in some neoplasias correlates with poor prognosis (26). However, it is not known if overexpression of peroxiredoxins is due to changes in a rhythmic profile of activation by NADPH that contribute to tumor development in a protective redox role of peroxiredoxins.

On the other hand, Myc family oncoproteins (c-Myc, N-Myc, and L-Myc) regulate the transcription of several genes, some of them implicated in the shuttling of glucose to activate the PPP, resulting in the generation of large amounts of NADPH and the biosynthesis of various macromolecules (27). In addition, it has been demonstrated that Myc disrupts the circadian molecular clock. Specifically, it activates the Bmal1–Clock heterodimer, thus disrupting circadian metabolic oscillation (28). This effect occurs through constitutive activation of Rev-erbα, the expression of which could be related to poor clinical outcome in human neuroblastoma (28). These observations highlight the interplay between redox state and circadian clock in cancerous processes.

TOWARD A CIRCADIAN CHARACTERIZATION OF THE WARBURG EFFECT?

The onset and development of carcinogenic growth involves a multistep process characterized by a set of biological features known as hallmarks of cancer. The initial list of hallmarks encompassed characteristics such as replicative immortality, angiogenesis, and metastasis (29). Recently, new cellular and biochemical parameters were incorporated into the list of hallmarks (30). One of them, the cancer-associated reprogramming of energy metabolism, also known as the Warburg effect, is characterized by predominant glycolytic activity despite aerobic conditions and functional mitochondria. In cancer, the Warburg effect is related to an upregulation of WNT/β-catenin signaling and a concomitant downregulation of the PPARγ-associated actions (31). The rationale of the Warburg effect in oncology is that cancerous cells are programmed for high cellular proliferation; hence, the continuous entry into the cell cycle involves a constant input of new molecules for the synthesis of biological membranes, genetic material, and all the cellular elements needed for the newly formed tumoral cells (32). This anabolic commitment is fulfilled by an upgraded availability of NADPH during neoplastic growth, since this coenzyme is required by the reductive biosynthetic reactions of a duplicating cell (33). NADPH can be generated by the activities of (1) glucose-6-phosphate dehydrogenase and 6-gluconate phosphate dehydrogenase (redox and decarboxylating steps in the PPP); (2) $NADP^+$-dependent isocitrate dehydrogenase (mitochondrial isozyme that provides NADPH for antioxidant activity); (3) $NADP^+$-dependent malic enzymes (important cytosolic enzymes during the β-reduction reactions); and (4) transhydrogenase (mitochondrial enzyme that is also part of the antioxidant defense mechanisms). Along with higher NADPH formation, the anabolic response associated with the Warburg effect requires the enzymatic activity of citrate lyase, which provides carbon skeletons for fatty acid synthesis (12).

The relationship between circadian rhythms and the Warburg effect has been scarcely explored (from 1,911 entries in PubMed in December 2017 with the keyword "Warburg effect," only 9 are related to circadian rhythms). For example, in 2017, Cao and Wang reported on the potential connection between circadian responses, providing examples of metabolic reprogramming and offering interesting insights into the onset and development of tumors (34). Four other reports specifically explored the effect of light exposure and the concomitant increase in melatonin as cancer suppressors by disrupting the Warburg effect in human breast and prostate cancer xenografts, as well as in leiomyosarcoma (35–37). Dr. Vallee's group has made interesting thermodynamic considerations regarding the equilibrium between WNT/β-catenin and PPARγ signaling in fibrosis and glioma; the first favors the Warburg effect by reprogramming cellular energy metabolism, and the second promotes a reduction in circadian physiology upon its inactivation (31, 38, 39).

A more formal approach to analyze the importance of 24-h fluctuations in the Warburg effect and the availability of NADPH in cancer needs to consider that redox influence of NADPH and NADH is completely dissimilar (40). Given that the former is a coenzyme for anabolic reactions and the latter for catabolic reactions, the metabolic context for the action of each one is necessarily different; hence, reports that refer to both coenzymes as NAD(P)H and claim a regulatory redox event are conceptually mistaken. Another common mistake is to consider that the cellular redox state can be inferred from the synthesis, presence, or activity of nicotinamide phosphoribosyltransferase. This enzyme allows the formation of nicotinamide adenine dinucleotides; however, the redox state is necessarily defined by the ratio of the redox couple (reduced/oxidized) (40).

Indirectly, the reduced role of PPARγ in cancerous cells (31) could be associated with the damped circadian rhythms mentioned in the previous section. Specifically, there are few reports on circadian regulation of the enzymes responsible for NADPH availability during the Warburg effect, and almost none regarding

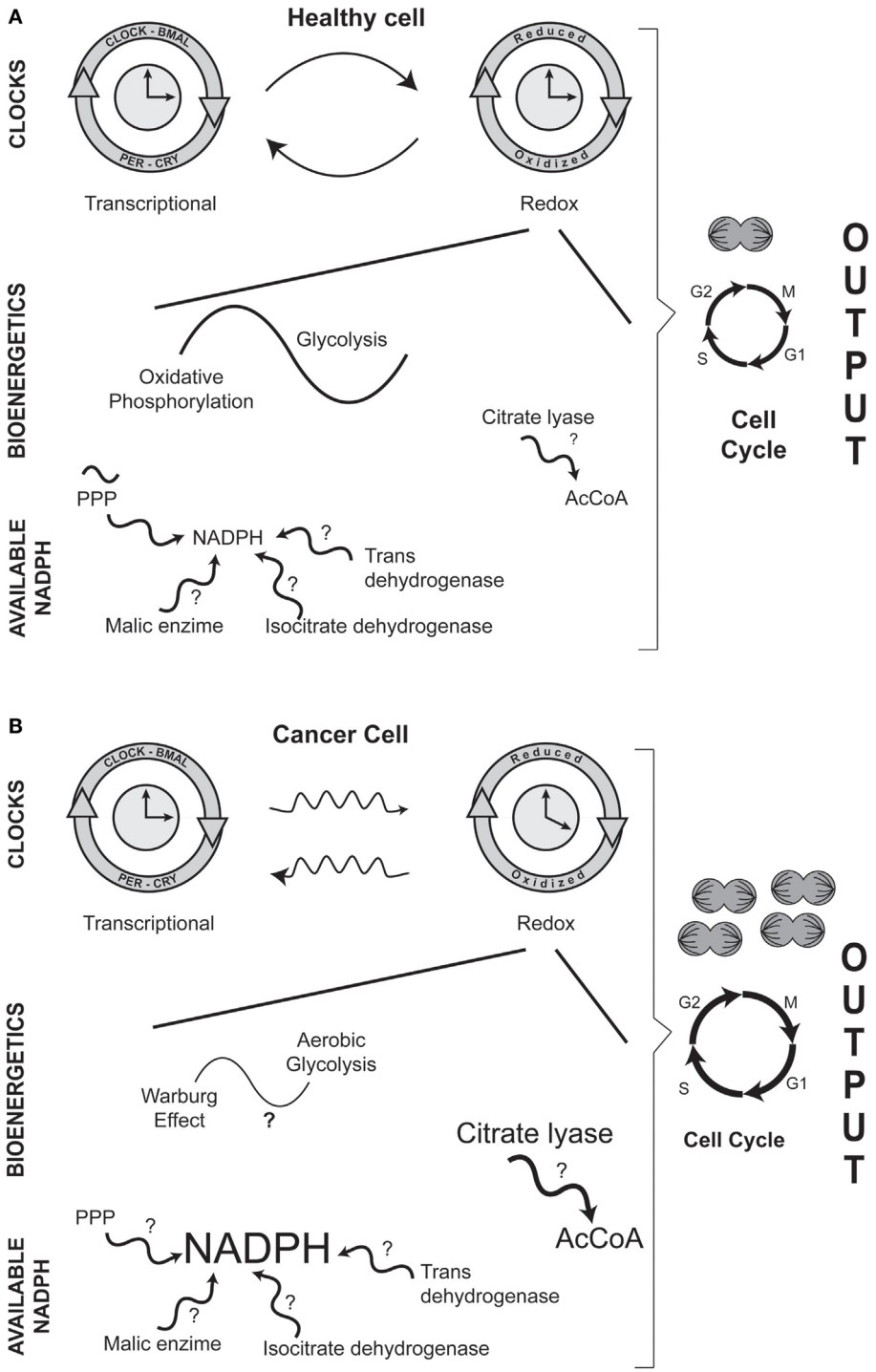

FIGURE 1 | Comparison between healthy **(A)** and cancer **(B)** cells: circadian and metabolic differences in NADPH cellular handling. In both entities, there is a dual interaction between the transcriptional and the redox circadian clocks. However, in cancerous cells, the daily variations in biochemical and molecular phenomena are disrupted. A major difference between a normal and a transformed cancer cell is the bioenergetic adaptation in which the main mitochondrial activity is no longer ATP production (glycolysis/oxidative phosphorylation en healthy cells) but the constant input of metabolic intermediaries (Warburg effect, or aerobic glycolysis, in cancerous cells) that are needed for cellular duplication. A distinctive biochemical characteristic in carcinogenesis is the increased availability of the anabolic coenzyme, NADPH; NADPH can be potentially be formed by various enzymatic reactions. Regarding the higher levels of NADPH, the production of acetyl-CoA (AcCoA) is also increased by the activity of the cytoplasmic enzyme citrate lyase. In the figure, events or enzymatic reactions that show circadian rhythmicity are depicted by the symbol ~; for example, circadian rhythmicity of the pentose phosphate pathway (PPP) in normal cells. Question marks (**?**) indicate events or enzymatic reactions that have not been characterized as showing putative daily rhythmicity in either healthy or cancer cells.

their daily rhythms in cancerous cells or tumors. As an exception, the activity of the PPP, one of the major generators of NADPH, has been recognized as an element of circadian physiology in various cell systems (13).

SUMMARY

The conceptual message of this minireview is outlined in **Figure 1**. (1) Carcinogenesis involves altered circadian physiology and a modified relation between transcriptional and redox clocks; (2) the main role of mitochondria is no longer ATP synthesis; (3) bioenergetic is transformed from glycolysis/oxidative phosphorylation into aerobic glycolysis (Warburg effect); (4) the metabolic networks are oriented toward anabolic reactions; (5) NADPH is more available to ensure biosynthetic reactions; (6) cellular replicative function is enhanced.

Although daily variations in redox mechanisms are well established in healthy cells, some reactions associated with NADPH metabolism have not been well characterized in terms of 24-h rhythmicity (question marks in **Figure 1**). The situation is even more accentuated in cancerous cells, since few reports have approached the onset and development of NADPH availability and the Warburg effect from a circadian perspective. Undoubtedly, the characterization of circadian rhythmicity of NADPH formation in healthy and neoplastic cells, as well as the Warburg effect in cancer, will be promising fields of opportunity for laboratories interested in studying redox adaptations in the physiopathology of the circadian timing system.

AUTHOR CONTRIBUTIONS

MD-M and IM participated in the conceptual aspect of the manuscript, wrote several sections of the minireview, and agreed with the final version of the manuscript.

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Circadian Rhythms and Clock Genes in Reproduction: Insights From Behavior and the Female Rabbit's Brain

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Clock gene oscillations are necessary for a successful pregnancy and parturition, but little is known about their function during lactation, a period demanding from the mother multiple physiological and behavioral adaptations to fulfill the requirements of the offspring. First, we will focus on circadian rhythms and clock genes in reproductive tissues mainly in rodents. Disruption of circadian rhythms or proper rhythmic oscillations of clock genes provoke reproductive problems, as found in clock gene knockout mice. Then, we will focus mainly on the rabbit doe as this mammal nurses the young just once a day with circadian periodicity. This daily event synchronizes the behavior and the activity of specific brain regions critical for reproductive neuroendocrinology and maternal behavior, like the preoptic area. This region shows strong rhythms of the PER1 protein (product of the Per1 clock gene) associated with circadian nursing. Additionally, neuroendocrine cells related to milk production and ejections are also synchronized to daily nursing. A threshold of suckling is necessary to entrain once a day nursing; this process is independent of milk output as even virgin does (behaving maternally following anosmia) can display circadian nursing behavior. A timing motivational mechanism may regulate such behavior as mesolimbic dopaminergic cells are entrained by daily nursing. Finally, we will explore about the clinical importance of circadian rhythms. Indeed, women in chronic shift-work schedules show problems in their menstrual cycles and pregnancies and also have a high risk of preterm delivery, making this an important field of translational research.

Keywords: maternal behavior, lactation, PER1 protein, suckling, pregnancy, parturition, preoptic area, oxytocin

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INTRODUCTION

Few studies have explored the relation between circadian rhythms and reproduction. Most of the early works focused on lactation and maternal behavior (MB), largely in rodents. However, the discovery of functional molecular clock machinery in reproductive tissues, and the use of clock gene mutant models have revealed that such genes play a main role in orchestrating reproductive processes in mammals. First, we will focus on circadian rhythms and clock genes in reproductive tissues, from implantation through lactation, mainly in rodents. Then, we will focus on the rabbit, a lagomorph with a striking circadian rhythm of lactation, unique to this class of mammals. Our studies in this animal are revealing, entraining of behaviors and neuroendocrine processes in specific

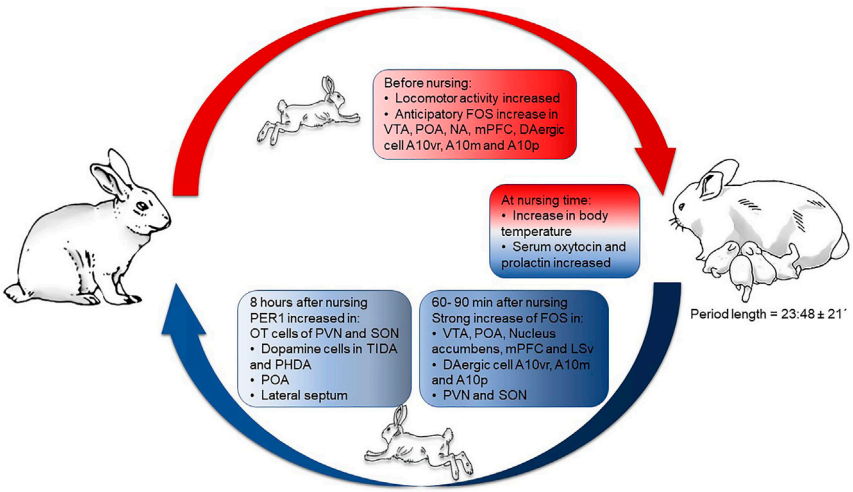

FIGURE 1 | Behavioral, physiological, and neural changes throughout circadian lactation in the rabbit doe. Abbreviations: A10vr, A10 ventral rostral; A10m, A10 medial; A10p, A10 posterior; FOS, c-Fos protein; mPFC, medial prefrontal cortex; NA, nucleus accumbens; OT, oxytocin; PHDA, periventricular hypophysial dopaminergic cells; POA, preoptic area; PVN, paraventricular nucleus of the hypothalamus; SON, supraoptic nucleus; TIDA, tuberoinfundibular dopaminergic cells; VTA, ventral tegmental area. In non-pregnant, non-lactating females FOS protein rhythms reach a peak at different hours in different structures, but in lactating does all of these rhythms shift to the hour of nursing. Figure derived from data previously published in Ref. (44, 49, 62–64, 66, 79, 80).

brain structures as a consequence of suckling by pups (**Figure 1**). Finally, we will explore the translational importance of a "healthy" circadian clock for proper rhythms in reproduction.

CIRCADIAN RHYTHMS AND CLOCK GENES IN REPRODUCTIVE PROCESSES

Many physiological processes and behaviors in mammals are rhythmic. The most evident daily change is the sleep/wake cycle, but there are clear changes in the blood concentration of several hormones and specific metabolites throughout the day (1). These changes allow organisms to adapt to the environmental light/dark cycle and consequently to the resources available at specific times of day or night. These rhythms are controlled by an endogenous molecular clock within the suprachiasmatic nucleus (SCN), located in the forebrain of mammals, which is entrained by the light/dark cycle. The molecular clockwork is composed of a group of core clock genes, *Per*, *Cry*, *Clock,* and *Bmal1*, organized in a transcription–translation feedback loop that oscillates every 24 h. Their oscillations are associated with self-sustaining redox rhythms, known as nontranscriptional clocks as well as metabolic rhythms in an organ-specific manner [Reviewed in Ref. (1)]. Reproductive tissues have also functional molecular clocks and, although at the top of the hierarchy are the SCN oscillations, it is now recognized that the circadian system is organized along several axes of a redundant network that exchanges bidirectional timing information among the components (2, 3). An early study found that lesions to the SCN completely eliminated phasic LH release (4), and in recent years much information has accumulated to support the importance of the clockwork mechanism in reproduction by using mutant mouse models with various disruptions of the molecular clockwork. Recently, in *Clock/Clock* mutant mice it was demonstrated that few of these animals became pregnant, they had a high rate of fetal reabsorption and severe dystocia and the fetuses showed morphological abnormalities (5, 6). However, it is possible that this is an effect not only of the *Clock/Clock* mutation as *Per1*, *Per2,* and *Bmal1* knockout mice, but also shows several abnormalities during pregnancy and parturition (7, 8). Very little is known about the possible mechanisms involved. In *Clock/Clock* mutants, serum progesterone levels are twofold lower and estradiol is significantly lower in mid-pregnancy compared to wild-type females, differences that have been associated with a high incidence of pup reabsorption (5). Indeed, impaired steroidogenesis appears to be a common problem in clock gene mutants as pregnant *Bmal1* (−/−) mice also have lower progesterone serum levels than *Bmal1* (+/±) and reduced embryo implantation (9). Moreover, in rats, deletion of ovarian *Bmal1* gene affected genes critical for progesterone production, leading to implantation failure; these effects were reversed by the implantation of a single wild-type ovary (10). Regarding *Per1* and *Per2* mutants, although fertile, they exhibit lower reproductive success than the control group, as occurs in aged wild-type mice (7). Together, the above information indicates that proper oscillations of the core clock genes in reproductive tissues are necessary for successful ovulation, embryo implantation, and steroidogenesis (11). In **Table 1,** we summarize some effects on reproduction provoked by alterations in specific clock genes. These reproductive disorders are observed in clock gene-deficient animals. Thus, it remains to be determined at which specific levels of control clock genes act, as the functions described in **Table 1** are complex and have a multifactorial regulation. Moreover, as clock genes control transcription in a tissue-specific manner and recently nontranscriptional metabolic clocks have been discovered [Reviewed in Ref. (1)], the possibility exists that endocrine factors (i.e., specific

TABLE 1 | Some effects in reproductive success by changes in clock genes genotype in mammals.

Clock gene	Species	Effect	Reference
Gonads			
Bmal 1$^{-/-}$	Mouse	Ovarian size reduced	(8)
Bmal 1$^{-/-}$	Mouse	Low testosterone and high luteinizing hormone in serum; reduction in esteroidogenic genes in testes, reduced sperm count. Infertility	(12, 13)
Clock	Human polymorphism	Semen volume reduction, low sperm motility, and idiopathic infertility. Alteration in serum levels of testosterone and FSH	(14, 15)
Cry1	Mouse KD	Reduction of meiotic process and maturation in oocytes	(16)
Bmal1$^{flx/flx}$	Mouse	Changes in phasic LH sensitivity of theca cells in ovary	(17)
Bmal1$^{flx/flx}$	Mouse	Failure to mate with receptive females. Low secretion of FSH and GnRH. Tyrosine hydroxylase in brain decreased	(18)
Estrous and menstrual cycles			
Clock$^{\Delta 19}$	Mouse	Higher proportion of irregular estrous cycles	(19–21)
Clock	Mouse$^{clock/clock}$	Irregular estrous cyclicity and failure to have a coordinated LH surge on proestrus	(22)
Bmal 1$^{-/-}$	Mouse KO	Changes in daily pattern of estrogen receptor β in tissues implicated in female reproductive functions	(23, 24)
Clock	Human polymorphism	Irregular menstrual cycles	(25)
Gestational/parturition			
Per 1$^{-/-}$ and *Per 2*$^{-/-}$	Mouse	Successful parturition reduced	(7)
Bmal 1$^{-/-}$	Mouse	Lack of implantation and embryonic development. Impaired steroidogenesis, low progesterone levels and embryo implantation reduced. Alterations in delivery times	(8–10, 26)
Clock	Mouse$^{clock/clock}$	Elevated rates of fetal reabsorption	(5)
Bmal 1	Human polymorphism	Miscarriages increased	(27)
Postpartum success			
Per 1$^{-/-}$ and *Per 2*$^{-/-}$	Mouse KO	Number of pups weaned reduced	(7)
Clock$^{\Delta 19}$	Mouse	Postnatal mortality increased and low prolactin levels and reduced milk production	(19, 28)

hormones) could play a main role in the expression of reproductive disorders related to clock gene disruption.

In rats, delivery occurs at daytime, i.e., during the rest period [Reviewed in Ref. (29)], and destruction of the SCN disrupts the timing of birth (30). Takayama et al. (31) explored the role of the pineal gland hormone melatonin (MEL) and found that pinealectomized rats gave birth at either day or night and that MEL replacement at night (but not during the day), across pregnancy, restored the timing of parturition during the day in most subjects. Interestingly, in rodents, the placenta expresses functional clock genes and also glucocorticoid receptors (32) and MEL receptor MT1 (33), which are rhythmically expressed. Thus, it is possible that maternal central hormonal secretions also drive the activity of the placenta in pregnancy and parturition (34). By contrast, in primiparous rabbits kept under laboratory conditions (14 h light:10 h dark) parturition occurs throughout the day, regardless of litter size delivered (35).

Regarding lactation, mother rats nurse more often during the resting phase, i.e., across daytime (36, 37). In mice, maternal crouching (nursing posture) peaks during the day and is less frequent during the night and, concomitantly, prolactin serum levels are higher during the day (28). By contrast, *Clock* mutant mice do not have a significant peak of either crouching or prolactin, and the amount of milk secreted from mutant mice is lower (as calculated by a significant lower body weight of pups) when compared to wild-type dams (28). Additionally, pups from homozygous *Bmal1* null mice are 30% lighter at weaning (8), supporting the importance of a circadian molecular clock in timing MB and lactation. In cows, the mammary gland's demand for nutrients in early lactation is several-fold increased over that seen during pregnancy and this demand is not met just by increasing food intake (38), a finding from which a compensatory circadian mechanism was proposed. During the transition from pregnancy to lactation, there is an upregulation of the positive limb of the core clockwork as well as of clock regulatory genes in specific metabolic pathways of the rat's mammary gland, liver, and adipose tissues to support the increased nutritional demands of lactation [Reviewed in Ref. (39)]. Accordingly, in mice *Per1* and *Bmal1*, mRNA levels are elevated in late pregnant and lactating mammary tissues supporting their role in mammary gland development and differentiation (40).

NURSING WITHIN A CIRCADIAN CONTEXT: THE RABBIT MODEL

Doe rabbits nurse the young once a day, for approximately 3 min, inside a nest constructed by the mother across pregnancy (41). This invariability in the nursing pattern is observed throughout lactation (ca. 30 days), despite a marked increase in milk output across the first 20 days and a gradual decline thereafter (42). Nursing occurs at night, under light:dark or continuous light conditions, with circadian periodicity (43, 44). A threshold of suckling stimulation is essential for this regulation as reducing litter size below six kits disrupts the circadian expression of nursing (35). Although deliveries occur throughout the day, a population of parturient rabbits becomes synchronized to initiate and maintain nursing at around the same time from lactation day 1 onward. A Rayleigh analysis of the hour of nursing in the population of studied does indicated that, despite the hour of

delivery most nursing episodes occurred during the night, at 03:51 h, from postnatal days 1–15 (35). This adjustment is possible because a negative correlation exists between time of delivery and time of nursing on lactation day 1, i.e., mothers giving birth in the early morning show longer "parturition-nursing" intervals than does delivering at later hours.

A normal duration of nursing bouts also depends on a threshold of suckling as mothers given four kits or less spend longer times inside the nest box (45). Yet, milk output *per se* is not essential to display a normal nursing *behavior* as virgins induced to behave maternally (by lesioning the main olfactory system) can enter the nest box, crouch over the litter, allow suckling, and exit ca. 3 min later. This behavioral pattern is observed with circadian periodicity in 55% of maternal virgins (46).

PER1 PROTEIN RHYTHMS SHIFT BY THE TIMING OF NURSING

Suckling induces oxytocin (OT) secretion in all mammals and, in rabbits, the amount secreted is directly related with the number of suckling kits (47). Does OT participate in translating the suckling stimulus received at the nipple to the brain regions regulating nursing periodicity and duration? The number and size of OT-immunoreactive (IR) neurons increases in the paraventricular hypothalamic nucleus (PVN) from estrus, through pregnancy, and into lactation (48). Following suckling, the total number of c-FOS-IR cells increases significantly in this structure (49). Bilateral lesions to the PVN of lactating rabbits abolish or disrupt the circadian display of nursing, but do not modify duration of suckling bouts (50). Although in rabbits no OT receptors are evident in the PVN, they are abundant in the prefrontal cortex, preoptic area (POA), and lateral septum [LS (51)], regions that participate in regulating specific aspects of the doe's MB (52, 53).

The doe's circadian nursing pattern is, in turn, a timing signal for the kits (54). By scheduling the hour of nursing we have shown that this predictable event entrains rhythms of locomotor behavior, metabolic parameters, plasma corticosterone hormones, and also several brain structures in 7–9-day-old kits (55–57). From these findings, we proposed that rabbit kits are a natural model of food entrainment (57, 58). The synchronization of brain structures was determined by quantifying the expression of the PER1 protein, product of the *Per1* clock gene. The rhythm of this protein can be synchronized to a particular stimulus, e.g., food cues, in specific brain regions (59). Thus, while the clockwork oscillations of the SCN are synchronized to the light/dark cycle, the rhythm of clock genes in peripheral tissues and in the brain can be entrained by stimuli other than light, like food (60, 61). From the findings that: (a) single or multiple entrances to the nest depend on the number of suckling kits (35, 45); (b) preventing suckling by kits on lactation days 7–9 significantly decreased the amount of PER1 protein at peak time in both PVN and supraoptic nucleus (62, 63), we consider that suckling can be an entraining signal for PER1 protein rhythms on particular neuroendocrine populations, specifically oxytocinergic and also in dopaminergic (DAergic) cells. Thus, in estrous does maintained under light:dark conditions [12:12; lights on at 07:00 = time (ZT) 0], PER1 protein in the PVN peaks at ZT15, as occurs in tyrosine hydroxylase (TH)-IR cells that co-express PER1. By contrast, in lactating rabbits the peak of PER1 and PER1/TH appears 4 h after the timing of scheduled nursing. DAergic populations from the tuberoinfundibular and periventricular hypophysial regions, related to the control of prolactin release in the hypophysis, also shift their rhythm of co-expression with PER1 protein according to the timing of suckling. In contrast, no change was observed in incertohypothalamic DAergic cells, which are not related to the control of prolactin secretion (63). Therefore, our results suggest that periodic suckling is a time signal for the synthesis and/or secretion of OT and prolactin at a predictable time.

The daily spontaneous return of the mother to the nest coincides with an increase in locomotor behavior (62), suggesting that she is in a state of high arousal to access the kits. Indeed, DAergic cells of the A10 mesolimbic system increase their cellular activity, anticipating daily nursing, supporting the assumption that she is in a high motivational state to visit the kits for nursing (64). Moreover, timing the suckling stimulus also synchronizes the POA and LS, essential for the expression of MB (65), as indicated by rhythms of PER1 (66). These results, together with those of the mesolimbic system (64), suggest the establishment of a "maternal entrainable circuit" where suckling seems to be the entraining signal. Taken together, the entraining of PER1 oscillations points to the importance of the *Per1* gene in specific brain regions for uncoupling their oscillations from the master clock to fulfill a specific reproductive demand, the care, and nourishment of the litter.

TRANSLATIONAL IMPORTANCE OF CIRCADIAN RHYTHMS AND CLOCK GENES DISRUPTION

Disruption of circadian rhythms has profound consequences in humans. Light during the day is the main synchronizer for our circadian rhythms and controls the timing of our neuroendocrine system. For example, the hormone melatonin is secreted only during the night and seems to be a humoral entraining signal for peripheral organs to show proper circadian rhythms (1). Epidemiological studies were the first to indicate that the exposure to artificial light during the night, which disrupts the normal secretion of melatonin (67), is associated with circadian disruptions and to breast cancer [Reviewed in Ref. (68)]. Regarding reproduction, women shift-workers (in which the master clock is exposed to artificial light at night) have an increased risk of endometriosis, irregular menstrual cycles (with pain and unusual menstrual bleeding), delayed ovulation, increased miscarriage rate, preterm delivery, and infant low birth weight (69, 70). It has also been proposed that MEL can be a zeitgeber for the timing of parturition in women (29). The above evidence highlights the importance of central signals from the master clock and pineal MEL to peripheral reproductive organs for proper fetus development, as shown in rats (71). Besides, other organs (e.g., placenta) may play a direct role. Full-term placenta expresses circadian rhythms of *Clock*

and *Bmal1* (72), and clock gene polymorphisms are associated with placental abruption (73) and even a single polymorphism of *Bmal1* is associated with an increase in miscarriages (27). Finally, RNA microarray analysis of human milk fat globules indicates differential daily expression of 7% of transcripts (74). Moreover, there are daily changes in the concentration of antibodies and complement proteins of the immune system among several other cellular and soluble components of human milk (75). Interestingly, baby milk formula and food enriched with tryptophan (a precursor of MEL) helps to improve infant sleep when consumed at night (76, 77). This is an emerging area of research known as "chrononutrition" (78).

CONCLUSION

Clock genes in reproductive tissues, together with those in the SCN and other brain structures, play a central role in orchestrating circadian rhythms in all reproductive processes from implantation to lactation. Lesion studies of the SCN as well as alterations of the molecular clockwork using mutant mice models have revealed multiple disruptions in all reproductive processes. In contrast, very little is known about circadian rhythms and reproduction in wild-type animals, except in the rabbit. This species offers an extraordinary opportunity for exploring this issue, particularly during lactation as, in lagomorphs, nursing usually occurs once a day with circadian periodicity, a unique characteristic among mammals. Consequently, it is possible to explore in neuroendocrine cells of this species the relevance of particular components of the circadian clockwork with minimal manipulations to the animals, as opposed to rodents, that nurse several times a day. The translational importance of circadian rhythms in reproduction was first recognized through studies of women in shift-work and recently through the finding of differences in the components of breast milk across the circadian cycle, results that could improve the health and well-being of infants.

AUTHOR CONTRIBUTIONS

MC, GG-M, and EM contributed to the writing of the manuscript and approved the final version.

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Histamine Regulates Molecular Clock Oscillations in Human Retinal Pigment Epithelial Cells *via* H_1 Receptors

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Vertebrate eyes are known to contain circadian clocks, but their regulatory mechanisms remain largely unknown. To address this, we used a cell line from human retinal pigment epithelium (hRPE-YC) with stable coexpression of reporters for molecular clock oscillations (*Bmal1-luciferase*) and intracellular Ca^{2+} concentrations (*YC3.6*). We observed concentration-dependent increases in cytosolic Ca^{2+} concentrations after treatment with histamine (1–100 μM) and complete suppression of histamine-induced Ca^{2+} mobilizations by H_1 histamine receptor (H_1R) antagonist *d*-chlorpheniramine (*d*-CPA) in hRPE-YC cells. Consistently, real-time RT-PCR assays revealed that H_1R showed the highest expression among the four subtypes (H_1–H_4) of histamine receptors in hRPE-YC cells. Stimulation of hRPE-YC cells with histamine transiently increased nuclear localization of phosphorylated Ca^{2+}/cAMP-response element-binding protein that regulates clock gene transcriptions. Administration of histamine also shifted the *Bmal1-luciferase* rhythms with a type-1 phase-response curve, similar to previous results with carbachol stimulations. Treatment of hRPE-YC cells with *d*-CPA or with more specific H_1R antagonist, ketotifen, blocked the histamine-induced phase shifts. Furthermore, an H_2 histamine receptor agonist, amthamine, had little effect on the *Bmal1-luciferase* rhythms. Although the function of the *in vivo* histaminergic system within the eye remains obscure, the present results suggest histaminergic control of the molecular clock *via* H_1R in retinal pigment epithelial cells. Also, since *d*-CPA and ketotifen have been widely used (e.g., to treat allergy and inflammation) in our daily life and thus raise a possible cause for circadian rhythm disorders by improper use of antihistamines.

Keywords: antihistamine, cytosolic calcium, human, molecular clock, retina, transcriptional regulation

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INTRODUCTION

The histaminergic system in the central nervous system controls diverse physiological functions including sleeping–waking, thermoregulation, and feeding (1). To achieve these functions, histaminergic neurons in the tuberomammillary nucleus (TMN) of the posterior hypothalamus send long-distance axons into diverse brain areas (2). Interestingly, histaminergic projections from the brain to the retina have been shown to exist in rodents and primates (3–6), but knowledge on

Abbreviations: ANOVA, analysis of variance; CPA, chlorpheniramine; CREB, Ca^{2+}/cAMP-response element-binding protein; CT, circadian time; H_1R, H_1 histamine receptor; H_2R, H_2 histamine receptor; HDC, histidine decarboxylase; DAPI, 4′,6-diamidino-2-phenylindole; pCREB, phosphorylated CREB; PRC, phase-response curve; RPE, retinal pigment epithelial; SCN, suprachiasmatic nucleus; TMN, tuberomammillary nucleus.

their physiological functions remains limited. In baboon eyes, histamine reduced flash sensitivity in ON ganglion cells (7). In macaque eyes, H_1 histamine receptor (H_1R) was expressed in horizontal cells, and H_2 histamine receptor (H_2R) was expressed in cone photoreceptors (8). Histamine significantly reduced hyperpolarization-activated currents recorded from cones in monkeys (8) and modulated retinal ganglion cell firings in rats and monkeys (9). Furthermore, dopaminergic amacrine cells in mice expressed H_1R and displayed histamine-induced cytosolic calcium mobilizations (10). Source of histamine within the retina may not be only from the TMN projections but also from local synthesis because genes encoding histamine synthetic enzyme, histidine decarboxylase (HDC), were expressed in the outer nuclear layer of mice retina (11). Meanwhile, no apparent changes in retinal structures and functions were identified in HDC knockout ($HDC^{-/-}$) mice (11), and thus retinal histaminergic regulations remain controversial.

Histamine release from histaminergic neurons is generally coupled with vigilance states, being active during wakefulness and inactive during sleep (1, 12). Daily rhythms of sleep and wakefulness are strongly regulated by the central circadian pacemaker located within the hypothalamic suprachiasmatic nucleus (SCN; **Figure 1A**) (13). Meanwhile, SCN neuronal activity rhythms are directly regulated by histaminergic projections (1, 14). This suggests the presence of a histaminergic feedback system between the SCN clock and histaminergic sleep–wake mechanisms (**Figure 1B**). In addition, it is well known that the mammalian retina contains a circadian clock, because rhythmic clock gene expressions have been reported in various retinal cells (15–18). It has also been shown that photoreceptor disk shedding (19–21), dopamine synthesis (22), melatonin release (23), and retinal electrical responses to light (24) are all under circadian clock control. SCN neurons were reported to receive axons from intrinsically photosensitive retinal ganglion cells for photoentrainment of circadian rhythms (25). Thus, it is reasonable to hypothesize that the histaminergic system within the eye may function as an additional feedback system that intermediates between the retinal and central circadian clocks (**Figure 1B**). However, substantial evidence is lacking to prove this hypothesis. In the earlier works, circadian rhythms in clock gene transcriptional levels and adenylyl cyclase activities were identified in retinal pigment epithelial (RPE) cells (26–28). Based on these findings, a cell line was recently generated from human retinal pigment epithelium, hRPE-YC (29), that stably coexpresses reporters for clock gene transcriptions (*Bmal1-luciferase*) and intracellular Ca^{2+} concentrations (*YC3.6*). Using this model cell line, this study provides evidence for functional expression of H_1R and histaminergic control of the molecular clock within the eye.

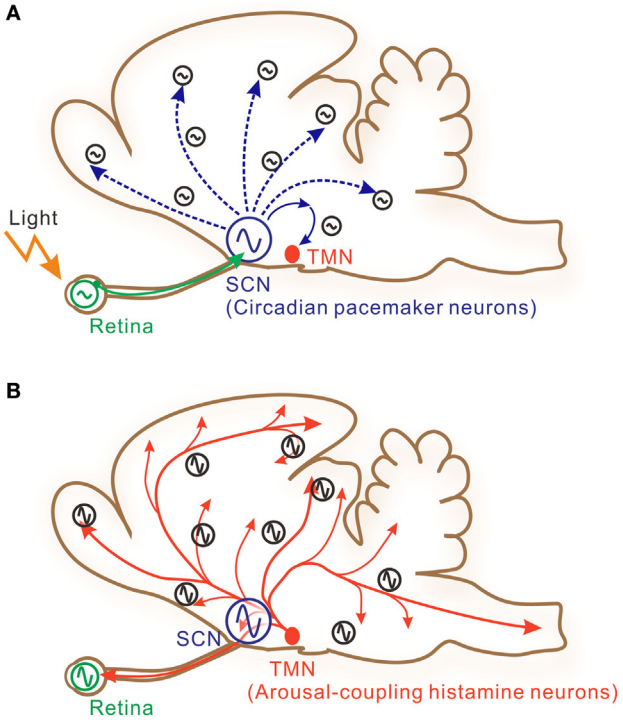

FIGURE 1 | A schematic illustration of interactive signaling underlying photic and histaminergic regulation of the circadian clock. **(A)** The circadian pacemaker neurons located within the hypothalamic suprachiasmatic nucleus (SCN) are directly controlled by retinal projections (solid green arrow). The retina contains independent circadian oscillators controlling photic sensitivity. Clock gene oscillations are observed in many other cells in the brain, although system-level oscillations are strongly dependent on core SCN oscillations. These peripheral clock oscillations are controlled by indirect neural connections (dashed blue arrows) or humoral factors from the SCN. The tuberomammillary nucleus (TMN) of the posterior hypothalamus, which contains histamine neurons, may also be under the control of the SCN *via* hypothalamic neural networks. **(B)** The TMN sends long-distance axons directly to diverse brain areas and to the retina (solid red arrows). SCN neurons and retinal cells also receive histaminergic fibers and may be under the control of the TMN. Taken together, these features suggest that histaminergic signaling may function as a feedback signal to stabilize or boost system-wide circadian oscillations.

MATERIALS AND METHODS

Cell Cultures

hRPE-YC cells (less than five passages) were cultured in Dulbecco's modified Eagle's medium/F12 supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), sodium bicarbonate (1.2 g/L), and 1% penicillin/streptomycin antibiotics (Invitrogen) under constant temperature (37°C) and 5% CO_2.

Ca^{2+} Imaging

The Ca^{2+} imaging protocols were described previously (30). Briefly, cells were seeded onto 35-mm glass-bottom dishes. The culture medium was gently rinsed from the dishes using buffered salt solution. Fluorescence images were obtained under perfusion of buffered salt solution using an upright microscope (Axioplan 2; Carl Zeiss, Thornwood, NY, USA) with a water-immersion objective (Achroplan ×20 NA0.5w; Carl Zeiss). Pairs of fluorescent images (535 ± 15/480 ± 15 nm) were produced with a light pulse of 440 ± 5 nm generated by a dual filter wheel system (Lambda 10-3; Sutter Instruments, Novato, CA, USA) and acquired using a cooled charge-coupled device camera (CoolSnap Fx; Photometrics, Tucson, AZ, USA). The timings of shutter gating and image acquisitions at 6-s intervals were regulated by digital imaging software (MetaFluor ver. 6.0; Japan Molecular Devices, Tokyo, Japan). Histamine and *d*-chlorpheniramine (*d*-CPA)

(both from Sigma-Aldrich, St. Louis, MO, USA) were perfused onto the cells by switching the perfusate.

Bmal1-Luciferase Assay

The *Bmal1-luciferase* rhythms were analyzed as described (29) using culture medium supplemented with 50 μM beetle luciferin (Promega, Madison, WI, USA) and a multichannel chemiluminescence analyzer (Kronos-Dio, Model AB-2550; ATTO Co. Ltd., Tokyo, Japan) set at 37°C. The time point with the peak chemiluminescence level in the *Bmal1-luciferase* rhythms was regarded as circadian time (CT) 20. To analyze phase-response curves (PRCs) against pharmacological stimulations, Kronos recordings were paused for 5 min. During the pause, 10% of culture medium (100 μL) was collected from each dish. Histamine, amthamine dihydrobromide, ketotifen fumarate (Sigma-Aldrich), or *d*-CPA was added to the collected culture medium and gently returned to the culture dish (final diluted concentration: 50 μM for histamine, 50 μM for amthamine dihydrobromide, 10 μM for *d*-CPA, and 10 μM for ketotifen). Although hRPE-YC cells represented little sensitivity to light (29), above medium exchanges were carefully conducted under dim red light (<3 lx). The PRCs were eye fitted by three experienced investigators.

Immunofluorescence Confocal Imaging

To examine the effects of histamine on the phosphorylation levels of Ca^{2+}/cAMP response element-binding protein (CREB), hRPE-YC cells plated on 35-mm glass-bottom dishes were stimulated with histamine (100 μM) for 10 min during subjective night-time. Immediately after the stimulations, hRPE-YC cells were fixed in 4% phosphate-buffered paraformaldehyde for 15 min and washed three times with phosphate-buffered saline. The samples were immunostained with 1:100 diluted affinity-purified rabbit anti-P-CREB ($pSer^{133}$) (Sigma-Aldrich) and embedded in Vectashield (Vector Laboratories, Burlingame, CA, USA) containing 4′,6-diamidino-2-phenylindole (DAPI) as described (29). Images were acquired using a confocal laser-scanning microscope (A1MP plus; Nikon, Tokyo, Japan).

Real-Time RT-PCR Assay

The mRNAs for the four histamine receptor subtypes (H_1–H_4) and HDC were quantified by referring a housekeeping gene (human β-*actin*) in hRPE-YC cells using a real-time RT-PCR system (Roter-Gene Ver. 6.0 software, Corbett Research, Sydney, NSW, Australia). The cell cultures and RNA extraction procedures were described previously (29). The PCR primers for histamine receptors (31) and HDC (32) were designed elsewhere. Each primer (100 μM) was used with Rotor-Gene SYBR Green RT-PCR Master Mix (Qiagen, Germantown, MD, USA) in the 72-well rotor of the PCR system (Rotor Gene 3000A; Corbett Research) as described (29). mRNA levels were expressed as $2^{-\Delta Ct}$ using β-*actin* mRNA level as internal standard.

Statistical Analysis

Data are presented as mean ± SEM. One-way analysis of variance followed by Duncan's multiple range test and four-parameter Hill function were used to analyze concentration–response curve for histamine. Kruskal–Wallis test followed by Steel–Dwass test was used to compare gene expression profiles. A two-tailed Student's *t*-test was used for pairwise comparisons. A 95% confidence level was considered to indicate statistical significance.

RESULTS

Histamine mobilized intracellular Ca^{2+} in hRPE-YC cells in a concentration-dependent manner with an EC_{50} value of 10.4 μM (**Figures 2A–C**). At 100 μM, histamine consistently evoked a Ca^{2+} response in nearly all cells tested (97 ± 1.4%; 397 of 410 cells in 18 dishes). The histamine-induced Ca^{2+} response was significantly inhibited by pretreatment with 10 μM *d*-CPA (**Figure 2B**), suggesting that the response was primarily mediated by H_1R. Intracellular Ca^{2+} mobilizations after continuous bath application of 50 μM histamine were also examined, because this application was used for the *Bmal1-luciferase* assays. Bath application of 50 μM histamine produced a transient Ca^{2+} response that almost recovered to the baseline during a 30-min exposure period (**Figure 2D**).

To characterize the histamine receptor subtypes (H_1–H_4) expressed in hRPE-YC cells, cells were collected for real-time RT-PCR assays at early subjective daytime (CT1–CT3; number of dishes = 7) and early subjective night-time (CT13–CT15; number of dishes = 4) following on-line monitoring of the *Bmal1-luciferase* rhythms. The results revealed that H_1R showed the highest expression among the four histamine receptor subtypes with no difference in the levels between subjective daytime and subjective night-time. The expression levels of H_3 and H_4 histamine receptors were near to the detection limits. Therefore, the gene expression levels were further analyzed regardless of sampling time using a non-parametric test (**Figure 3**). The expression levels of H_1R ($P < 0.01$) and H_2R ($P < 0.05$) were significantly larger than those of H_3 and H_4 histamine receptors (Kruskal–Wallis test followed by Steel–Dwass test). In addition to these analyses, capability of histamine synthesis in hRPE-YC cells was analyzed by monitoring HDC expression. However, HDC expression was negligible in these cells (**Figure 3**).

To estimate the gene transcriptional regulations by histamine, immunofluorescence staining of phosphorylated CREB (pCREB) in hRPE-YC cells after histamine stimulation was examined. Compared with unstimulated controls (optical density = 31 ± 0.2, number of cells = 328, number of dishes = 3), treatment with 50 μM histamine for 10 min doubled the pCREB staining levels in the nucleus (optical density = 65.5 ± 1.9, number of cells = 312, number of dishes = 3; **Figure 4**). Consistent with the nuclear pCREB inductions, the same histamine stimulation produced circadian phase-delays or advances in the *Bmal1-luciferase* rhythms (**Figure 5A**). Accordingly, the type-1 PRC was eye fitted on the phase-shifting profiles dependent on the CT (**Figure 5B**). The histamine-induced phase shifts at CT14 (−2.8 ± 0.6 h, number of dishes = 5 in histamine-stimulated group) and CT20 (+5.4 ± 0.5 h, number of dishes = 6 in histamine-stimulated group) were almost completely inhibited by *d*-CPA or ketotifen treatment (**Figure 5B**). Compared with the phase responses to histamine stimulations, the H_2R-specific

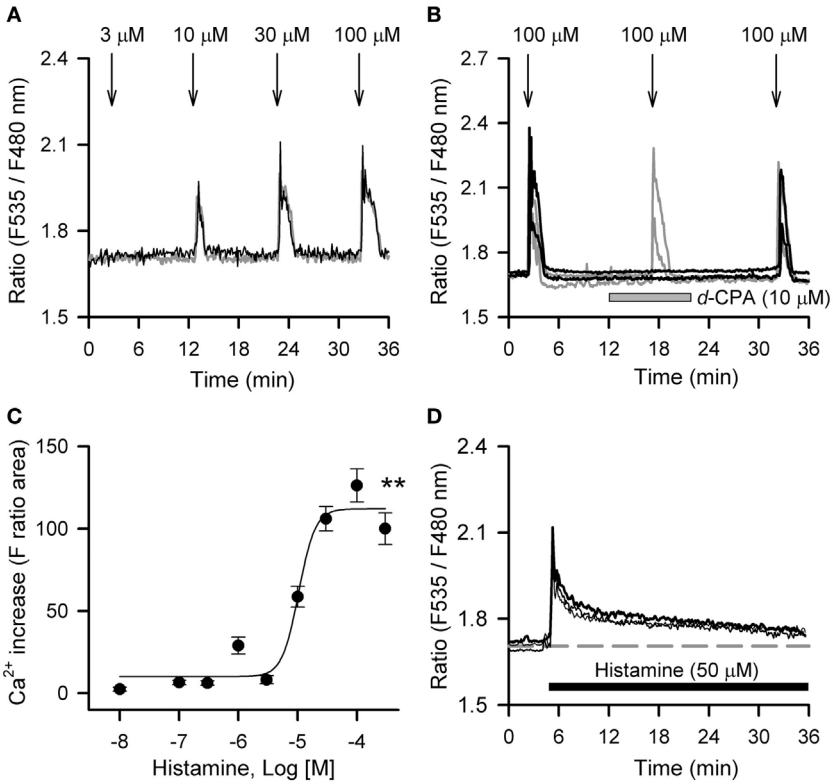

FIGURE 2 | Histamine-induced calcium mobilizations in hRPE-YC cells. **(A)** Histamine (3–100 μM) evoked dose-dependent increases in cytosolic Ca^{2+} concentrations in hRPE-YC cells. Two representative cell responses are shown. Arrows denote the onsets of 1-min histamine stimulations. **(B)** Repeated 100-μM histamine stimulations with 15-min intervals consistently elevated cytosolic Ca^{2+} in hRPE-YC cells (gray traces). In this experiment, the second histamine stimulation was also examined under perfusion of 10 μM *d*-chlorpheniramine (*d*-CPA) (black traces). Note that complete inhibition of Ca^{2+} responses by *d*-CPA and recovery of Ca^{2+} responses after rinsing out of *d*-CPA were observed. All of the above experiments were reproducible in at least three independent trials in separate culture dishes. **(C)** Concentration-response curves for histamine. $^{**}P < 0.01$ by one-way analysis of variance. **(D)** Continuous perfusion of histamine (50 μM, black bar) mobilized Ca^{2+} depending on the onset of stimulations without further amplification of Ca^{2+} responses during stimulations. The gray dashed line denotes the mean baseline Ca^{2+} level in the three representative cells.

agonist amthamine (50 μM) produced smaller phase delays (18.6% of histamine responses, $P < 0.01$ by Student's *t*-test, number of dishes = 3 at CT16) and phase advances (10.3% of histamine responses, $P < 0.01$ by Student's *t*-test, number of dishes = 3 at CT20; **Figure 5B**).

DISCUSSION

In this study, we explored the functions of histamine signaling in RPE cells using a human cell line. The results for Ca^{2+} imaging and real-time RT-PCR clearly demonstrated functional expression of H_1R in hRPE-YC cells. As H_1R couples with G_q proteins and links with the phosphatidylinositol signaling pathway to mobilize cytosolic Ca^{2+}, the conventional intracellular signaling pathway reported for RPE cells (33, 34) could be the trigger for phase shifts of *Bmal1* transcriptional rhythms. Indeed, nuclear pCREB expression was observed following the histamine stimulations. These results are consistent with a previous finding that stimulation of G_q-coupled *M3* muscarinic acetylcholine receptors in hRPE-YC cells resulted in transient Ca^{2+} increases, nuclear pCREB expressions, and phase shifts of *Bmal1-luciferase* rhythms with a type-1 PRC (29). Meanwhile, this study indicated gene expression of G_s-coupled H_2R in hRPE-YC cells but failed to demonstrate apparent phase shifts of *Bmal1-luciferase* rhythms by amthamine. In the previous study, forskolin, a pharmacological activator of adenylate cyclase, produced apparent phase shifts in hRPE-YC cells (29). Taken together, it is suggested that functional H_2R expression and activation of the downstream adenylate cyclase pathway could be limited in hRPE-YC cells. Histamine-induced circadian phase shifts have been studied in SCN slice preparations by reference to action potential firing rhythms (14), and this study indicates that similar histaminergic regulations may be present in the retinal circadian clock.

Retinal pigment epithelial cells have multiple functions within the retina. Among these, it should be emphasized that RPE cells are involved in the daily photoreceptor disk shedding critical for circadian rhythms in photic sensitivities. Phagocytosis of the photoreceptor outer segment by RPE cells is directly triggered by light or by intrinsic circadian clock mechanisms given that the rhythm is sustained under constant darkness (21). Although innervations from central histaminergic neurons have been

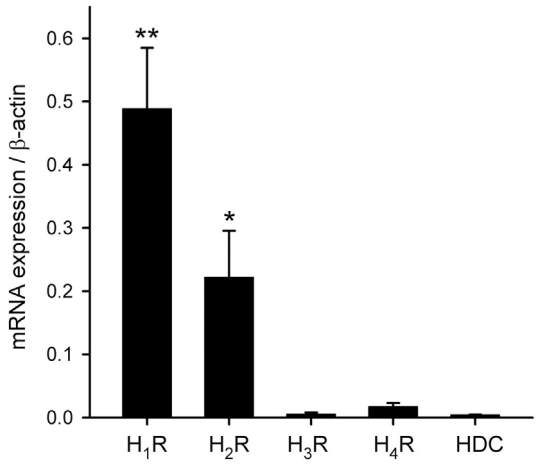

FIGURE 3 | Transcriptional profiles of histamine receptor subtypes and histamine synthetic enzyme. H_1–H_4Rs and histidine decarboxylase (HDC) mRNAs were quantified in hRPE-YC cells by quantitative RT-PCR with $2^{-\Delta Ct}$ using β-actin as internal control. H_1 histamine receptor (H_1R) exhibited the highest expression among the four subtypes. There were also detectable levels of H_2 histamine receptor (H_2R) expression in these cells. HDC was not expressed in hRPE-YC cells. $^{**}P < 0.01$ and $^{*}P < 0.05$ by Kruskal–Wallis test followed by Steel–Dwass test. Data represent mean ± SEM from 11 dishes.

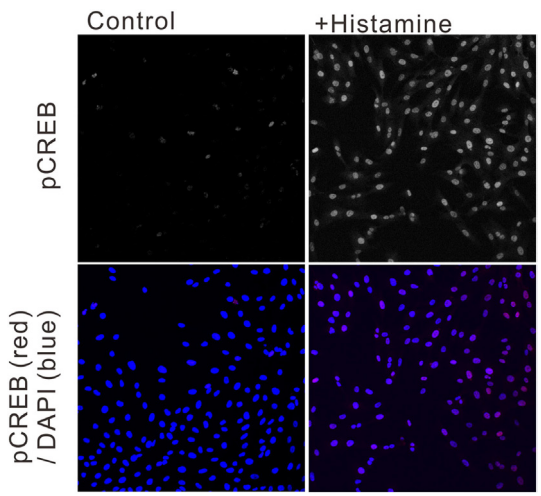

FIGURE 4 | Ca^{2+}/cAMP-response element-binding protein phosphorylation following histamine exposure. Immunofluorescence staining of phosphorylated CREB (pCREB) in unstimulated control hRPE-YC cells (left two images) and hRPE-YC cells after exposure to 50 μM histamine for 10 min (right two images). Counter-staining with 4′,6-diamidino-2-phenylindole (blue color in merged picture) demonstrated the nuclear localization of pCREB signals in hRPE-YC cells following the histamine stimulation.

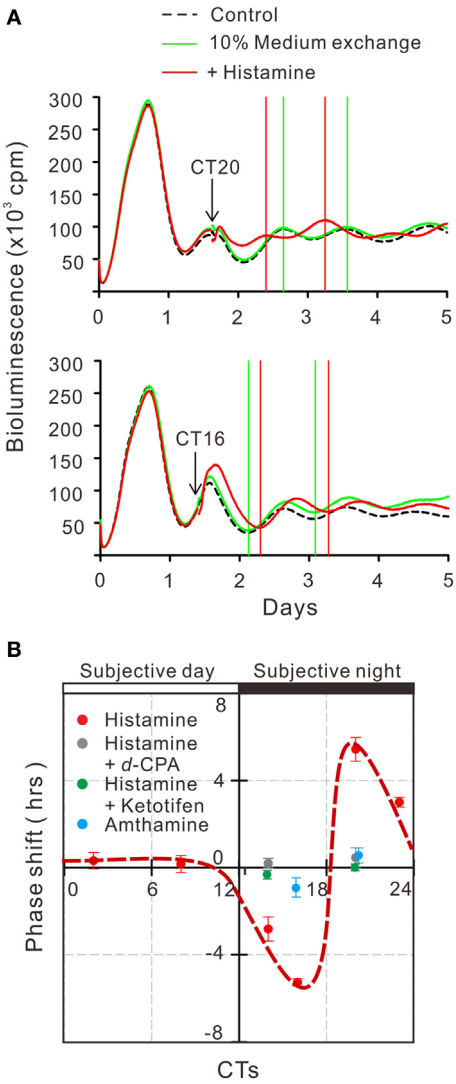

FIGURE 5 | Circadian phase shifts in *Bmal1* transcriptional rhythms following histamine exposure. **(A)** The mean *Bmal1-luciferase* intensities in 35-mm dishes were quantified using a multichannel chemiluminescence analyzer. The arrows indicate onset of 50 μM histamine exposures at CT20 and CT16. Subsequent troughs or peaks of circadian waves were compared with groups of non-treated control cells. **(B)** Based on the histamine-induced phase shifts at various time points, a type-1 phase-response curve was eye fitted (red circles with dotted line). Similar stimulation of cells by the H_2 histamine receptor agonist amthamine at CT20 or CT16 produced significantly smaller phase shifts (blue circles). Treatment with 10 μM *d*-chlorpheniramine (*d*-CPA) or ketotifen almost completely abolished the histamine-induced phase-advances at CT20 and delays at CT14 (gray circles for *d*-CPA treatment and green circles for ketotifen treatment). Data represent mean ± SEM from three to six dishes.

identified primarily in the ganglion cell layer and inner plexiform layer in the retina (4–9), histamine could be a paracrine modulator for various retinal cells. In addition, it has shown that the outer nuclear layer of mice retina express HDC genes using a laser microdissection technique (11), although the type of retinal cells synthesizing histamine has not yet been characterized. Thus, it is possible that histaminergic control of RPE cells, if any in *in vivo*, could be involved in the regulation of photoreceptor disk shedding rhythms. It was also shown that $HDC^{-/-}$ mice with recovery of the *Crb1* mutation exhibit normal retinal structures and functions, including the outer segment (11). However, these analyses were conducted under 12-h/12-h light/dark cycles and paid no particular attention to the tissue sampling time. Under

these circumstances, the direct light information was presumably sufficient to determine the phenotypes. Histamine release from histaminergic neurons is coupled with sleep–wake states (1, 12). Importantly, significant reductions in clock gene (*Per1* and *Per2*) transcriptional rhythms have been shown in many brain regions outside the SCN in HDC$^{-/-}$ mice (35). This suggests remote control of peripheral clock gene transcriptional rhythms by the brain histaminergic system (**Figure 1**). Thus, it is of particular interest whether changes in histaminergic tones and sleep–wake status can exert feedback on retinal clock regulations and ultimately on circadian clock systems. Further studies are needed to clarify these possibilities.

In relation to the effect of the H_1R antagonist observed in this study, we would like to emphasize the possible influence on human circadian clock regulations because H_1R antagonists are widely used in daily life. First-generation H_1R antagonists, such as *d*-CPA, are permeable to the brain and induce sedation and/or slow-wave sleep following systemic administration in rats (36, 37). Based on these effects, one of the first-generation H_1R antagonists, diphenhydramine, is currently sold as a sleeping aid in Japan. In addition, doxepin, another first-generation H_1R antagonist known to induce sleep, has been approved by the FDA for treatment of insomnia in the United States (38). Furthermore, numerous H_1R antagonists are currently sold as eye drops to treat ocular allergies (39). Despite the widespread use of H_1R antagonists, their influence on circadian clock regulations has not been analyzed in detail. The effects of daily systemic injections of ketotifen (an early phase second-generation H_1R antagonist) were recently evaluated in rats, with significant effects observed on their circadian locomotor activity rhythms (40). In addition, we preliminary observed reduction in *Per2* transcriptional rhythms in the SCN and hippocampus by daily systemic injections of ketotifen in rats (unpublished data). Numerous antihistamines, including *d*-CPA and ketotifen, represent affinity to muscarinic receptors to block acetylcholine signaling (41, 42) and thus use of antihistamines especially at high doses may also exert their influence on cholinergic clock regulations (29, 43). Together with the present results showing complete suppression of histamine-induced circadian phase shifts in hRPE-YC cells by *d*-CPA or ketotifen, we suggest that further clinical studies to analyze the influence of antihistamines on human circadian rhythms, with a special focus on circadian visual functions, are warranted.

In conclusion, the present results suggest histaminergic control of the molecular clock *via* H_1R in a model cell line for human RPE cells and thus raise a possible cause for circadian rhythm disorders by daily use of antihistamines.

AUTHOR CONTRIBUTIONS

MI designed the study, wrote and edited the manuscript, and directed the project. EM, YK, HK, and TM performed the experiments. EM analyzed the data.

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A Free-Choice High-Fat High-Sugar Diet Alters Day–Night *Per2* Gene Expression in Reward-Related Brain Areas in Rats

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Under normal light–dark conditions, nocturnal rodents consume most of their food during the dark period. Diets high in fat and sugar, however, may affect the day–night feeding rhythm resulting in a higher light phase intake. *In vitro* and *in vivo* studies showed that nutrients affect clock-gene expression. We therefore hypothesized that overconsuming fat and sugar alters clock-gene expression in brain structures important for feeding behavior. We determined the effects of a free-choice high-fat high-sugar (fcHFHS) diet on clock-gene expression in rat brain areas related to feeding and reward and compared them with chow-fed rats. Consuming a fcHFHS diet for 6 weeks disrupted day–night differences in *Per2* mRNA expression in the nucleus accumbens (NAc) and lateral hypothalamus but not in the suprachiasmatic nucleus, habenula, and ventral tegmental area. Furthermore, short-term sugar drinking, but not fat feeding, upregulates *Per2* mRNA expression in the NAc. The disruptions in day–night differences in NAc *Per2* gene expression were not accompanied by altered day–night differences in the mRNA expression of peptides related to food intake. We conclude that the fcHFHS diet and acute sugar drinking affect *Per2* gene expression in areas involved in food reward; however, this is not sufficient to alter the day–night pattern of food intake.

Keywords: *Per2*, fat and sugar, clock-genes, obesity, reward, nucleus accumbens

INTRODUCTION

The suprachiasmatic nucleus (SCN) controls the circadian (24-h period) rhythms in behavior and physiology (1, 2). In the SCN and in all cells of the body, a feedback loop of genes (known as clock genes) are expressed and repressed with a 24-h period. The positive limb of the loop consists of the genes *Clock* and *Bmal1* of which the protein dimer promotes *Per* and *Cry* expression, and genes from the negative limb which protein products repress *Clock* and *Bmal1* activity (3). Environmental light is the main synchronizer for the SCN (4), whereas other brain circadian clocks are more sensitive to internal hormonal and metabolic signals. Thus, feeding cues are also able to modify the day/night physiological variation. Circadian eating patterns can be altered by high-energy diets (5–7) such as the free-choice high-fat high-sugar (fcHFHS) diet, consisting of the choice between

tap water, chow-food, fat, and sugar (8). Rodents exposed to a fcHFHS diet show smaller day–night differences in food intake. Especially intake of fat and sugar components of the diet does not show day–night variations, whereas the intake of the nutritionally balanced chow diet remains rhythmic with a higher intake in the dark period when animals are active (8, 9). Moreover, we previously reported changes in the molecular clock properties of the lateral habenula (LHb) in fcHFHS diet-exposed mice, an area involved in reward-related behavior, whereas clock proteins in the arcuate nucleus, an important area for homeostatic feeding, were unchanged (9). It remains, however, to be determined whether molecular clock-gene expression in food-related reward circuitry, such as striatum and lateral hypothalamus (LH), are affected by a diet high in fat and sugar and if these effects are involved in disruption of the day/night feeding rhythm. We hypothesize that the obesogenic diet-induced disruption of day–night palatable intake is linked to nutrients (such as fat and sugar) affecting the brain oscillators within the food reward circuitry. In this study, we exposed rats to a fcHFHS diet for 6 weeks and measured clock-genes and food-related peptide gene expression in different reward-related brain areas. Subsequently, we evaluated the acute effects of sugar intake on *Per2* gene expression in the nucleus accumbens (NAc) of rats.

MATERIALS AND METHODS

Male Wistar rats weighing ~250 g were single-housed in Plexiglas cages in a temperature and light-controlled room with 21–23°C and a 12:12 h light:dark-cycle ZT0 at 7:00 a.m. (Zeitgeber Time: ZT0 onset of light and ZT12 when lights are off). Animals were fed with regular chow and water *ad libitum* during baseline. All experiments were approved by the Animal Ethics Committee of the Royal Netherlands Academy of Arts and Sciences (Amsterdam).

fcHFHS-Diet Effects on Clock-Gene and Output-Genes Expression in Feeding-Related Areas

Rats were either fed chow ($n = 14$) or the fcHFHS diet ($n = 14$): tap water, chow-food, 30% sucrose-water bottle, and a dish with fat (beef tallow, Vandemoortele, Belgium). Food intake was measured 3 times/week and 1 time/week at the beginning and the end of the day and night phases to assess the day–night food intake. Body weight was measured at least twice/week. After 6 weeks, rats from both groups were divided and euthanized at two different time points: ZT4 (day point) and ZT16 (night point) by sedation in a CO_2-chamber and immediately decapitated. Brains were quickly removed, frozen, and stored at −80°C. Epididymal and perirenal white adipose tissue (WAT) was dissected and weighted.

Sugar Intake Effect on *Per2* mRNA Expression in NAc

Rats were divided into two groups. During 7 days at ZT 10 (2 h before lights off), one group received an extra bottle of water ($n = 8$) and the other group a bottle with 30% sugared water ($n = 9$) during 2.5 min to consume ~5 kcal of sugar. To determine exact sugar intake, the bottle was weighted before and after drinking. Rats were sacrificed 30 min after last water or sugar intake. Animals were sedated and decapitated and brains were harvested as described above.

mRNA Extraction and Quantitative Real-Time PCR

Punches from frozen brains were taken using a small needle dissecting NAc, SCN, LH, habenula (Hb, containing both the medial and lateral parts), and ventral tegmental area (VTA) according to the Paxinos Altlas (10). Tissue was placed in TRIzol (QIAGEN) and homogenized using an ULTRA THURRAX homogenizer (IKA, Germany). RNA extraction and RT-PCR was performed for *Per2*, *Bmal1*, *Vglut2*, *Orexin* (11), *Cry1* (F primer: AAGTCATCGTGCGCATTTCA; R primer TCATCA TGGTCGTCGGACAGA), and *pre-pro-enkephalin*(F primer: CTTGTCAGAGACAGAACGGGT; R primer CCTTGCAGGT CTCCCAGATTT) as described previously (12).Reference genes: Cyclophilin (F primer ATGTGGTCTTTGGGAAGGTG; R primer GAAGGAATGGTTTGATGGGT), β-Actin (F primer ACAACC TTCTTGCAGCTCCTC; R primer CTGACCCATACCCACCAT CAC).

Statistics

All results are expressed as mean ± SEM. Statistical analysis was performed using Graphpad Prism. *T*-tests were performed for two group measures. Two-way ANOVA was performed to detect effects of diet, time or diet, and time interaction on gene expression. When detecting an interaction effect, a Tukey's HSD *post hoc* test was performed. Results were considered statistically significant at $p < 0.05$.

RESULTS

During all 6 weeks of the experiment, fcHFHS-fed rats were hyperphagic, cumulatively consuming 3,884 ± 56.23 kcal, compared with 3,041 ± 50.39 kcal ingested by the chow group [$t_{(26)} = 11.16$, $p < 0.001$]. Chow intake in the control group, and chow, fat, and sugar intake in the fcHFHS diet group were significantly higher at night compared with day (**Table 1**). At the end of the experiment, fcHFHS-fed rats were heavier and more obese than chow-fed rats [BW: 411.3 ± 4.2 vs. 429.7 ± 4.9 g; $t_{(26)} = 2.38$, $p < 0.001$; WAT: 5.6 ± 0.2 vs. 9.9 ± 0.5 g; $t_{(26)} = 8.34$, $p < 0.001$].

In all brain areas from the chow-fed group, *Per2* mRNA was higher at ZT16 (night) than at ZT4 (day). In fcHFHS-fed rats, however, this day–night difference was absent in the NAc and LH; i.e., no significant difference between day and night in animals fed the fcHFHS diet (**Figure 1A**). *Cry1* and *Bmal1* expression also showed significant day/night differences in most brain areas investigated (**Table 1**). Interestingly, the loss of day–night differences in the fcHFHS group was restricted to *Per2* (**Table 1**). We also measured day–night expression of *Vglut2* in all areas, *orexin* in the LH and *pre-pro-enkephalin* in NAc to investigate whether the observed changes in *Per2* were reflected in feeding-regulating genes. No significant changes were observed for *Orexin* [ANOVA: diet $F_{(1,22)} = 2.83$, $p = 0.1$; time $F_{(1,22)} = 0.008$, $p = 0.9$; Int. $F_{(1,22)} = 0.04$, $p = 0.8$], but *Vglut2* was altered in the

TABLE 1 | Eating patterns from chow-fed and free-choice high-fat high-sugar (fcHFHS) diet fed groups and mRNA expression from clock-genes *Cry1*, *Bmal1, Per2,* and the *Vglut2* gene.

Eating patterns

	Chow		fcHFHS					
	Chow		Chow		Fat		Sugar	
%	100		44.4 ± 1.4		13.5 ± 1.2		41.9 ± 2.2	
Day/night feeding	Day	Night	Day	Night	Day	Night	Day	Night
	16.2 ± 1.1	83.8 ± 1.1	15.7 ± 1.0	84.3 ± 1.0	8.4 ± 1.2	91.5 ± 1.2	22.1 ± 1.0	77.9 ± 1.1
Two-way ANOVA	Time $F_{(1,78)} = 5{,}894$, $p < 0.001$, diet component $F_{(2,78)} = 0$, $p > 0.99$, interaction $F_{(2,78)} = 76.6$, $p < 0.001$							
T-test day vs. night	$t_{(26)} = 44.9$, $p < 0.001$		$t_{(26)} = 49.3$, $p < 0.001$		$t_{(26)} = 48.1$, $p < 0.001$		$t_{(26)} = 36.2$, $p < 0.001$	

Gene expression

Gene	Group	Brain area:	Suprachiasmatic nucleus	Nucleus accumbens	Lateral hypothalamus	Habenula	Ventral tegmental area
Cry1	Chow	Day	4.6 ± 0.3	1.9 ± 0.2	9.3 ± 0.5	5.8 ± 1.5	2.6 ± 0.3
		Night	6.0 ± 0.5	2.4 ± 0.2	11.5 ± 1.4	9.4 ± 2.2	3.2 ± 0.1
	fcHFHS	Day	4.8 ± 0.5	2.2 ± 0.2	8.4 ± 0.5	5.7 ± 0.9	2.6 ± 0.4
		Night	6.5 ± 0.4	2.5 ± 0.2	10.9 ± 0.6	9.9 ± 1.9	2.9 ± 0.2
	Two-way ANOVA	Interaction	$F_{(1,23)} = 0.11$; $p = 0.7$	$F_{(1,24)} = 0.4$; $p = 0.5$	$F_{(1,21)} = 0.06$; $p = 0.7$	$F_{(1,23)} = 0.03$; $p = 0.8$	$F_{(1,24)} = 0.1$; $p = 0.7$
		Diet	$F_{(1,23)} = 0.65$; $p = 0.4$	$F_{(1,24)} = 1.0$; $p = 0.3$	$F_{(1,21)} = 0.8$; $p = 0.3$	$F_{(1,23)} = 0.007$; $p = 0.9$	$F_{(1,24)} = 0.3$; $p = 0.6$
		Time	**$F_{(1,23)} = 14.2$; $p < 0.01$**	**$F_{(1,24)} = 4.4$; $p < 0.05$**	**$F_{(1,21)} = 9.5$; $p < 0.01$**	**$F_{(1,23)} = 5.5$; $p < 0.0$**5	$F_{(1,24)} = 3.4$; $p = 0.07$
Bmal1	Chow	Day	3.7 ± 0.1	1.9 ± 0.1	9.0 ± 0.6	1.8 ± 0.2	1.5 ± 0.1
		Night	4.2 ± 0.4	1.6 ± 0.1	7.0 ± 0.5	1.7 ± 0.3	1.1 ± 0.1
	fcHFHS	Day	4.4 ± 0.2	1.5 ± 0.3	8.9 ± 0.6	1.9 ± 0.2	1.3 ± 0.2
		Night	3.9 ± 0.2	1.7 ± 0.1	6.6 ± 0.4	1.8 ± 0.3	1.1 ± 0.04
	Two-way ANOVA	Interaction	$F_{(1,24)} = 2.53$; $p = 0.1$	$F_{(1,22)} = 2.47$; $p = 0.1$	$F_{(1,22)} = 0.08$; $p = 0.7$	$F_{(1,23)} = 0.001$; $p = 0.9$	$F_{(1,24)} = 0.7$; $p = 0.3$
		Diet	$F_{(1,24)} = 0.7$; $p = 0.4$	$F_{(1,22)} = 1.4$; $p = 0.2$	$F_{(1,22)} = 0.3$; $p = 0.5$	$F_{(1,23)} = 0.19$; $p = 0.6$	$F_{(1,24)} = 1.1$; $p = 0.2$
		Time	$F_{(1,24)} = 0.006$; $p = 0.9$	$F_{(1,22)} = 0.4$; $p = 0.4$	**$F_{(1,22)} = 16.5$; $p < 0.01$**	$F_{(1,23)} = 0.03$; $p = 0.8$	**$F_{(1,24)} = 9.1$; $p < 0.01$**
Per2	Chow	Day	2.3 ± 0.2	0.7 ± 0.05	2.4 ± 0.1	0.5 ± 0.1	0.6 ± 0.07
		Night	3.3 ± 0.4	1.2 ± 0.1	4.0 ± 0.4	1.2 ± 0.1	0.9 ± 0.1
	fcHFHS	Day	3.0 ± 0.2	0.7 ± 0.06	2.6 ± 0.2	0.4 ± 0.06	0.6 ± 0.07
		Night	3.3 ± 0.3	0.8 ± 0.05	3.1 ± 0.2	1.2 ± 0.2	1.0 ± 0.08
	Two-way ANOVA	Interaction	$F_{(1,24)} = 1.1$; $p = 0.29$	**$F_{(1,22)} = 5.0$; $p < 0.03$**	**$F_{(1,22)} = 4.3$; $p < 0.04$**	$F_{(1,23)} = 0.08$; $p = 0.7$	$F_{(1,24)} = 0.9$; $p = 0.3$
		Diet	$F_{(1,24)} = 1.0$; $p = 0.3$	$F_{(1,22)} = 2.6$; $p = 0.12$	$F_{(1,22)} = 1.1$; $p = 0.2$	$F_{(1,23)} = 0.05$; $p = 0.8$	$F_{(1,24)} = 0.1$; $p = 0.6$
		Time	$F_{(1,24)} = 3.4$; $p = 0.07$	**$F_{(1,22)} = 11.7$; $p < 0.01$**	**$F_{(1,22)} = 14.2$; $p < 0.01$**	**$F_{(1,23)} = 14.9$; $p < 0.01$**	**$F_{(1,24)} = 12.5$; $p < 0.01$**
Vglut2	Chow	Day	2.2 ± 0.5	0.1 ± 0.02	21.6 ± 1.1	14.7 ± 2.5	11.6 ± 1.4
		Night	2.9 ± 0.6	0.1 ± 0.03	26.8 ± 1.5	22.3 ± 3.7	10.5 ± 0.8
	fcHFHS	Day	1.9 ± 0.2	0.06 ± 0.01	26.9 ± 2.7	16.6 ± 1.6	10.6 ± 1.6
		Night	2.1 ± 0.4	0.07 ± 0.01	24.2 ± 1.4	19.0 ± 3.7	11.7 ± 0.6
	Two-way ANOVA	Interaction	$F_{(1,24)} = 0.31$; $p = 0.5$	$F_{(1,23)} = 0.22$; $p = 0.6$	**$F_{(1,22)} = 4.38$; $p < 0.05$**	$F_{(1,23)} = 0.76$; $p = 0.3$	$F_{(1,22)} = 0.83$; $p = 0.3$
		Diet	$F_{(1,24)} = 0.9$; $p = 0.3$	**$F_{(1,23)} = 7.16$; $p < 0.05$**	$F_{(1,22)} = 0.56$; $p = 0.4$	$F_{(1,23)} = 0.06$; $p = 0.8$	$F_{(1,22)} = 0.0$; $p = 0.9$
		Time	$F_{(1,24)} = 0.8$; $p = 0.3$	$F_{(1,23)} = 0.005$; $p = 0.9$	$F_{(1,22)} = 0.45$; $p = 0.5$	$F_{(1,23)} = 2.7$; $p = 0.1$	$F_{(1,22)} = 0.0$; $p = 0.9$

The upper part of the table shows feeding day–night patterns of chow and fcHFHS rats. Results of the two-way ANOVA comparing daytime (day, nigh) vs. diet component (chow, sugar, fat) on the percentage of caloric intake are shown for the fcHFHS group. Results from the t-test analysis of day–night intake are shown under every diet component for chow and fcHFHS groups. In the lower part of the table, the different brain areas are shown in columns. In rows are presented: the studied gene; the diet condition: chow/fcHFHS and; and daytime: day/night. Results from the two-way ANOVA analysis (diet condition vs. daytime) per brain area are shown under every gene description. Significant statistical effects are highlighted in bold letters. Data are presented as mean ± SEM.

LH and NAc of the fcHFHS-fed group (**Table 1**). In the LH, we observed an interaction effect; however, the *post hoc* analysis did not detect differences between night and day in the chow or in the fcHFHS group. In the NAc, *Vglut* mRNA was significantly lower at both day and night in fcHFHS diet-fed rats compared with chow-fed rats (**Table 1**). *Pre-pro-enkephalin* expression was higher during the light period in both chow-fed (0.052 ± 0.002) and fcHFHS-fed (0.057 ± 0.002) groups compared with the dark period [chow 0.044 ± 0.003; fcHFHS 0.042 ± 0.002; time $F_{(1,22)} = 27.0$, $p < 0.001$], but no significant diet or interaction

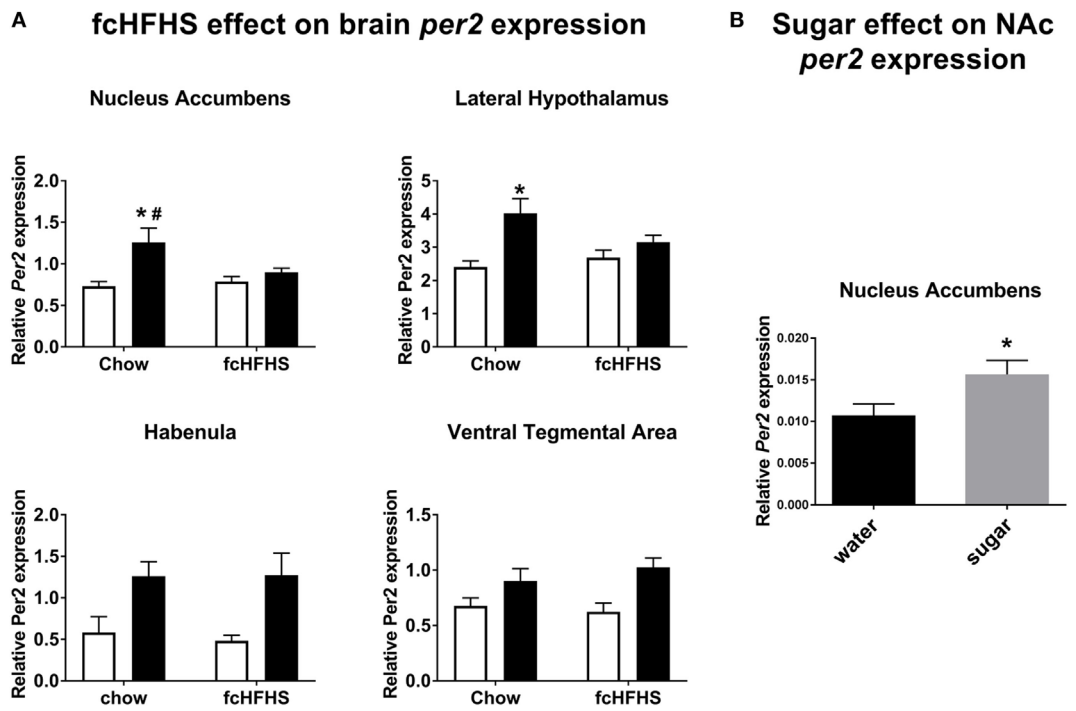

FIGURE 1 | *Per2* mRNA expression in nucleus accumbens (NAc) and lateral hypothalamus, but not habenula or ventral tegmental area, is altered by free-choice high-fat high-sugar (fcHFHS) diet exposure. **(A)** Day (white bars) night (black bars; time factor) expression of *Per2* in chow diet vs. fcHFHS diet groups (diet factor). All the structures showed significant day–night variations, and when an interaction was observed. * indicates a significant day–night difference of *Per2* expression; and # indicates a significant effect of diet (chow vs. fcHFHS) on *Per2* expression at night. **(B)** *Per2* mRNA expression in the NAc is significantly higher after sugar drinking compared with water drinking in chow-fed rats. * indicates a significant difference in *Per2* expression after water intake vs. sugar intake. Data are presented as mean ± SEM.

effects were observed [diet $F_{(1,22)} = 0.25$, $p = 0.61$; interaction $F_{(1,22)} = 1.6$, $p = 0.2$].

Next, we determined the direct effect of sugar intake on *Per2* mRNA expression in the NAc and observed that *Per2* mRNA was significantly increased by sugar ingestion (4.7 ± 0.1 kcal) compared with drinking water (**Figure 1B**).

DISCUSSION

We show that the fcHFHS diet produced a specific disruption in day–night *Per2* expression in the NAc and LH, which was not observed for *Cry1* and *Bmal1* mRNA expression. In the LH and NAc, *Per2* mRNA disruption caused by the fcHFHS diet exposure coincided with alterations of *Vglut2* mRNA (**Table 1**), a marker of glutamatergic activity and excitatory neuronal functions (13), suggesting a relation between the loss of daily *Per2* variation when consuming a fcHFHS diet and changes in neuronal activity. In none of the brain areas studied, we observed a day–night difference in *Vglut2* expression. This could be due to the timing of sampling, missing the trough or peak, or to the neuronal heterogeneity in the studied areas. However, we did observe a clear overall diet effect on *Vglut2* mRNA in the NAc at both time points measured. Given the importance of glutamate in the NAc for dopamine signaling and the previously reported effects of high energy diets on dopamine receptor binding (14), it might be that this reflects a dampening of neuronal activity of NAc dopamine neurons.

The changes in *Per2* mRNA expression, without changes in *Bmal1* and *Cry* mRNA in the NAc and LH of rats fed fcHFHS diet in this study are similar to previous results described in mice where the fcHFHS diet produced changes only in *PER2* but not in BMAL1 protein expression in the LHb (9). Also, after chronic alcohol intake in mice, a specific *Per2* mRNA acrophase shift was observed in the liver while *Cry* and *clock* remained unaffected (15). *In vitro*, the period length and acrophase of *Per2* mRNA expression in cultured hypothalamic neuronal cells are altered after glucose enrichment to the media, whereas *Bmal1* rhythmicity remained unaffected (16). The specific alteration of *Per2* could indicate that this gene is more sensitive than other clock genes to changes in the physiological state (e.g., hypercaloric feeding or chronic alcohol intake), as for instance, the ablation of dopaminergic cells of the VTA decrease *Per2* mRNA expression as well as its protein product (17) which could reflect a direct response to the microenvironment independent of a clock mechanism. On the other hand, it remains to be determined whether this specific *Per2* alteration might be due to an intra-cellular clock-gene desynchronization that could be reflecting an aberrant clock function.

We also showed that acute sugar consumption when given at the end of the light period increased *Per2* mRNA expression

in the NAc. Interestingly, mice with *ad libitum* access to a 5 and 10% sugared water solution consume it mainly during the night phase and this did not disturb *Per2* gene expression in the NAc (18). Taken together, these data suggest that time of sugar intake is an important factor to produce *Per2* alterations in the NAc and that intake at the "wrong" time disturbs the day–night expression of this clock gene. Furthermore, we observed in this study that rats with chronic access to the fcHFHS diet exhibited reduced *Per2* expression in the NAc and LH at night compared with the chow-fed rats. This could indicate that sugar ingestion, in behaviorally rhythmic animals, has to be accompanied with fat ingestion to produce the *Per2* reduction in NAc at night since in the experiment of Bainier et al. (18), where mice ingested only sugar (mainly during the night) *Per2* mRNA expression was similar compared with animals ingesting water. When chronically exposed to the fcHFHS diet which combines sugar and fat, also metabolic changes appear, including high basal blood glucose (19), thus it might be that this prolonged hyperglycemia impacts cell functioning and consequently, produces a clock-gene disruption in the NAc and LH, two areas with no self-sustained oscillations, in which normal rhythmicity could be overridden by abnormal physiological factors such as hyperglycemia. In line with such direct effects of glucose, the NAc and LH contain glucose-sensitive cells (20, 21).

Although we clearly show effects of the fcHFHS diet on *Per2* mRNA in NAc and LH, these changes were not accompanied by changes in feeding rhythm or expression of genes involved in feeding behavior. For example, *pre-pro-enkephalin* mRNA in chow-fed animals showed a clear difference between ZT4 and ZT16, but this was not affected by fcHFHS-diet feeding. Apparently, the changes in *Per2* alone in these areas are not sufficient to induce changes in the daily feeding pattern. Of note, an overall *Per2* mutation in mice does result in loss of the daily rhythm in sucrose drinking (18), pointing to a role for *Per2* in other areas of the brain (or body), or to developmental effects of *Per2* in feeding behavior.

The LH has direct glutamatergic projections to the LHb (22), which could have predicted changes in the Hb as well. We did not find, however, an effect of the fcHFHS diet on rhythmic *Per2* gene expression in the Hb. Possibly light is a stronger *zeitgeber* than food in the Hb, as there are clear light inputs to Hb (23) like is known for the SCN (which also still showed a day/night difference for clock genes). Earlier we showed, in mice, that PER2 protein in the LHb was affected by the fcHFHS diet (9); however, these mice showed clear changes in the daily feeding rhythm of fat and sugar. These results highlight the hierarchical organization of the circadian system; when disturbances are in "weak" brain oscillators (NAc and LH) this does not affect behavior. It remains to be confirmed when a spontaneous change of feeding patterns toward day time does occur in rats, whether this would be accompanied by the same *Per2* disruptions in the LHb as shown for day-snacking mice. We cannot discard that disruptions of *Per2* in NAc and LH could reflect a progressive alteration of the circadian system and with more profound obese state, other areas like LHb would also be compromised.

In this study, the fcHFHS diet did not result in high-fat and/or sugar intake during the light period, as we had previously observed in mice and rats (8, 9). This discrepancy might be due to the amounts of sugar and fat consumed. In previous studies, mice and rats consumed more fat (>30%) than sugar (25%) when fed a fcHFHS diet. In the current experiment, rats consumed only 10% of their total caloric intake as fat, whereas sugar intake was higher than shown before. It is unclear what caused this difference in intake; however, it does point to a role for dietary intake in feeding patterns. Previously we observed that rats, consuming more than 30% fat on the fcHFHS diet, consumed 40% of their sugar intake during the light period (8). Nonetheless, when rats were exposed to only sugar *ad libitum* in addition to chow (fcHS diet), sugar intake was mainly restricted to the dark period (8). The animals in the current experiment drank similar amounts of sugar as animals on the fcHS diet (8), thus, it could well be that although sugar can influence *Per2* in the reward circuitry, this is not sufficient to induce behavioral effects. This points to an additional factor linked to fat feeding that together with altered *Per2* expression mediates disruptions in palatable intake patterns, but only when the total fat intake exceeds a minimum amount. It is clear that sugar intake or fat intake alone does not disrupt behavioral rhythms in rats (8).

Taken together, we show that the fcHFHS diet and acute sugar drinking affect *Per2* gene expression in areas involved in food reward. These *Per2* expression changes, however, were not sufficient to alter feeding-related peptides or feeding behavior.

ETHICS STATEMENT

All experiments were approved by the Animal Ethics Committee of the Royal Netherlands Academy of Arts and Sciences (Amsterdam).

AUTHOR CONTRIBUTIONS

AB-V and SF designed experiments. AB-V, UU, LE, and LK performed experiments. AB-V and SF prepared the manuscript. UU, LE, LK, AK, and JM edited the manuscript. SF supervised the entire study.

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Time-of-Day Effects on Metabolic and Clock-Related Adjustments to Cold

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Background: Daily cyclic changes in environmental conditions are key signals for anticipatory and adaptive adjustments of most living species, including mammals. Lower ambient temperature stimulates the thermogenic activity of brown adipose tissue (BAT) and skeletal muscle. Given that the molecular components of the endogenous biological clock interact with thermal and metabolic mechanisms directly involved in the defense of body temperature, the present study evaluated the differential homeostatic responses to a cold stimulus at distinct time-windows of the light/dark-cycle.

Methods: Male Wistar rats were subjected to a single episode of 3 h cold ambient temperature (4°C) at one of 6 time-points starting at Zeitgeber Times 3, 7, 11, 15, 19, and 23. Metabolic rate, core body temperature, locomotor activity (LA), feeding, and drinking behaviors were recorded during control and cold conditions at each time-point. Immediately after the stimulus, rats were euthanized and both the soleus and BAT were collected for real-time PCR.

Results: During the light phase (i.e., inactive phase), cold exposure resulted in a slight hyperthermia ($p < 0.001$). Light phase cold exposure also increased metabolic rate and LA ($p < 0.001$). In addition, the prevalence of fat oxidative metabolism was attenuated during the inactive phase ($p < 0.001$). These metabolic changes were accompanied by time-of-day and tissue-specific changes in core clock gene expression, such as DBP ($p < 0.0001$) and REV-ERBα ($p < 0.01$) in the BAT and CLOCK ($p < 0.05$), PER2 ($p < 0.05$), CRY1 ($p < 0.05$), CRY2 ($p < 0.01$), and REV-ERBα ($p < 0.05$) in the soleus skeletal muscle. Moreover, genes involved in substrate oxidation and thermogenesis were affected in a time-of-day and tissue-specific manner by cold exposure.

Conclusion: The time-of-day modulation of substrate mobilization and oxidation during cold exposure provides a clear example of the circadian modulation of physiological and metabolic responses. Interestingly, after cold exposure, time-of-day mostly affected circadian clock gene expression in the soleus muscle, despite comparable changes in LA over the light–dark-cycle. The current findings add further evidence for tissue-specific actions of the internal clock in different peripheral organs such as skeletal muscle and BAT.

Keywords: circadian, locomotor activity, thermoregulation, thermogenesis, gene expression, skeletal muscle, brown adipose tissue

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INTRODUCTION

Daily cyclic changes in environmental conditions are key signals for the adaptive and anticipatory activity of most living species, including mammals. The mammalian thermoregulatory system is fairly adapted to periodic changes in ambient temperatures that may reach high amplitudes depending on the geographic location (1–3). Intriguingly, in mammals, a role for daily body temperature cycles in the internal synchronization has been demonstrated, *in vivo, ex vivo*, as well as *in vitro* (1, 4, 5), indicating that the circadian timing system and the thermoregulatory system reciprocally influence each other.

In general, during the dark phase, ambient temperature decreases to its lowest daily levels. Lower ambient temperatures induce metabolic changes aimed to defend internal body temperature. Considering this, it is well known that a cold environment induces autonomic, cardiovascular, metabolic, and behavioral adjustments that depend on the synchronized activation of multiple independent pathways resulting in thermal adaptation/acclimation (6). These adjustments include physiological changes such as vasoconstriction (heat retention or storage) and thermogenesis from both the activation of brown adipose tissue (BAT) (non-shivering) and skeletal muscle involuntary contraction (shivering) (6). Synchronized activation of the autonomic innervation to BAT and white adipose tissue (WAT), liver, adrenal, and skeletal muscle is necessary to produce the necessary amount of energy and heat to keep body temperature within safe levels during cold exposure (7).

Contractile skeletal muscle activity acts as an important heat source during environmental cold exposure in rodents (6, 8) and humans (9, 10). Increased ADP/ATP ratio, 5′adenosine monophosphate-activated protein kinase (AMPK) activity, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α) activity, and consequently, free-fatty acid (FFA) uptake and oxidation, increases intracellular substrate availability for heat production (11). Notably, in BAT, the requirement of a functional molecular clock has been demonstrated, as a deficiency in the expression of the BMAL-1 and PER2 clock gene leads to activation of compensatory heat production mechanisms (12, 13). In humans, it has recently been demonstrated that BAT glucose uptake might be associated with the heat production rhythm (14). The latter data indicate that the central body clock has an important role in the tuning of the cold-evoked response, probably by setting the basal metabolic rate to a new reference level or balancing the autonomic tonus.

Metabolic rate increases in response to cold through shivering and non-shivering thermogenesis. Both processes are centrally regulated (6) and result in increased lipid and carbohydrate oxidation in involved tissues (mainly skeletal muscle and BAT) (8, 15). Therefore, lipid mobilization (from WAT *lipolysis* and liver *de novo* lipogenesis) increases to provide the main substrate used for the thermogenic activity. Interestingly, the molecular clock influences substrate oxidation (16, 17). Therefore, modulation of the central thermoregulatory pathways by the biological clock in the suprachiasmatic nuclei (SCN) might result in changes in cold exposure adjustments depending on the time-of-day. In line with this hypothesis, the master clock projects to the major brain areas involved in metabolic/thermal balance (18, 19).

Core components of the molecular circadian clock are expressed throughout the body, both centrally and peripherally and interact with intracellular pathways directly related to metabolism and heat production (12–14, 16, 17, 20). In view of the above, the present study aimed to investigate how time-of-day modulates the peripheral adjustments induced by cold exposure at a physiological and a molecular level.

MATERIALS AND METHODS

Animals

All experiments were performed in adult male Wistar rats (Charles River Breeding Laboratories, Sulzfeld, Germany). After arrival at the animal facility, animals were housed in individual cages (25 cm × 25 cm × 35 cm), with a 12/12-h light–dark (L/D) schedule [lights on at 0700 h, defined as Zeitgeber Time 0 (ZT0)]. Animals were allowed to adapt to the new environment for 1.5 weeks before the first experiments. All rats were kept under constant temperature (22 ± 2°C) and humidity (50 ± 5%) conditions. Food and water were available *ad libitum*. The animal care committee of the Royal Netherlands Academy of Arts and Sciences (DEC/KNAW) approved all experiments.

Experimental Procedures

To verify where would be a good site to monitor internal body temperature, a pilot study group was completed before the described experiments. In this preliminary group, each animal was implanted with two data loggers: one in the dorsal subcutaneous area, caudal to the BAT and the other one inside the peritoneal cavity, stitched to the abdominal wall. Since these loggers are not radio-telemetry based, interference between them was not observed, as it would be expected for other available models. After recovery from surgery, animals were exposed to episodes of lowered ambient temperatures during light and dark phases of the L/D cycle. With this initial study, we were able to identify that the intraperitoneal loggers produced the most stable and reliable results.

Experimental animals were anesthetized with isoflurane, which guaranteed a rapid recovery from the small surgery necessary to insert the temperature sensors. A ventral incision at the *linea alba* was made to introduce a data-logger probe (DST nano-T, StarOddi, Iceland) into the peritoneal cavity. Each logger was sutured to the inner musculature before the incision was closed. This procedure allowed continuous monitoring of core body temperature (T_{core}) with a decreased risk of internal displacement of the sensor, which could cause misleading readings due to its position. These probes recorded internal T_{core} with 5-min intervals.

For the main study (presented in **Figure 1**), on the fourth day after surgery, the basal 24-h locomotor activity (LA), energy expenditure (EE), and food and water intake were continuously recorded for each animal with an indirect calorimetry system (PhenoMaster/LabMaster, TSE Systems, Bad Homburg, Germany). LA was assessed as beam-breaks recorded during 15 min intervals. Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were assessed every 15 min for 100 s. Respiratory exchange ratio (RER) was calculated according to the formula:

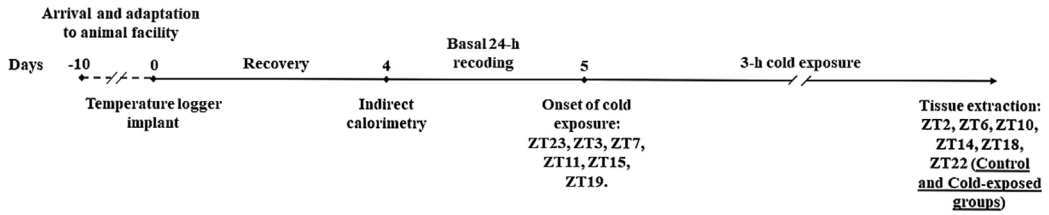

FIGURE 1 | Graphical representation of experimental paradigm.

VCO_2/VO_2. EE was calculated with the formula: $(CVO_2 \times VO_2 + CVCO_2 \times VCO_2)/1{,}000$, considering CVO_2 and $CVCO_2$ preset reference values given by the manufacturer ($CVO_2 = 3.941$ and $CVCO_2 = 1.106$). Carbohydrate (CHO) and lipid oxidation were calculated with the adapted *formulae* (21, 22) previously used (23): $(4.585 \times VCO_2 - 3.226 \times VO_2)/60{,}000$ for CHO oxidation and $(1.695 \times VO_2 - 1.701 \times VCO_2)/60{,}000$ for lipid oxidation. After the basal recordings, the ambient temperature was lowered to 0–5°C at different times of the day: ZT23–2, ZT3–6, ZT7–10, ZT11–14, ZT15–18, and ZT19–22. The average rate of ambient cooling was −0.8°C/10 min during the period of 3 h of cooling activity. Peak rates of −2.3°C/10 min were observed during the first 45 min of the experimental protocol, while rates lower than −0.5°C/10 min were observed and maintained after 90 min of cooling. By the end of each episode of lowered ambient temperature, animals were rapidly anesthetized with 80% CO_2 and immediately sacrificed by decapitation. For room temperature controls, animals were kept in the same experimental room, but outside the temperature controlled TSE chamber without any previous manipulation and were sacrificed at the same time points. After decapitation, the brain was removed, snap frozen on dry ice, and stored at −80°C. Soleus skeletal muscle and interscapular BAT were collected, frozen in liquid nitrogen, and then stored at −80°C.

Tissue Processing

RNA Extraction and cDNA Synthesis

Total RNA was extracted and purified with the TRIzol reagent protocol (Macherey-Nagel, Oensingen, Switzerland). The quality of RNA was examined by Agilent 2100 Bioanalyzer equipped with Nano chips (Agilent Technologies, Palo Alto, CA, USA) and concentrations were determined by Nanodrop spectrophotometer (ThermoScientific Technologies, Wilmington, DE, USA). A fixed amount of total RNA was reverse-transcribed with SensiFAST cDNA Synthesis Kit (Bioline, Taunton, MA, USA). For the control of genomic DNA contamination, we employed a minus reverse transcriptase sample (−RT).

Real-Time PCR (RT-PCR)

The expression of clock, metabolic, and thermogenesis-related genes was evaluated by RT-PCR (LightCycler ® 480, Roche) with the following reaction system: 2 µl of cDNA was incubated with 50 ng of both reverse and forward primer from gene of interest (see **Table 1** for primer sequences) and SensiFAST no-ROX Mix (Bioline, Taunton, MA, USA) for a final volume of 10 µl. The relative amount of each gene was normalized against the geometric mean of three housekeeping genes: hypoxanthine-guanine phosphoribosyl transferase (HPRT), ribosomal protein S18 (S18), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for the BAT; GAPDH, S18, and cyclophilin for the soleus muscle. The reference genes mentioned (HPRT, GAPDH, S18, and cyclophilin) were selected based on their constant expression under the various experimental conditions (i.e., time of the day and/or ambient temperature). The relative expression level of clock genes and other genes of interest in each sample was obtained by dividing the absolute amount of the target gene by the average of the reference genes values. Each gene/tissue RT-PCR was performed in a single plate.

Statistical Analysis

The effects of cold exposure and duration in T_{core}, LA, VO_2, RER, EE, CHO, and lipid oxidation, as well as food and water intake, were assessed with a repeated measures ANOVA two-way followed by an appropriate *post hoc* test. The net area under the curve (AUC) for each condition and parameter was calculated, i.e., the absolute change of the physiological variable during the analyzed experimental period compared to $t = 0$. "Net" AUC in this case means that the area of the negative peaks (i.e., decrease compared to $t = 0$) was subtracted from that of the positive peaks (i.e., increases compared to $t = 0$). To analyze the combined effect of cold exposure and time-of-day an ANOVA two-way was applied to the AUC data. The time-of-day effect was assessed with an independent ANOVA one-way for control and cold-exposed situations followed by *post hoc* analysis when appropriate. The effects of cold exposure and time-of-day on mRNA expression were assessed with ANOVA two-way followed by an appropriate *post hoc* test. Data are expressed as mean ± SEM. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Daily Oscillations of Body Temperature, LA, and Energy Metabolism

During the basal data collection, 24 h before the actual experiment, ambient temperature inside the calorimetric chamber was kept close to the typical animal facility temperature of 22.18 ± 0.21°C (**Figure 2A**), although a time-of-day effect was apparent (**Table 2**). Animals exhibited a clear day/night oscillation in all the studied variables, despite the high variability observed for both food and water intake (**Table 2**; **Figure 2**). Core body temperature (**Figure 2B**) and spontaneous LA (**Figure 2C**) peaked during the active phase. The increased activity was

TABLE 1 | Information about gene primers.

Genes	Reference no.	Reverse	Forward
Clock genes			
PER1	NM_001034125.1	TGGCCAGGATCTTGAACACTGCTA	ATGCAGAAACAACAGCCACGGTTC
PER2	NM_031678.1	CAACGCCAAGGAGCTCAAGT	CACCCTGAAAAGAAAGTGCGA
CRY1	NM_198750.2	TCATCATGGTCGTCGGACAGA	AAGTCATCGTGCGCATTTCA
CRY2	NM_133405.2	TGTACAAGTCCCACAGGCGGTA	TGGATAAGCACTTGGAACGGAA
ARNLT/BMAL-1	NM_024362.2	TGCAGTGTCCGAGGAAGATAGC	CCGATGACGAACTGAAACACCT
CLOCK	NM_021856.2	TTGCAGCTTGAGACATCGCT	CGATCACAGCCCAACTCCTT
DBP	NM_012543.3	TGCCTTCTTCATGATTGGCTG	CCTTTGAACCTGATCCGGCT
REV-ERBα	NM_001113422.1	CATGGGCATAGGTGAAGATTTCT	ACAGCTGACACCACCCAGATC
Metabolic genes			
CREB1	NM_031017.1	ACTCTGCTGGTTGTCTGCTC	GCAGTGACTGAGGAGCTTGT
PGC1-α	NM_031347.1	GGTCATTTGGTGACTCTGG	TGCCATTGTTAAGACCGAG
PGC1-β	NM_176075.2	AGGAGGGCTCATTGCGTTTT	AAAAGGCCATCGGTGAAGGT
PPAR-α	NM_013196.1	GGCCTTGACCTTGTTCATGT	TCACACAATGCAATCCGTTT
PPAR-γ	NM_013124.3	GGGGGTGATATGTTTGAACTTG	CAGGAAAGACAACAGACAAATCA
HSP90	NM_001004082.3	ACCGAATCTTGTCCAGGGCATCA	CGGGCCCACCCTGCTCTGTA
UCP1	NM_012682.2	GCTTTGTGCTTGCATTCTGA	AATCAGCTTTGCTTCCCTCA
UCP2	NM_019354.3	GGGCACCTGTGGTGCTAC	GACTCTGTAAAGCAGTTCTACACCAA
UCP3	NM_013167.2	ATAGTCAGGATGGTACCGAGCA	GCACTGCAGCCTGTTTTGCTGA
CIRBP	NM_031147.2	TAACCACCACCCCTCCAGAA	GCGTTAGGAAGCTTGGGTGT
CPT1-α	NM_031559.2	AAAGACTGGCGCTGCTCA	ACAATGGGACATTCCAGGAG
CPT1-β	NM_013200.1	TGCTTGACGGATGTGGTTCC	GTGCTGGAGGTGGCTTTGGT-
AMPK	NM_019142.2	TAGAGAATGACCCCGCTGCT	TGTCACAGGCATATGGTGGTC
NAMPT	NM_177928.3	TCGACACTATCAGGTGTCTCAG	ACAGATACTGTGGCGGGAATTGCT
FAT/CD36	NM_001109218.1	CCTTGGCTAAATAACGAACTCTG	ACAGTTTTGGATCTTTGACGTG
GLUT4	NM_012751.1	CAGCGAGGCAAGGCTAGA	GGGCTGTGAGTGAGTGCTTTC
HSL	NM_012859.1	CCACCCGTAAAGAGGGAACT	TCACGCTACATAAAGGCTGCT
LPL	NM_012598.2	AGCAATTCCCCGATGTCCA	CAAAACAACCAGGCCTTCGA
ADR-β2	NM_012739.3	CGACCGCTATGAGCGTGTAG	CGCTTCACGTTCGTGCTGGC
ADR-β3	NM_013108.2	CCTTGCTAGATCTCCATGG	CTTCCCAGCTAGCCCTGTT
GR	NM_012576	GGAGCAAAGCAGAGCAGGTTT	ACCTGGATGACCAAATGACCC
FOXO-1	NM_001191846.2	GTAGGGACAGATTGTGGCGAA	ACGAGTGGATGGTGAAGAGTG
ACC1	NM_022193.1	CAGGCTACCATGCCAATCTC	GATGATCAAGGCCAGCTTGT
ACC2	NM_053922.1	GCTTCCGCTCCAGGGTAGAGT	GCACGAGATTGCTTTCCTAG
mTOR	NM_019906.1	CCCGAGGAATCATACAGGTG	AGCAGCATGGGGTTTAGGT
CamK2a	NM_012920.1	AAGGCTGTCATTCCAGGGTC	TGGCGTGAAGGAATCCTCTG
Housekeeping genes			
S18	NM_213557.1	TGGCCAGAACCTGGCTATACTTCC	CTCTTCCACAGGAGGCCTACACG
HPRT1	NM_012583.2	AACAAAGTCTGGCCTGTATCCAA	GCAGTACAGCCCCAAAATGG
GAPDH	NM_017008.4	TCCACCACCCTGTTGCTGTA	TGAACGGGAAGCTCACTGG
Cyclophilin	NM_017101.1	GAAGGAATGGTTTGATGGGT	ATGTGGTCTTTGGGAAGGTG

associated with increased food and water intake (**Figures 2D,E**) and resulted in augmented oxygen consumption (**Figure 2F**) and heat production (**Figure 2G**). During the active phase, substrate utilization was shifted toward carbohydrate oxidation, while during the rest phase, lipid metabolism was predominant as observed through RER (**Figure 2H**). The baseline levels of thermal, behavioral, and metabolic parameters at the onset of our cooling paradigm were similar to those observed during the previous day and both exhibited time of the day related changes (Table S1 in Supplementary Material).

Effect of Time-of-Day on Body Temperature and EE During Acute Environmental Cooling

Average ambient temperature after 2 h of cooling was 4.01 ± 0.07°C, regardless of time of the day. Body temperature was affected by cold exposure in a time-of-day-dependent fashion (**Figures 3A,B**). When experimental cooling started during the light phase (ZT3, 7, and 11), a slight and transient hyperthermia was observed ($p < 0.001$). Interestingly, during the ZT15–18 protocol, core body temperature stayed elevated during the final 120 min of cold exposure ($p < 0.001$), despite the normal decrease in this parameter observed in the control animals. Cold exposure during the ZT19–22 and ZT23–2 protocols did not affect core body temperature as compared to baseline values. The time-of-day-dependent body temperature responses were confirmed by the AUC analysis (**Figure 3B**; **Table 3**; Table S2 in Supplementary Material), showing a significant effect of both ZT and cold exposure.

Body temperature is the result of heat production and heat dissipation. Therefore, understanding heat production dynamics during cold exposure at different phases of the light/dark daily cycle would be of value for the present analysis. A good

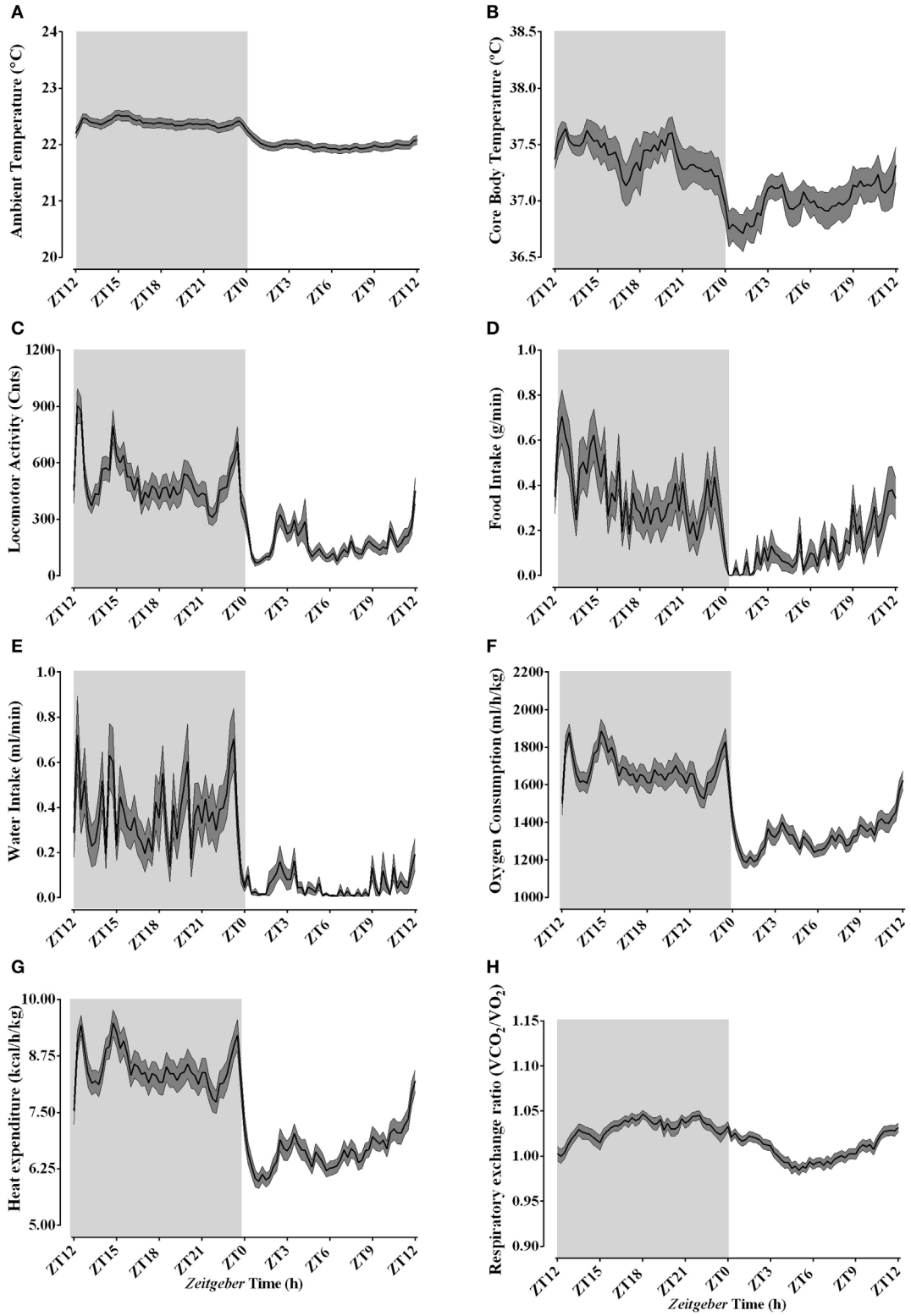

FIGURE 2 | Daily rhythms of thermal, behavioral, and metabolic parameters under regular animal facility conditions. Light phase begins at Zeitgeber Time 0 (ZT0) and dark phase (shaded areas) begins at ZT12. Regular daily oscillatory levels of the ambient temperature **(A)**, body temperature **(B)**, locomotor activity **(C)**, food intake **(D)**, water intake **(E)**, oxygen consumption **(F)**, heat production **(G)**, and respiratory quotient **(H)** were monitored approximately 24 h before the cold episodes. Results are presented as mean ± SEM (n = 34–50).

TABLE 2 | Mean basal 24-h and light/dark levels of thermal, behavioral, and metabolic parameters under control conditions (24 h before environmental cooling).

	$Mean_{24\text{-}h} \pm SD$	$Mean_{light\ phase} \pm SD$	$Mean_{dark\ phase} \pm SD$	*n*	Time-of-day effect	*p* Value
Ambient temperature (°C)	22.18 ± 0.21	21.99 ± 0.07	22.39 ± 0.06	50	$F(96, 4.753) = 7.059$	<0.0001
Core body temperature (°C)	37.20 ± 0.25	37.00 ± 0.14	37.40 ± 0.14	34	$F(96, 3.201) = 2.695$	<0.0001
Locomotor activity (Cnts)	337.1 ± 194.8	175.5 ± 81.1	502.1 ± 125.6	50	$F(96, 4.753) = 12.54$	<0.0001
Oxygen consumption (ml/h/kg)	1,500 ± 196.1	1,327 ± 86.65	1,677 ± 86.64	50	$F(96, 4.753) = 14.44$	<0.0001
Respiratory exchange ratio (VO_2/CO_2)	1.02 ± 0.02	1.03 ± 0.01	1.01 ± 0.01	50	$F(96, 4.753) = 8.036$	<0.0001
Heat Expenditure (kcal/h/kg)	7.56 ± 1.00	6.67 ± 0.44	8.47 ± 0.42	50	$F(96, 4.753) = 15.51$	<0.0001
Food intake (g/15 min)	0.24 ± 0.17	0.12 ± 0.10	0.36 ± 0.12	50	$F(96, 4.749) = 4.241$	<0.0001
Water intake (ml/15 min)	0.21 = 0.19	0.05 ± 0.05	0.37 ± 0.15	49	$F(96, 4.749) = 5.263$	<0.0001

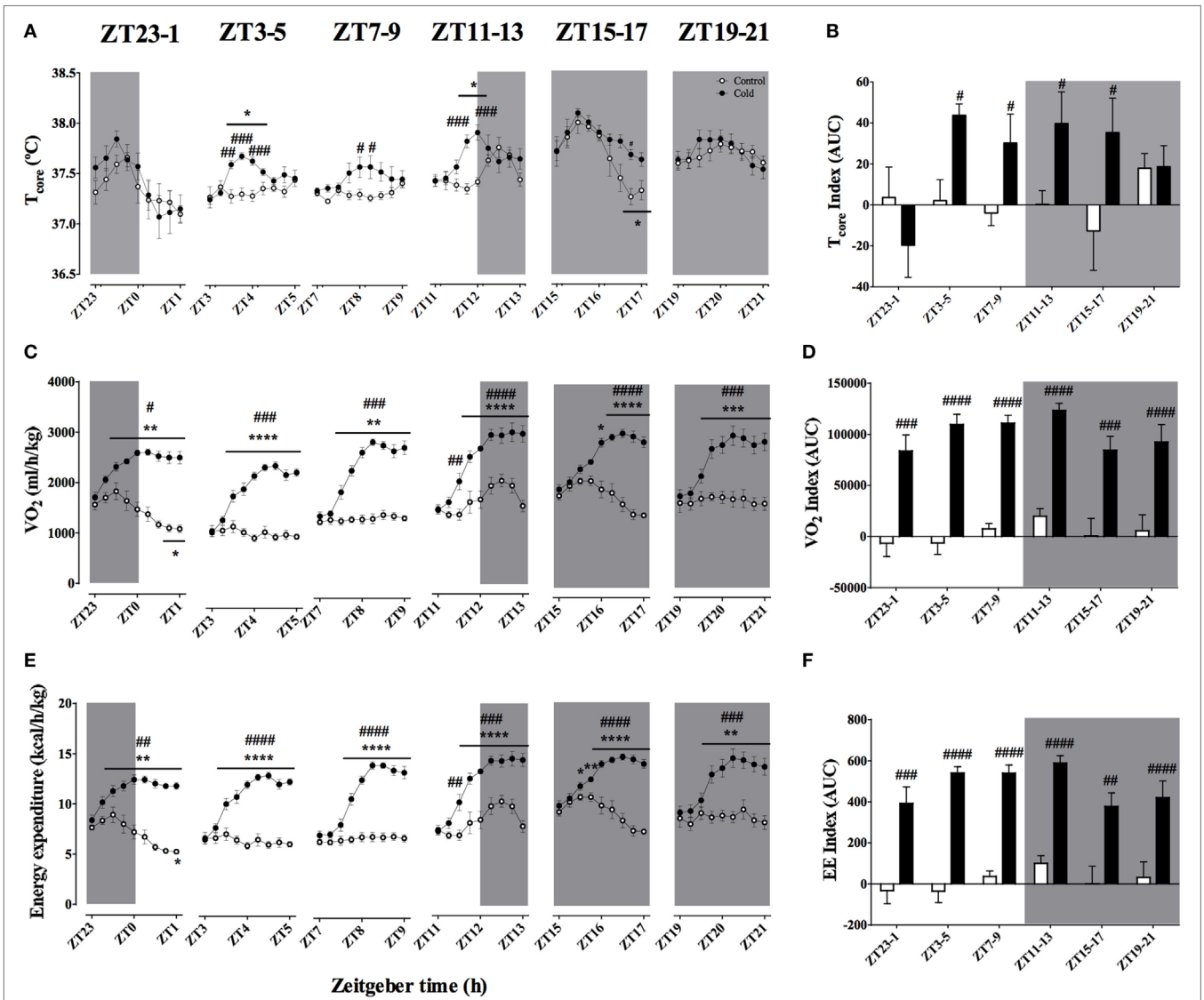

FIGURE 3 | Effect of time-of-day on body temperature **(A,B)**, VO_2 **(C,D)**, and energy expenditure **(E,F)** changes induced by environmental cooling. Results are presented as averages of 15 min bins during 2 h of environmental cooling **(A,C,E)** and as the area under the curve (AUC) **(B,D,F)** calculated as the net change from the basal levels before cold exposure. Light phase began at Zeitgeber Time 0 (ZT0) and dark phase (shaded areas) began on ZT12. Results are presented as mean ± SEM. * indicates differences between the first and the other time-points within the control or the cold-exposed group, $p < 0.05$. # indicates differences between control and cold conditions, $p < 0.05$. $n = 5$–11/group/ZT.

proxy for heat production is oxygen consumption. Oxygen consumption showed a strong increase during cold exposure ($p < 0.0001$; **Figures 3C,D**), however, despite the observed effect of ZT, there was no interaction between the two factors (**Table 3**). As soon as the ambient temperature dropped, VO_2 rose until reaching a steady state, usually about 60 min after

TABLE 3 | ANOVA two-way table (ZT × ambient temperature) demonstrating the effect of cold exposure and time of the day on net area under the curve (in arbitrary units for each parameter) for thermal, behavioral, and metabolic parameters.

	Effect of ZT		Effect of cold		Interaction	
	F(DFn, DFd)	*p* Value	*F*(DFn, DFd)	*p* Value	*F*(DFn, DFd)	*p* Value
Body temperature	$F(5, 56) = 3.734$	0.0055	$F(1, 56) = 4.487$	0.0386	$F(5, 56) = 2.183$	0.0689
Locomotor activity	$F(5, 74) = 0.372$	0.8665	$F(1, 74) = 42.21$	<0.0001	$F(5, 74) = 0.839$	0.5265
VO_2	$F(5, 88) = 3.743$	0.0040	$F(1, 88) = 201.2$	<0.0001	$F(5, 88) = 0.3105$	0.9055
Respiratory exchange ratio	$F(5, 88) = 3.404$	0.0074	$F(1, 88) = 107.6$	<0.0001	$F(5, 88) = 5.558$	0.0002
CHO oxidation	$F(5, 88) = 3.314$	0.0087	$F(1, 88) = 1.619$	0.2067	$F(5, 88) = 4.435$	0.0012
Lipid oxidation	$F(5, 88) = 1.051$	0.3931	$F(1, 88) = 72.19$	<0.0001	$F(5, 88) = 2.017$	0.0839
Energy expenditure	$F(5, 88) = 3.889$	0.0031	$F(1, 88) = 230.2$	<0.0001	$F(5, 88) = 2.405$	0.0430
Food intake	$F(5, 86) = 0.074$	0.9960	$F(1, 86) = 2.434$	0.1224	$F(5, 86) = 1.018$	0.4123
Water intake	$F(5, 88) = 1.065$	0.3851	$F(1, 88) = 0.037$	0.8470	$F(5, 88) = 1.433$	0.2203

the start of the protocol ($p < 0.0001$). Together with the rise in metabolic rate (VO_2), EE significantly increased with cold exposure (**Figures 3E,F**), independent of the time-of-day (**Figure 3F**; **Table 3**).

Effect of Time-of-Day on LA and Food/Water Intake During Acute Environmental Cooling

In view of the observed effects of time-of-day on the changes in body temperature and heat production in response to a cold environment, it was necessary to investigate whether cold-induced changes in LA or food and/or water intake could play a role. Despite their intrinsic relationship, the changes in T_{core} were not strictly accompanied by changes in LA in a time-of-day-dependent manner (**Figures 4A,B**; **Table 3**). Cold exposure induced an increase in LA (**Figures 4A,B**) that was independent of *Zeitgeber* time (**Table 3**; Table S2 in Supplementary Material). Regarding food and water intake, regardless of time-of-day cold exposure did not affect these parameters (**Figures 4C–F**; **Table 3**).

Effect of Time-of-Day on Respiratory Quotient and Substrate Oxidation During Acute Environmental Cooling

The respiratory quotient, an index of substrate oxidation prevalence, decreased during every cold exposure, an effect that was modulated by time-of-day (**Figures 5A,B**; **Table 3**). In line with a decrease in RER, cold exposure evoked a consistent increase in lipid oxidation (**Figures 5E,F**). Interestingly, the higher lipid oxidation's AUC was apparent only at ZT7–9, 11–13, 15–17, and 19–21, indicating that this rise was accentuated during the dark phase (**Figure 5F**). Although carbohydrate (CHO) oxidation was not significantly affected by cold exposure, it showed a time-of-day association (**Figures 5C,D**; **Table 3**). Increased CHO oxidation was observed only during the ZT3–6 protocol and the first hour of the ZT11–14 protocol ($p < 0.001$). On the other hand, CHO oxidation decreased when animals were exposed to cold during the dark phase, especially at ZT15–18 and 19–22 ($p < 0.05$), when also the largest decreases in RER were observed.

Effect of Time-of-Day on Clock Genes mRNA Expression in Skeletal Muscle and BAT After Acute Environmental Cooling

The mRNA expression of eight clock genes was investigated in both soleus muscle and BAT (**Tables 4** and **5**; **Figure 5**). Time-of-day affected the expression of five of eight and six of eight clock genes in soleus muscle and BAT, respectively. Expression of CRY2 and CLOCK genes was not significantly affected by time-of-day in either tissue, although CLOCK gene expression showed a tendency in the BAT ($p = 0.054$). Cold exposure caused a significant change in most (seven of eight) of the clock genes studied in BAT and half of the clock genes studied in the soleus muscle. In the BAT, time-of-day interacted with cold exposure only for DBP and REV-ERBα expression. In soleus muscle, time-of-day interacted with cold exposure for clock gene expression of five of eight of the genes studied (CLOCK, PER2, CRY1, CRY2, and REV-ERBα). BMAL-1 and DBP were significantly affected by cold exposure in the BAT ($p < 0.001$ and $p < 0.0001$), but not in soleus muscle ($p = 0.479$ and $p = 0.313$). While BMAL-1 was increased by cold exposure especially during the light phase, DBP decreased after the ZT7–10 and ZT11–14 protocols. The negative repressor loop of the core clock (PER1, PER2, CRY1, and CRY2) was upregulated in both BAT and soleus muscle. Interestingly, time-of-day interacted with the increased mRNA expression in three of four of the genes in soleus muscle, but with none of the genes in BAT. Finally, REV-ERBα mRNA expression showed a strong interaction effect of time-of-day and cold exposure in both tissues. In fact, except for ZT3–6, REV-ERBα expression was significantly decreased by cold exposure, with a steep reduction after ZT7–10 in both tissues.

Effect of Time-of-Day on Metabolic Genes mRNA Expression in BAT After Acute Environmental Cooling

We studied 18 genes directly or indirectly related to metabolism or thermogenesis in the BAT (**Table 4**; **Figure 6**). Time-of-day affected the mRNA expression of 6 of 18 of the genes studied (PGC1-α, PPAR-α, UCP1, CIRBP, AMPK, and HSL) and just missed significance in 3/18 (PGC1-β, LPL, and ADR-β3). Cold exposure affected most of the BAT genes studied (13 of 18 genes) and just missed significance for GR mRNA expression ($p = 0.051$).

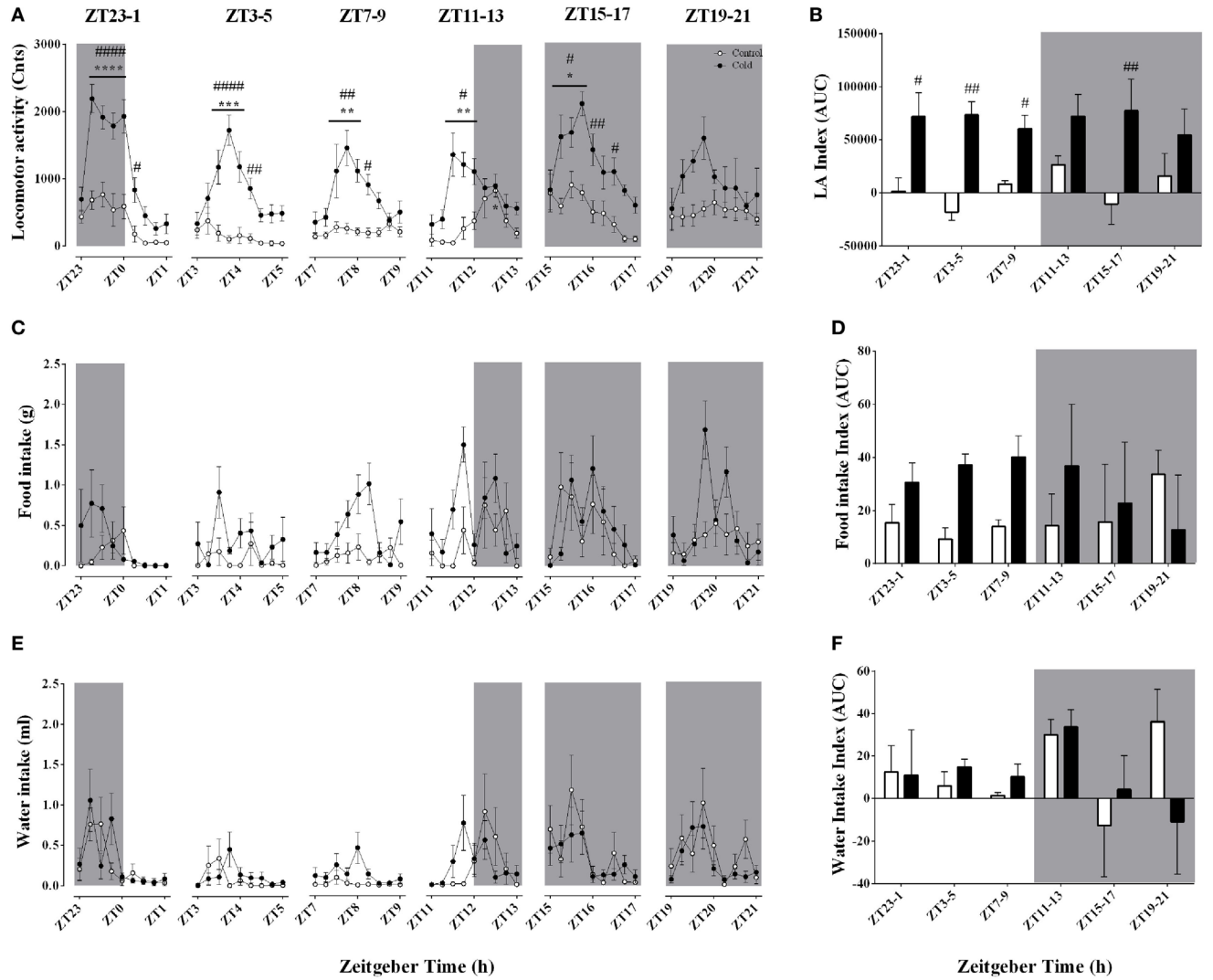

FIGURE 4 | Effect of time-of-day on locomotor activity **(A,B)**, food intake **(C,D)**, and water intake **(E,F)** changes induced by environmental cooling. Results are presented as averages of 15 min bins during 2 h of environmental cooling **(A,C,E)** and area under the curve (AUC) **(B,D,F)** calculated as the net change from the basal levels before cold exposure. Light phase began at ZT and dark phase (shaded areas) began on ZT12. Results are presented as mean ± SEM. * indicates differences between the first and the other time-points within the control or the cold-exposed group, $p < 0.05$. # indicates differences between control and cold conditions, $p < 0.05$. $n = 7–11$/group/ZT.

Only 4 of 18 of the investigated genes showed a significant interaction between time-of-day and cold exposure (PGC1-α, HSP90, CIRBP, and ACC2), while PGC1-β and PPAR-α almost reached significance. The genes that were most clearly affected by cold exposure were PGC1-α, HSP90, UCP1, CIRBP, and LPL ($p < 0.0001$).

Effect of Time-of-Day on Metabolic Genes mRNA Expression in Skeletal Muscle After Acute Environmental Cooling

We studied 23 genes directly or indirectly related to metabolism or thermogenesis in the soleus muscle (**Table 5**; **Figure 7**). Time-of-day affected the mRNA expression of 7 of 23 genes (PGC1-α, PPAR-α, HSP90, UCP3, CPT1-α, GLUT4, and ADR-β2) and just missed significance for NAMPT-1 expression ($p = 0.066$). Cold exposure affected mRNA expression of 17 of 23 of the studied genes and almost reached significance for FAT/CD36 levels ($p = 0.083$). The interaction between time-of-day and cold exposure was significant for 11 of 23 of the studied genes and just missed significance for 4 of 23 genes (CREB, PGC1-β, GR, and ACC2). The genes that were most clearly affected by cold exposure were PGC1-α, GLUT4, HSL, ADRβ2, and GR ($p < 0.0001$).

DISCUSSION

The main finding of the present study is that the cold-induced metabolic response and changes in gene expression in BAT and

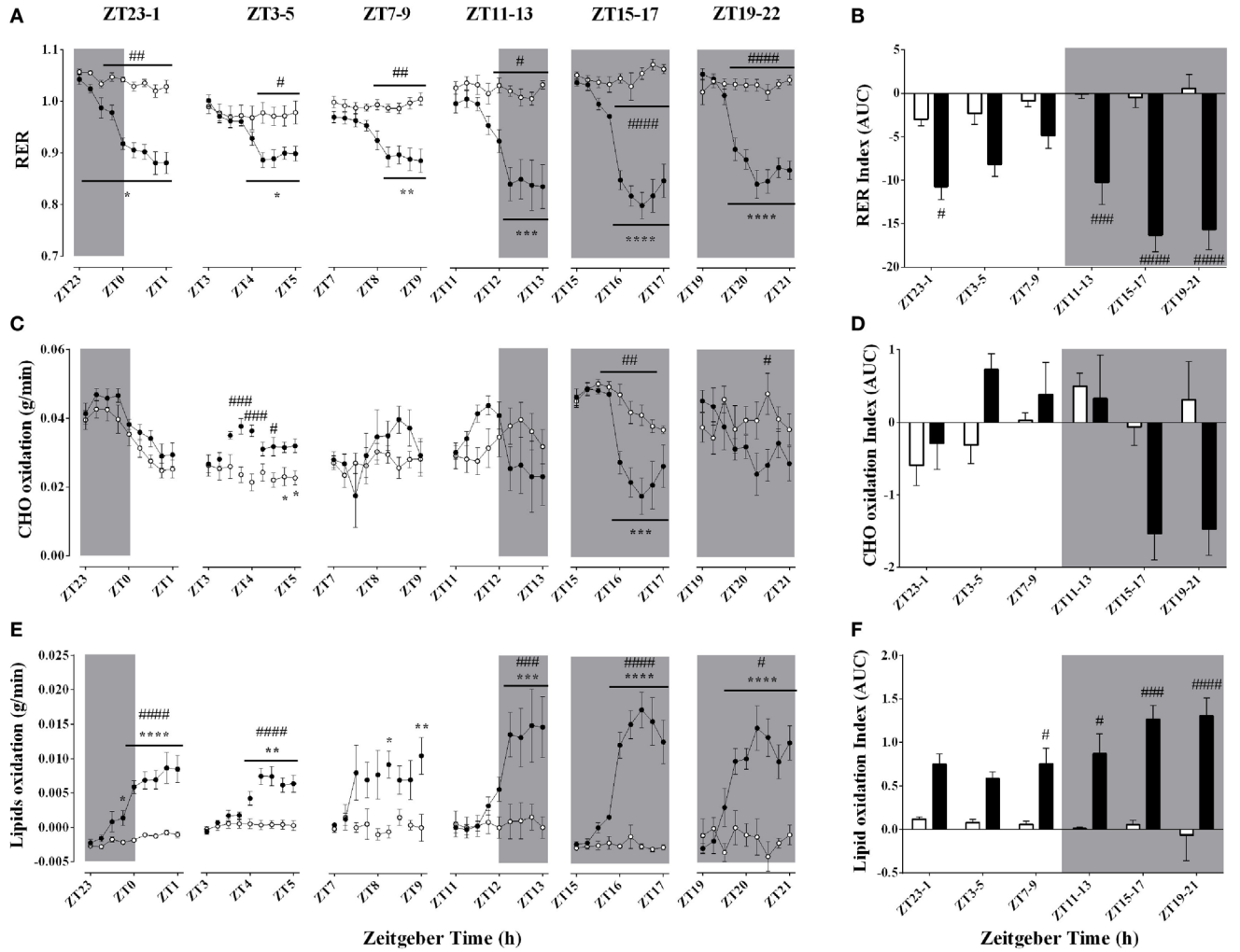

FIGURE 5 | Effect of time-of-day on respiratory quotient **(A,B)**, carbohydrate **(C,D)**, and lipid oxidation **(E,F)** changes induced by environmental cooling. Results are presented as averages of 15 min bins during 2 h of environmental cooling **(A,C,E)** and area under the curve (AUC) **(B,D,F)** calculated as the net change from the basal levels before cold exposure. Light phase began at Zeitgeber Time 0 (ZT0) and dark phase (shaded areas) began on ZT12. Results are presented as mean ± SEM * indicates differences between the first and the other time-points within the control or the cold-exposed group, $p < 0.05$. # indicates differences between control and cold conditions, $p < 0.05$. $n = 7–11$/group/ZT.

muscle differ depending on the time-of-day of the cold exposure. For instance, the cold-induced increase in lipid oxidation was mainly observed during the dark phase (**Figures 5E,F**), indicating a daily modulation of the cold-induced metabolic adaptations. In the soleus skeletal muscle, particularly active during shivering thermogenesis, cold exposure increased the expression of clock genes in both the negative and positive loop of the core clock mechanism, PER/CRY and BMAL-1/CLOCK, respectively (**Figure 6**). In addition, besides upregulating clock gene expression in both regulatory loops of the BAT molecular clock as well, cold exposure inhibited the expression of DBP and REV-ERBα in BAT (**Figure 6**). The current findings add further evidence for a tissue-specific action of the internal clock in peripheral tissues such as the skeletal muscle and BAT. Whether this differential modulation in response to environmental *stimuli* relies on the activity of the central clock, extra-SCN sites within the central nervous system or peripheral inputs remains to be further elucidated.

Effects of Cold Exposure During Different Times-of-Day on Thermogenesis and Substrate Oxidation

The thermal and metabolic adjustments induced by an acute exposure to a cold environment are well known for mice (24–29), rats (6, 8, 30–32), and humans (9, 33–35). Our experiments confirmed the effects of cold on the induction of increased heat production mostly through the concerted modulation of behavioral (LA, food, and water intake) and metabolic (T_{core}, VO_2, RER, EE, CHO, and lipid oxidation) processes. In accordance with our hypothesis, we found that time-of-day modulated the thermal, behavioral, and metabolic responses.

TABLE 4 | Significance levels observed for the two-way ANOVA analysis of gene expression in the brown adipose tissue.

	n	Time-of-day	Cold exposure	Interaction
BMAL-1	80	<0.0001****	0.0002***	0.4584
CLOCK	79	0.0542	0.0018**	0.4676
PER1	82	0.0657	<0.0001****	0.4003
PER2	80	<0.0001****	<0.0001****	0.1492
CRY1	82	0.0003***	<0.0001****	0.0580
CRY2	83	0.2550	<0.0001****	0.3824
DBP	82	<0.0001***	<0.0001****	<0.0001****
REV-ERBα	8C	<0.0001***	0.3983	0.0018**
CREB	83	0.3688	0.0010**	0.2376
PGCl-α	83	0.0022**	<0.0001****	0.0024**
PGC1-β	81	0.0673	0.8981	0.0568
PPAR-α	82	0.0196*	0.3069	0.0592
PPAR-γ	83	0.1241	0.1606	0.1145
HSP90	83	0.5366	<0.0001****	0.0321*
UCP1	82	0.0043**	<0.0001****	0.3767
CIRP	81	<0.0001****	<0.0001****	<0.0001****
CPT1-β	81	0.2195	0.0011**	0.2755
AMPK	82	0.0402*	0.0053**	0.2437
FAT/CD36	81	0.2195	0.0038**	0.3579
GLUT4	83	0.4685	0.0310*	0.4725
HSL	81	0.0203*	0.0390*	0.2161
LPL	80	0.0756	0.0001****	0.4909
ADR-β3	79	0.0572	0.0412*	0.2272
GR	82	0.2009	0.0506	0.2960
ACC1	73	0.4955	0.0272*	0.5446
ACC2	79	0.4957	0.3617	0.0221*

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

TABLE 5 | Significance levels observed for the two-way ANOVA analysis of the gene expression in the *soleus* muscle.

	n	Time-of-day	Cold exposure	Interaction
BMAL-1	80	<0.0001****	0.4785	0.6391
CLOCK	83	0.1868	0.0817	0.0488*
PER1	80	0.0010**	<0.0001****	0.2543
PER2	78	<0.0001****	<0.0001****	0.0183*
CRY1	81	<0.0001****	<0.0001****	0.0199*
CRY2	82	0.3535	0.0002***	0.0016**
DBP	81	<0.0001****	0.3126	0.1582
REV-ERBα	76	<0.0001****	0.7068	0.0308*
CREB	83	0.7026	0.0229*	0.0521
PGC1-α	80	<0.0001****	<0.0001****	0.0006***
PGC1-β	79	0.3707	0.0297*	0.0984
PPAR-α	82	0.0340*	0.0169*	0.0136*
PPAR-γ	80	0.7800	0.0023**	0.3825
HSP90	78	0.0401*	0.1641	0.0015**
UCP2	65	0.5029	0.0189*	0.5634
UCP3	77	<0.0001****	0.9447	0.0009***
CIRP	83	0.7462	0.4239	0.2013
CPT1-α	79	0.0084**	0.0009***	0.0285*
CPT1-β	82	0.1321	0.3225	0.0004***
AMPK	83	0.4686	0.0008***	0.0219*
NAMPT-1	82	0.0655	0.0071**	0.0234*
FAS/CD36	81	0.5285	0.0830	0.0067**
GLUT4	81	0.0071**	<0.0001****	0.0062**
HSL	80	0.3865	<0.0001****	0.1707
LPL	83	0.4184	0.0200*	0.0125*
ADR-β2	82	0.0170*	<0.0001****	0.2198
GR	83	0.3263	<0.0001****	0.0642
FOXO-1	80	0.3576	0.0005***	0.3377
ACC2	13	0.3244	0.0300*	0.0919
mTOR	74	0.5338	0.3857	0.2628
CamK2a	79	0.7143	0.0104*	0.3953

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Significant decrements of ambient temperature pose a primitive threat to body temperature regulation. In order to keep body temperature within physiological levels, the central nervous system modulates a number of physiological processes involved in heat conservation and production. In the present experiment, we observed that rat T_{core} is resilient to a temporally limited exposure to a reduced ambient temperature. In fact, after 120 min of environmental cooling, normal T_{core} was preserved for each time-of-day exposure (**Figure 3A**). Curiously, we even detected a small rise in T_{core} when the animals were challenged with a cold environment presented during the light phase, an effect that is not observed in smaller rodents, such as mice (24–29). This mismatch of heat production and heat dissipation (leading to body heating) might be due to a heat defensive state potentiated by the sudden decrease in ambient temperature during the sleep period.

Indeed, a lower T_{core} during the light phase is maintained through tail vasoconstriction and decreased EE (36–39). In such a condition, the T_{core} regulatory system likely presents a higher sensitivity to changes in locomotor and metabolic activity (40), resulting in increased T_{core} during the first hour of cold exposure in the light phase, when heat production was increased (**Figure 3A**). Conversely, during the dark phase, basal T_{core} is slightly raised as a function of the increased LA, EE, and circadian rhythm (39), making the immediate impact of the increased metabolic rate on T_{core} less perceptible.

Indeed, the well-described circadian-dependent decrease in T_{core} during the second half of dark phase seems to be counterbalanced by heat production induced by cold exposure (**Figure 3A**, ZT15–17). This cold-induced hyperthermia was also reported by others (6, 31, 41–43). Initially, heat dissipation is reduced to minimize heat loss to the colder environment (6). As cold exposure is maintained, heat production is increased and heat loss and production reach a steady state in which T_{core} can be successfully preserved. Therefore, as previously observed with other stressors (40, 44–47), it seems that the time-of-day-dependent effects of cold exposure on body temperature reflect the transitory disturbance in heat loss and heat production mechanisms elicited by mixed signals from the internal circadian time and the thermoregulatory pathways that participate in the homeostatic responses.

In line with this perspective, the increased metabolic rate (**Figures 3C,D**) and LA (**Figures 4A,B**) in response to cold exposure might also reflect environmental temperature gradient (intensity of the stimulus) rather than a time-of-day-dependent modulation. Interestingly, LA spiked during the first hour of the cooling protocol but decreased thereafter (**Figure 4A**). Similar results were described by others (43, 48, 49), suggesting that this probably reflects coupled mechanisms of heat conservation (cold avoidance behavior reflecting increased LA) and heat production (shivering thermogenesis reflecting the decreased LA). In fact, as the environment gets colder, the animals lessen their LA without dampening metabolic rate and EE (**Figures 3C,E**), which points toward increased shivering activity of thermogenic pathways. Moreover, both oxygen consumption and calculated EE reached

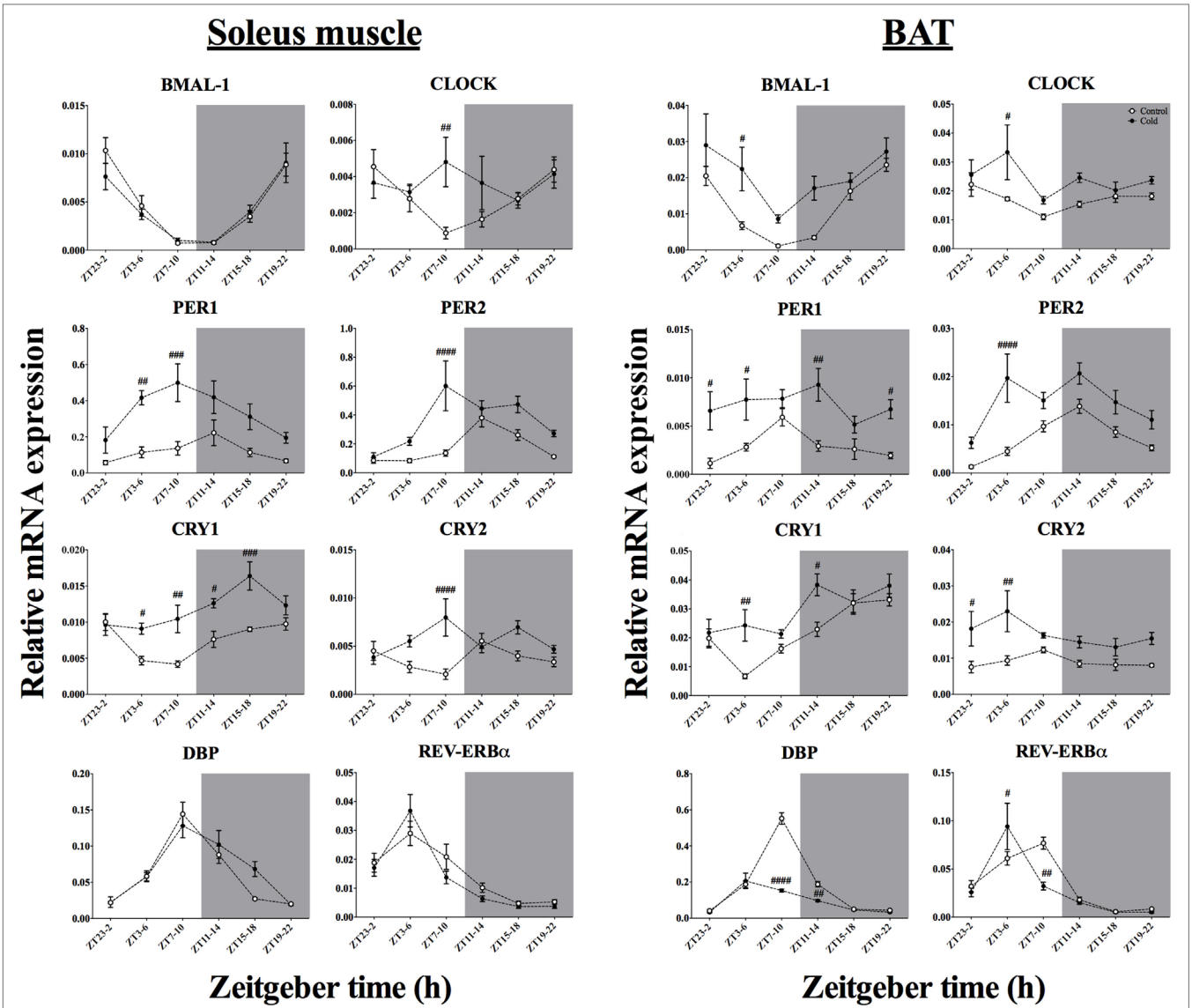

FIGURE 6 | Effect of time-of-day on clock gene expression in both soleus muscle and brown adipose tissue (BAT) after exposure to environmental cooling. Light phase began at Zeitgeber Time 0 (ZT0) and dark phase (shaded areas) began at ZT12. Results are presented as mean ± SEM. $p < 0.05$. # indicates differences between control and cold conditions, $p < 0.05$. $n = 5–7$/group/ZT.

steady states of increased activity regardless of time-of-day. This is in accordance with previous experiments in mice showing that the relative changes in metabolic rate were similar when the cooling protocol started at the beginning of the light or dark phase (27, 29). Interestingly, Tokizawa and colleagues (29) observed that the threshold for increased heat production was elevated during the dark phase, while behavioral curling was elicited earlier during the light phase. In rats, a similar effect of time-of-day on thermoregulatory thresholds was also observed during exercise (47). To our knowledge, this is the first time that a time-of-day effect on the cold defensive response is reported for the rat model. With the present results, it seems that the circadian system regulates the basal settings of body temperature, thereby indirectly establishing the level of the homeostatic response required to minimize or even neutralize the physical challenge on thermal homeostasis posed by the reduced ambient temperature.

Even though thermal balance was successfully defended during cold exposure regardless of time-of-day, lipid and CHO utilization were affected by both environmental time and temperature (**Figure 5**). It has been previously shown that environmental cooling provides a key signal to substrate utilization for shivering (involuntary muscle contractions) and non-shivering (generated by proton gradient within mitochondrial oxidative activity and controlled by uncoupling proteins) thermogenesis from skeletal muscle and BAT, respectively (6, 8, 30, 50–52). Environmental conditions and the intensity of cold exposure determine the metabolic rate and the prevalence of the thermogenic pathway activated to generate heat (6, 8, 51). Vaillancourt and colleagues

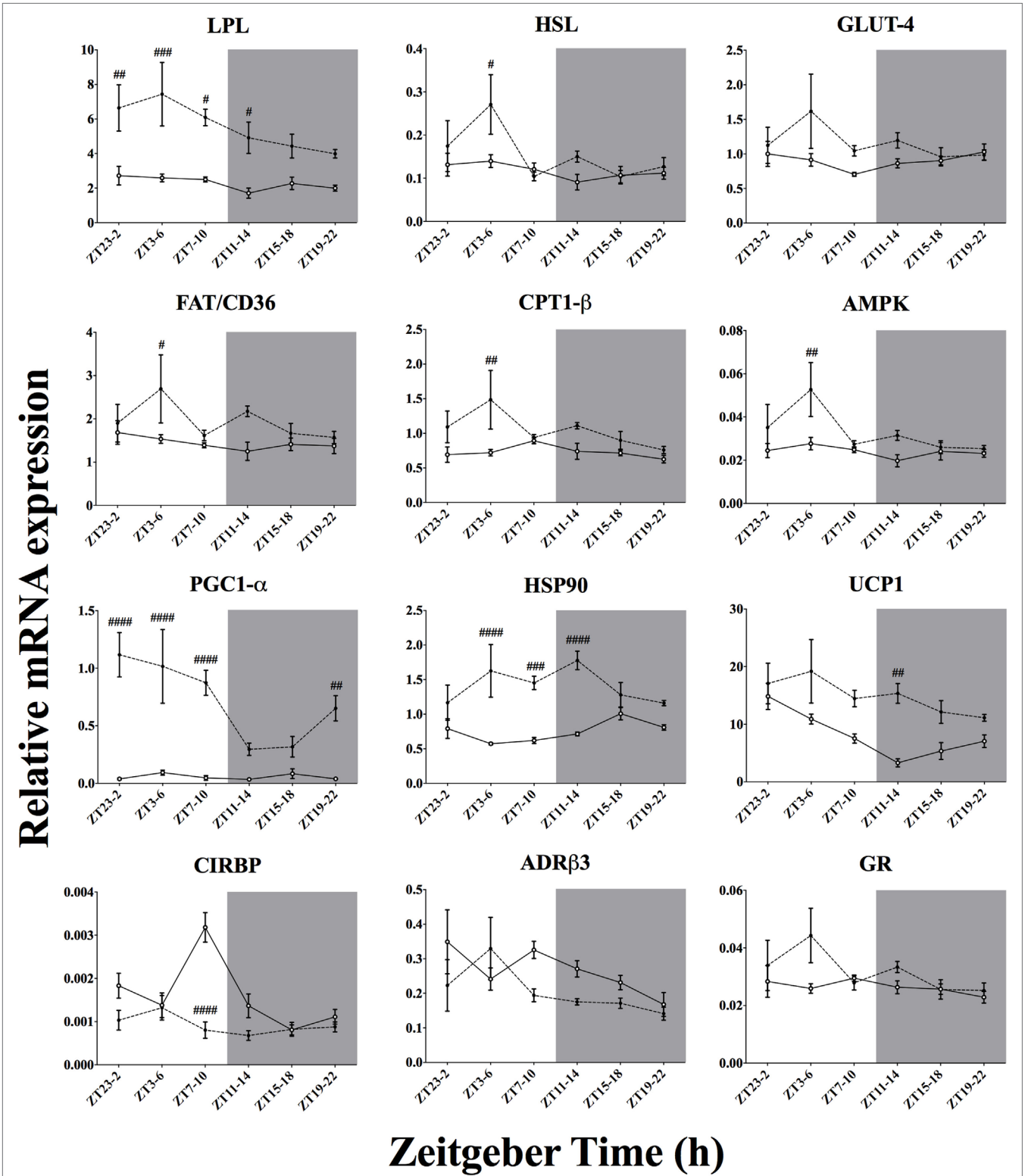

FIGURE 7 | Effect of time-of-day on metabolic gene expression in brown adipose tissue (BAT) after exposure to environmental cooling. Light phase began at Zeitgeber Time 0 (ZT0) and dark phase (shaded areas) began at ZT12. Results are presented as mean ± SEM. # indicates differences between control and cold conditions, $p < 0.05$. n = 5–7/group/ZT. Data for CREB, PGC1-β, PPAR-α, PPAR-γ, ACC1, and ACC2 are not shown (refer to **Table 4** for results).

(8) have previously shown that although affecting total EE; below a certain threshold (~15°C), the higher rates of CHO, lipid, and protein oxidations are not intensified by even lower environmental temperatures (10 and 5°C). To our knowledge, we are the first to report a clear time-of-day effect on substrate utilization during cold exposure.

Interestingly, we observed that during the active period, cold exposure caused a major switch by increasing lipid oxidation (**Figures 5E,F**) and lessening CHO oxidation (**Figures 5C,D**), whereas during the light phase both lipid and CHO oxidation rates were increased, although only transitory for CHO. This might reflect the first stage of cold defensive mechanisms in which in skeletal muscle shivering first consumes carbohydrates stocks in the glycolytic pathway to promptly generate ATP for muscular contraction and heat production (51, 52). As cold exposure persists, the participation of shivering in the thermal balance decreases and a primary role is appropriated by BAT non-shivering thermogenesis, with increased lipid uptake and oxidation (35, 52). Therefore, time-of-day influences the balance between CHO and lipids utilization during cold exposure, providing further evidence that the daily rhythms expressed through an internal clock in different body tissues might influence metabolic adjustments in heat-generating tissues.

Differences in the Metabolic and Thermal Adjustments to Cold Were Related to Distinct Changes in mRNA Expression of Clock and Clock-Controlled Genes

To increase metabolic heat production during exposure to a lower environmental temperature, activity in skeletal muscle and BAT is intensified through different pathways within the central nervous system (6). In the present study, we observed that after 3 h of cold exposure 13 of 18 and 17 of 23 of the chosen genes thought to be involved in cold-induced metabolic heat production were affected by our cold protocol in the BAT (**Figure 7**) and skeletal muscle (**Figure 8**), respectively.

In addition to the metabolic effects, cold exposure also affected clock and clock-controlled gene expression in BAT and soleus muscle, although to a different extent (**Figure 6**). In the BAT, seven of eight core clock genes studied were altered by the lower environmental temperature. Specifically, five of those genes were upregulated after cold exposure, without an interaction with time-of-day on this response. Others have already implicated PER1, PER2, CRY1, and CRY2 in BAT in cold exposure (12, 13). In fact, a functional clock is necessary to mediate molecular adjustments in the mitochondrial activity program (13, 17, 53, 54). However, the present study is the first to show this upregulation of BAT activity during cold exposure at different moments of the day, reinforcing the hypothesis that the thermogenic program, although it is intermingled with the core clock machinery (55), relies on the intensity of the stimulus rather than on time-of-day.

Interestingly, in BAT two of the clock genes, DBP and REV-ERBα, were downregulated by cold exposure with a time-of-day interaction. REV-ERBα is also downregulated after increased energetic demands and is acknowledged as an important repressor of mitochondrial activity and seems to be regulated by the positive loop of the core clock (16, 17, 53). In fact, we observed that the reduced REV-ERBα expression is accompanied by an augmented expression of BMAL-1 and PGC1-α, especially during the light phase (**Figures 6** and **7**), which points toward a pronounced shift in BAT mitochondrial activity in response to the light-to-dark transition (54) and change in ambient temperature, probably through a temperature compensation mechanism.

Regarding the soleus skeletal muscle, we observed that only the genes from the negative loop of the canonical core clock machinery were affected by cold exposure (**Figure 6**; **Table 5**), i.e., PER1, PER2, CRY1, and CRY2, that is half of the studied clock genes (four of eight). In line with this, resetting of the peripheral clock by cold exposure (13) and exercise (56) seems to be dependent of *Per1/2* (57), as is resetting by glucocorticoids fluctuations (58). These findings reinforce the idea that cold exposure can reset the peripheral clock by changes in metabolic activity, since genes encoding for mitochondrial activity, such as CPT1, PGC1α, PPAR, and FOXO1 were affected. In addition, genes that regulate substrate uptake (LPL and GLUT4), cellular energetic state (AMPK), and mitochondrial activity (CPT1, NAMPT, PGC1α and PPARs) showed an interaction with time-of-day, suggesting that key components of the cellular energetic balance are dependent on temporal input to establish the necessary shifts in molecular programs required to survive in a colder environment. On the other hand, cold exposure also changed GR and ADRβ receptor expression in both soleus muscle and BAT, indicating that also hormonal and autonomic, i.e., non-metabolic, factors might be involved in this shifting process.

In the skeletal muscle (**Figure 8**), the increased expression of LPL, HSL, and FAT/CD36 points toward boosted lipid uptake, which is supported by previous physiological and molecular expression data (59). Glucose uptake was also elevated by cold exposure as suggested by increased GLUT4 expression in the present experiment and those performed by others (10, 11). Taken together, increased lipid and glucose uptake suggests an augmented substrate oxidative state within the soleus muscle. Reinforcing this assumption, the expression of key metabolic regulators such as AMPK, CamK2a, and NAMPT-1 was also increased after cold exposure (**Table 5**). It is thought that AMPK activation due to increased energetic demand regulates the key mitochondrial transporter for β-oxidation, CPT1-α/β, as well as key transcription factors for mitochondrial activity, such as PPARs, PGC1-α, and FOXO1 (60–65). In fact, all these genes associated with mitochondrial activity were upregulated in the skeletal muscle. The present gene expression results thus support a role for the skeletal muscle in metabolic heat production. This idea is also supported by increased HSP90 mRNA expression, which might be related to higher oxidative activity demanding more chaperone protein content for protein stabilization (66).

Interestingly, despite the higher energetic demand, we did not observe a clear effect of cold exposure on UCP3 activity (**Figure 8**). It is thought that UCP3 could be directly involved in muscular heat production independent of shivering (35, 67). In line with our results, others (68) have shown that muscular UCP3 activity might not be involved in cold-induced metabolic heat production. Instead, uncoupling seems to constitute an intramuscular FFA buffering system during cold exposure, at

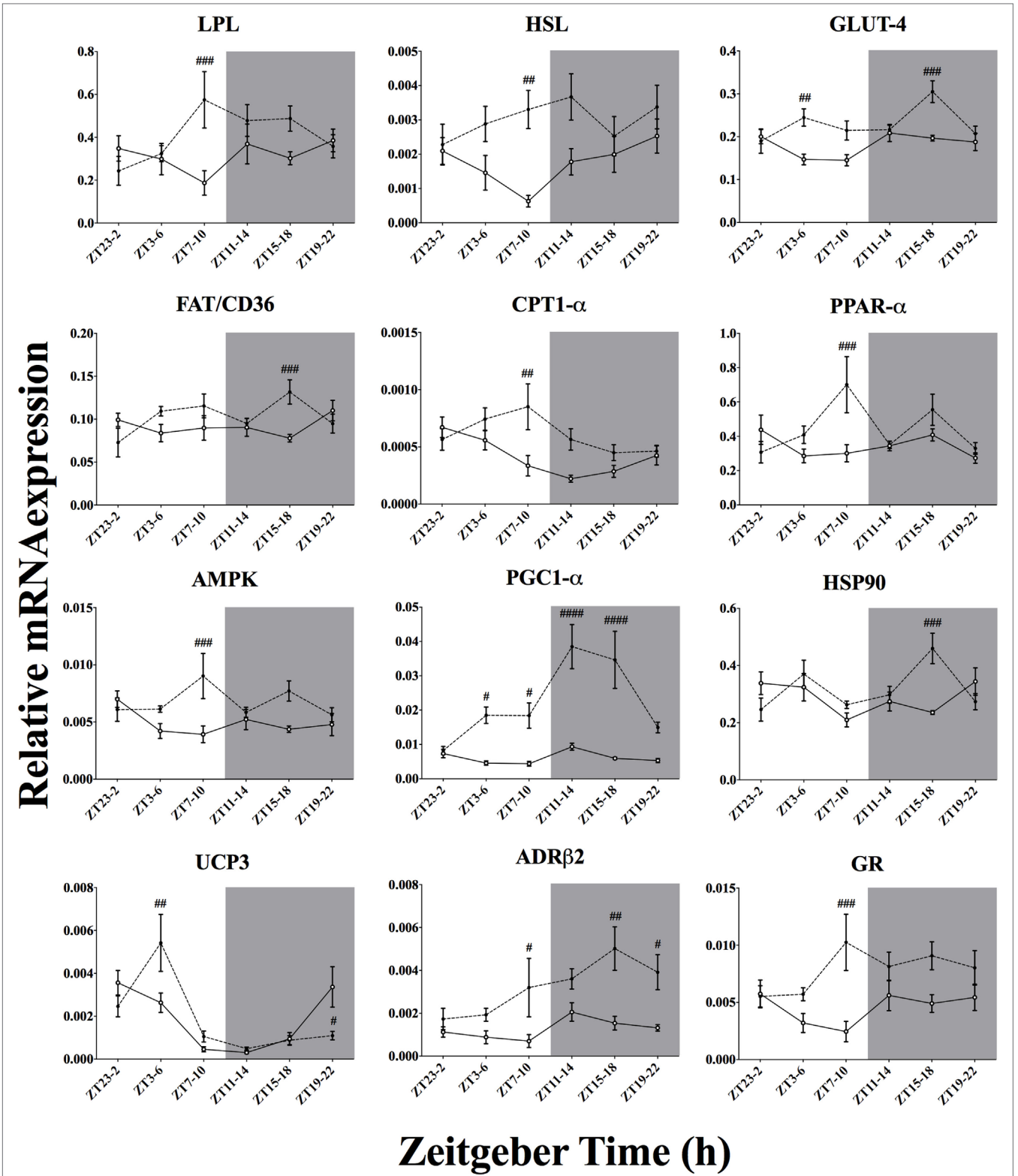

FIGURE 8 | Effect of time-of-day on metabolic gene expression in soleus muscle after exposure to environmental cooling. Light phase began at Zeitgeber Time 0 (ZT0) and dark phase (shaded areas) began at ZT12. Results are presented as mean ± SEM. # indicates differences between control and cold conditions, $p < 0.05$. n = 5–7/group/ZT. Data for CREB, PGC1-β, PPAR-γ, *UCP2*, CIRBP, *CPT-β,* NAMPT-1, FOXO-1, ACC2, CamK2a, and *mTOR* are not shown (refer to **Table 5** for results).

least in murine models (12, 69). In addition, we also observed a possible influence of neuronal and hormonal stimulation on these molecular adjustments since expression of ADRβ2 and GR was upregulated after cold exposure, pointing to a direct modulation of the cold-induced adjustments by the sympatho-cortico-adrenal system (12, 70–72).

In the BAT, cold exposure also upregulated the substrate uptake and mobilization program, as can be concluded from the increased mRNA expression of LPL, FAT/CD36, and GLUT4 (**Figure 7**; **Table 4**). Transcription of key enzymes for β-oxidation, such as CPT1-β and ACC1 was also upregulated by cold exposure. Aligned with this finding, mitochondrial activity, and biogenesis, expressed by the function of the key transcription factor PGC1α was increased by cold exposure. This increased mitochondrial activity state is reinforced by improved UCP1, increased HSP90 (66) and decreased CIRPB (73) expression after cold exposure. AMPK activity, which is thought to play a key role in mediating cellular metabolic flux, was further stimulated by cold exposure. Increased BAT activity is well supported by previous studies and accumulating evidence implicates both glucose and lipid oxidation in BAT non-shivering thermogenesis (14, 74–77).

It is clear that the initial metabolic state, as defined by the circadian system, is crucial for the physiological response to environmental stimuli and therefore should be taken into account to understand the intrinsic capacity to adapt to changes in ambient temperature. In the current experiment, the cold-induced metabolic shift toward lipid oxidation provided a clear example of such a time-dependency and further proof that the internal clock plays an important role in shaping such physiological responses. In the soleus skeletal muscle, cold exposure upregulated specifically the negative loop of the canonical clock (PER/CRY), whereas in the BAT both the negative and positive loop (BMAL-1/CLOCK) were upregulated. Thereby, the present study for the first time showed a tissue-specific effect of cold exposure on clock and clock-controlled genes and thus demonstrated the existence of an interaction between time-of-day and homeostatic adjustments elicited by acute cold exposure.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the animal care committee of the Royal Netherlands Academy of Arts and Sciences (DEC/KNAW). The protocol was approved by the animal care committee of the Royal Netherlands Academy of Arts and Sciences (DEC/KNAW).

AUTHOR CONTRIBUTIONS

FM, CC, and AK designed the experiments. FM, ZZ, YS, PG, RJ, and EF acquired the data. FM, ZZ, EF, CC, and AK contributed to the analysis and interpretation of data. FM and AK participated in the elaboration of the manuscript and gave final approval for submission and publication, being accountable for all aspects of the present work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fendo.2018.00199/full#supplementary-material.

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Daily Gene Expression Rhythms in Rat White Adipose Tissue Do Not Differ Between Subcutaneous and Intra-Abdominal Depots

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White adipose tissue (WAT) is present in different depots throughout the body. Although all depots are exposed to systemic humoral signals, they are not functionally identical. Studies in clock gene knockout animals and in shift workers suggest that daily rhythmicity may play an important role in lipid metabolism. Differences in rhythmicity between fat depots might explain differences in depot function; therefore, we measured mRNA expression of clock genes and metabolic genes on a 3-h interval over a 24-h period in the subcutaneous inguinal depot and in the intra-abdominal perirenal, epididymal, and mesenteric depots of male Wistar rats. We analyzed rhythmicity using CircWave software. Additionally, we measured plasma concentrations of glucose, insulin, corticosterone, and leptin. The clock genes (*Bmal1/Per2/Cry1/Cry2/RevErbα/DBP*) showed robust daily gene expression rhythms, which did not vary between WAT depots. Metabolic gene expression rhythms (*SREBP1c/PPARα/PPARγ/FAS/LPL/Glut4/HSL/CPT1b/leptin/visfatin/resistin*) were more variable between depots. However, no distinct differences between intra-abdominal and subcutaneous rhythms were found. Concluding, specific fat depots are not associated with differences in clock gene expression rhythms and, therefore, do not provide a likely explanation for the differences in metabolic function between different fat depots.

Keywords: circwave, visceral WAT, retroperitoneal WAT, lipid metabolism, circadian

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INTRODUCTION

Sustained disturbances in daily rhythmicity (e.g., shift work, jet lag) increase the risk to develop obesity and related metabolic disease (1). Storage in and release of lipids from white adipose tissue (WAT) are regulated processes that anticipate rest-activity and feeding cycles. WAT is abundantly present throughout the body in different fat depots. In male rats, the main depots are located underneath the skin in the inguinal area [subcutaneous white adipose tissue (sWAT)], and in the abdominal cavity (intra-abdominal depots): perirenal- (pWAT, retroperitoneal, next to the kidney), epididymal- (eWAT, connected to and lining the epididymis), and mesenteric WAT (mWAT, intraperitoneal, lining the gastrointestinal tract).

Abbreviations: ANS, autonomic nervous system; COG, centre of gravity (see method section); WAT, white adipose tissue; ZT, zeitgeber time.

Interestingly, although all depots are exposed to systemic humoral signals, such as circulating hormones and nutrients, subcutaneous and intra-abdominal WAT depots are not functionally identical (2, 3). For example, retroperitoneal WAT is more responsive to metabolic challenges (fasting/refeeding) compared to subcutaneous WAT (4). Additionally, in various lipodystrophy syndromes subcutaneous fat stores are depleted, while simultaneously intra-abdominal WAT accumulates (5), pointing to differential differentiation and proliferation of adipose depots. Moreover, excess storage of intra-abdominal WAT is associated with adverse health effects, whereas subcutaneous WAT accumulation might be beneficial (6–9). Moreover, effects of sex hormones (10) and glucocorticoid treatment differ between WAT depots (11). To date, it is unexplained where these differences originate and how they are integrated to ensure that the net effect of the WAT depots results in energy homeostasis.

Like most peripheral tissues, WAT depots encompass an intrinsic molecular clockwork based on a transcriptional–translational feedback loop. Since clock proteins regulate the expression of genes involved in many (metabolic) processes within a cell, clock rhythms play an important role in tissue function. The core loop of the molecular clock is formed by the Clock:Bmal1 heterodimer that upregulates expression of the Period 1–3 (Per 1–3) and Cryptochrome 1–2 (Cry1–2) proteins. Per's and Cry's subsequently heterodimerize, translocate to the nucleus, and inhibit Clock:Bmal1 activity. As a consequence, Clock:Bmal1 transcriptional activity drops, which reduces the transcription of *Per* and *Cry* genes, thereby activating Clock:Bmal1 again. The retinoic acid-related orphan nuclear receptors, RevErb and ROR, represent additional regulatory loops that enhance the robustness of the core loop, by binding to retinoic acid-related orphan receptor response elements on the Bmal1 promotor (12).

Studies in clock gene knockout animals and studies in shift workers suggest daily rhythms play an important role in lipid metabolism. For example, the arrhythmic CLOCKΔ19 C57BL/6J mouse is hyperglycemic, hyperlipidaemic, hyperleptinaemic, and hypoinsulinaemic, with increased body weight and visceral adiposity (13, 14). Moreover, disruption of the adipocyte clock by adipose tissue specific deletion of Bmal1, results in obesity, temporal changes in plasma concentration of fatty acids, and altered hypothalamic appetite regulation (15). In CLOCKΔ19 C57BL/6J mice, the impaired adipose tissue clock may directly affect diurnal transcriptional regulation of lipid homeostasis, reducing FFA/glycerol mobilization from WAT stores (16).

To determine whether differences in daily rhythmicity between WAT depots could explain differences in depot function, we analyzed rhythmicity of clock gene (*Bmal1, Per2, Cry1, Cry2, RevErbα*, and *DBP*) and metabolic gene expression (*SREBP1c, PPARα, PPARγ, FAS, LPL, Glut4, HSL, CPT1b, leptin, visfatin*, and *resistin*) in different intra-abdominal and subcutaneous WAT depots. We conclude that differences in the molecular clock or clock-controlled genes do not provide a major explanation for the differences in metabolic function between the different fat depots. Furthermore, our results suggest that in *ad libitum* feeding conditions the timing of subcutaneous WAT clock gene rhythms can be extrapolated to those of intra-abdominal WAT depots.

RESULTS

Overall Rhythmicity of Gene Expression in Adipose Tissue

To describe rhythmicity, we considered the following factors to be important; peak time [expressed as center of gravity; COG (see Materials and Methods)], robustness, and amplitude. Therefore, we analyzed variation between depots for these factors. We defined "robustness" of a rhythm as: uniformity between cycles and/or animals measured by three characteristics; period, phase, and shape of wave. R^2 values indicate goodness of fit on a scale from 0 to 1, i.e., how well the Circwave curve describes the data. Thus, r^2 values close to 1 indicate that individual samples deviate very little from the curve and, therefore, show little inter-animal variation in period, phase and shape of wave, and can be called robust. Clock gene and metabolic gene expression per WAT depot, r^2 (inter-individual variability) and amplitude are plotted for each gene in **Figures 1** and **2**. For all WAT depots, clock gene expression was highly rhythmic, with large amplitudes (range 125–272) and low variability (r^2 range 0.61–0.92) between animals. A clear exception was *Cry 2*, which showed much lower amplitude (range 37–61) and r^2 values (range 0.21–0.46) than the other five clock genes investigated. Metabolic genes on the other hand exhibited weak rhythmicity with lower amplitude (range 0–97) and high variability (r^2 range 0.21–0.71) between animals (**Figures 2** and **3**), similar to or lower than the values for *Cry 2*. Individual expression curves for each gene and WAT depot can be found in Figure S1 in Supplementary Material.

Clock Gene Expression Comparison Between WAT Depots

Clock gene expression showed pronounced daily rhythms in all WAT depots. R^2 values showed little variation between depots, and limited variation between genes (**Figure 1**). *Cry2* showed the most pronounced variation between WAT depots; r^2 values for pWAT (0.26) and eWAT (0.21) were about 50% smaller than for sWAT (0.46) and mWAT (0.44). Amplitude variations were limited between WAT depots (**Figures 1** and **3**). Of note, for most clock genes the lowest amplitude was found in pWAT. For *Per2* mRNA the amplitude in sWAT was clearly higher compared to the other depots. Peak time for the different clock gene curves (depicted as COG) was very similar between WAT depots (**Figure 1**, one-way ANOVA: ns). *Bmal1* peaked in the beginning of the light phase (ZT24) and as expected, *Per* and *Cry* rhythms were in antiphase, to *Bmal1*. *Per2* (ZT15–16), and *Cry2* (ZT14–17) peaked in the early dark period, whereas *Cry 1* (ZT19–20) mRNA peaked in the middle of the dark period. *RevERBα* (ZT9–10) and *DBP* (ZT11–12) mRNA were high at the end of the light phase (**Figure 1**).

Metabolic Gene Expression Comparison Between WAT Depots

Daily rhythms in metabolic gene expression were present; however, rhythmicity was not as robust (higher variability

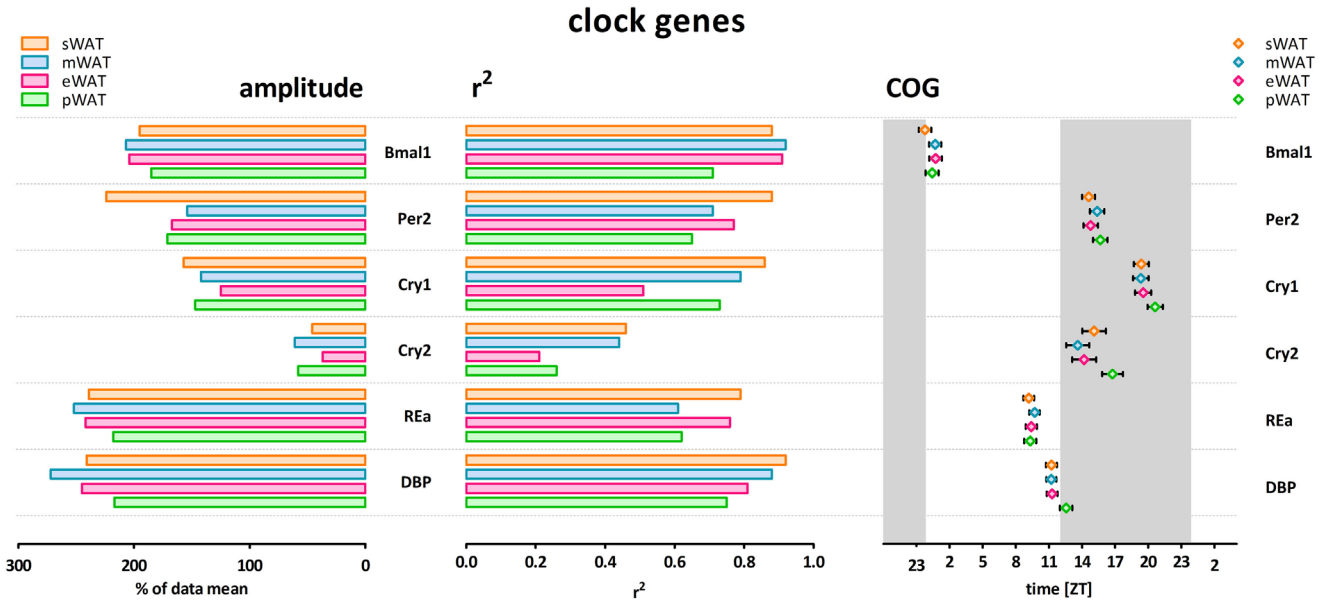

FIGURE 1 | Clock genes show high amplitude, high r^2 (low inter-individual variability) and low variability in center of gravity (COG) in subcutaneous and intra-abdominal white adipose tissue (WAT) depots. sWAT, subcutaneous; mWAT, mesenteric; eWAT, epididymal; pWAT, perirenal. Gray bars indicate the dark phase (ZT 12–24). Individual expression curves for each gene and WAT depot can be found in Figure S1 in Supplementary Material.

and lower amplitudes) as it was for clock genes (**Figure 2**). Rhythmicity was not apparent for every gene and for some genes not in every WAT depot. Absence of amplitude, R^2, and COG values in **Figure 2** indicates absence of significant rhythmicity, not absence of gene expression (see Figure S1 in Supplementary Material for individual gene expression curves). R^2 values were modest overall; r^2 was highest for *visfatin* in eWAT and sWAT (**Figures 2** and **3**). Similarly, amplitudes in metabolic genes were modest overall, i.e., <100%. Peak time (COG) for most metabolic genes did not differ between WAT depots. However, for *LPL* significant differences were detected (**Figure 2**). *LPL* peaked significantly earlier in pWAT compared to sWAT (two-tailed *t*-test $F = 1{,}133$; $p = 0.0342$; difference = 3.2 ± 1.5 h).

Daily Rhythms in Plasma Hormone and Substrate Levels

Plasma levels and COGs of glucose, insulin, corticosterone, and leptin are shown in **Figure 4**. Plasma glucose concentrations were modestly rhythmic and peaked at the transition from light to dark phase (~ZT14, amplitude 21%, ANOVA: $F = 4.78$; $p < 0.001$, CIRCWAVE: $r^2 = 0.35$; $p < 0.001$). Plasma insulin concentrations were not rhythmic, but showed a greater variation at the end of the light phase (ANOVA: $F = 1{,}867$; $p = 0.0923$). Plasma corticosterone concentrations were highly rhythmic and peaked slightly before the glucose peak (~ZT13, amplitude 237%, ANOVA: $F = 8{,}852$; $p < 0.001$, CIRCWAVE: $r^2 = 0.53$; $p < 0.001$). Plasma leptin concentrations were modestly rhythmic and peaked in the middle of the dark phase (~ZT17, amplitude 32%, ANOVA: $F = 4{,}073$; $p < 0.005$, CIRCWAVE: $r^2 = 0.22$; $p < 0.001$).

DISCUSSION

Different WAT depots have different functions, and increase and reduce their mass differentially, as illustrated by several metabolic disorders that result in loss of mainly subcutaneous or gain of mainly intra-abdominal (visceral) fat mass. Rhythmicity plays an important role in lipid metabolism, and clock gene rhythms have been described for some but not all WAT depots in rodents (17–20) and in humans (21, 22). We, therefore, hypothesized that differences in rhythmicity might explain differences in depot function and analyzed rhythmicity of gene expression in subcutaneous and different intra-abdominal WAT depots. However, in contrast to our hypothesis, we did not observe clear differences in clock gene rhythms between different WAT depots (**Figures 1**–**3**). Moreover, most metabolic genes only showed modest or non-significant rhythmicity. Therefore, differences in the molecular clock or clock-controlled genes do not provide a major explanation for the differences in metabolic function between the different fat depots.

We observed robust rhythms in clock gene expression in all four fat depots studied (*Bmal1, Per2, Cry1, Cry2, RevErbα, DBP*), with a peak time that was similar to what has been described previously for Wistar rats (20, 23, 24) and other rodent species (17–19). Only few studies have measured clock gene expression rhythms in both epididymal WAT and subcutaneous (inguinal) WAT; one found lower amplitudes in subcutaneous WAT compared to epididymal (19), whereas in the other study, amplitudes were marginally smaller in epididymal WAT compared to subcutaneous WAT (18). In our data set, amplitude, robustness, or timing (COG) were not significantly different between mesenteric-, perirenal-, epididymal-, and subcutaneous WAT depots. This is the first study to extensively compare clock gene

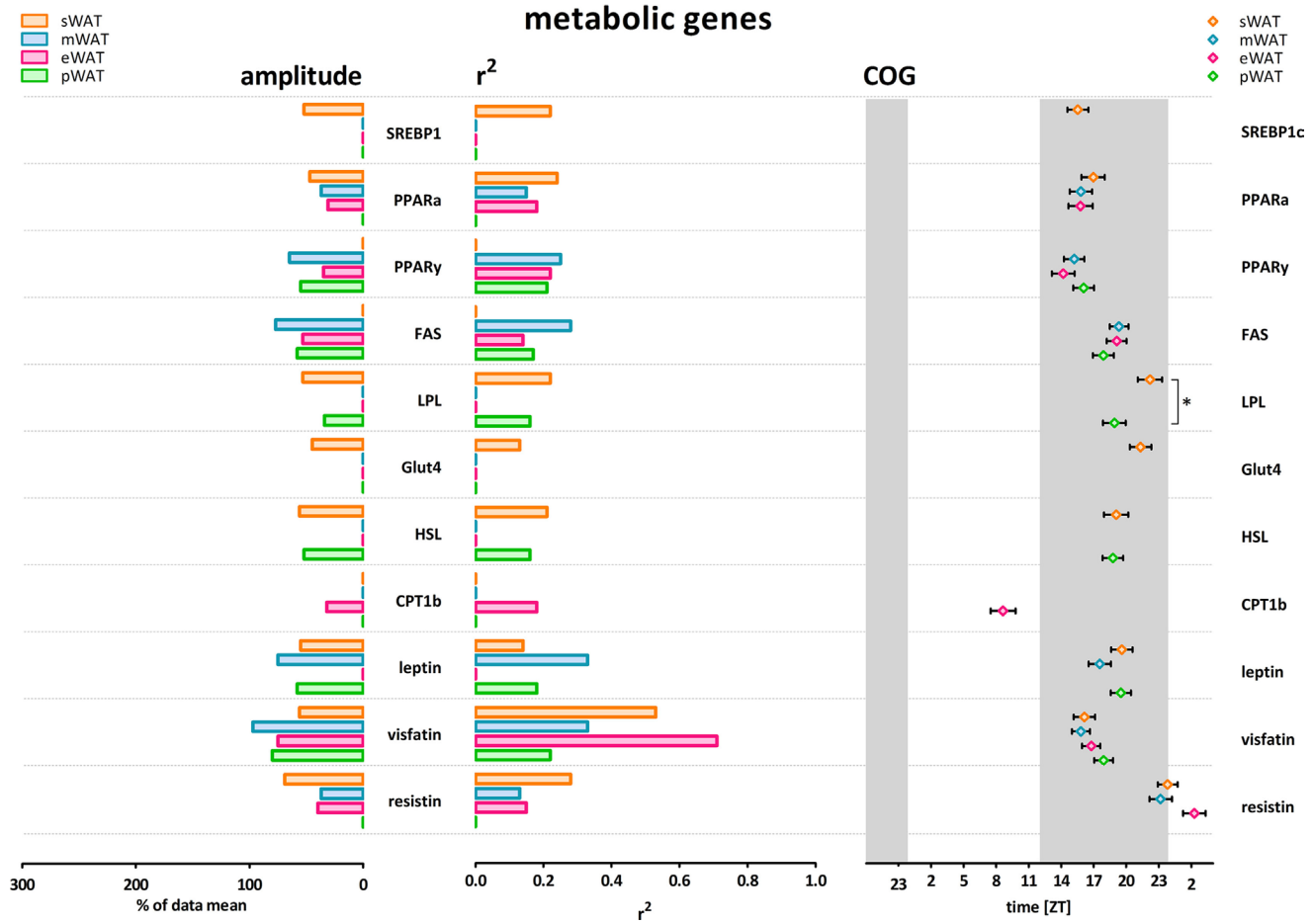

FIGURE 2 | Metabolic genes show modest amplitude, modest r^2 (inter-individual variability) and higher variability in center of gravity (COG) in subcutaneous and intra-abdominal white adipose tissue (WAT) depots. LPL peaked significantly earlier in pWAT compared to sWAT (two-tailed *t*-test $F = 1,133$; $p = 0.0342$; difference = 3.2 ± 1.5 h) sWAT; subcutaneous, mWAT; mesenteric, eWAT; epididymal, pWAT; perirenal. Gray bars indicate the dark phase (ZT 12–24). Absence of amplitude, R^2, and COG values indicates absence of significant rhythmicity, not absence of gene expression. Individual expression curves for each gene and WAT depot can be found in Figure S1 in Supplementary Material.

rhythms in subcutaneous and different abdominal WAT depots simultaneously. Because we did not observe pronounced differences between depots under these untreated, *ad libitum* feeding conditions, this suggests that with regard to clock gene expression rhythms the results from subcutaneous inguinal WAT—which in humans is far less invasive to biopsy compared to internal WAT depots—may be extrapolated to other depots.

In contrast to the overt day/night rhythms in clock gene expression, expression of metabolic genes showed no profound rhythmicity. Metabolic genes that did show significant rhythmicity mostly showed peak expression in the active (dark) phase. These findings are in line with data from mice (25). Metabolic genes are influenced by multiple circulating factors, such as corticosterone, insulin and nutrients, either directly (e.g., *via* a glucocorticoid response element) or *via* transcription factors (e.g., *SREBP1c*, *PPARs*) (26–28).

The daily rhythms in plasma corticosterone and glucose are independent of the daily rhythm in feeding behavior, whereas plasma levels of insulin and glucagon are mainly regulated by food intake (29, 30). Corresponding with previous data, we found that plasma concentrations of corticosterone and glucose peaked at the onset of the active phase. *PPAR*α and -γ are glucocorticoid sensitive transcription factors (31), and indeed for *PPARs* we observed an expression peak with a similar timing as that of plasma corticosterone. Plasma insulin concentrations did not show a significant day/night rhythm, but rather followed feeding activity with three spikes during the dark phase. Several genes encoding for proteins involved with energy storage in the fed state (*SREBP1c, PPARγ, LPL, FAS, Glut4, leptin, resistin*) showed a spiky expression pattern similar to the insulin curve (Figure S1 in Supplementary Material). These genes are likely influenced by feeding-induced insulin release, or by nutrients directly (e.g., *via* PPRE) (28).

LPL serves as a gatekeeper that controls local fatty acid uptake into cells by catalyzing the hydrolysis of circulating triglycerides. Transcription of *LPL* is upregulated by fatty acids, SREBP1c and PPARγ and downregulated and inactivated in the fasted state by glucocorticoids, catecholamines, and decreased levels of PPARγ

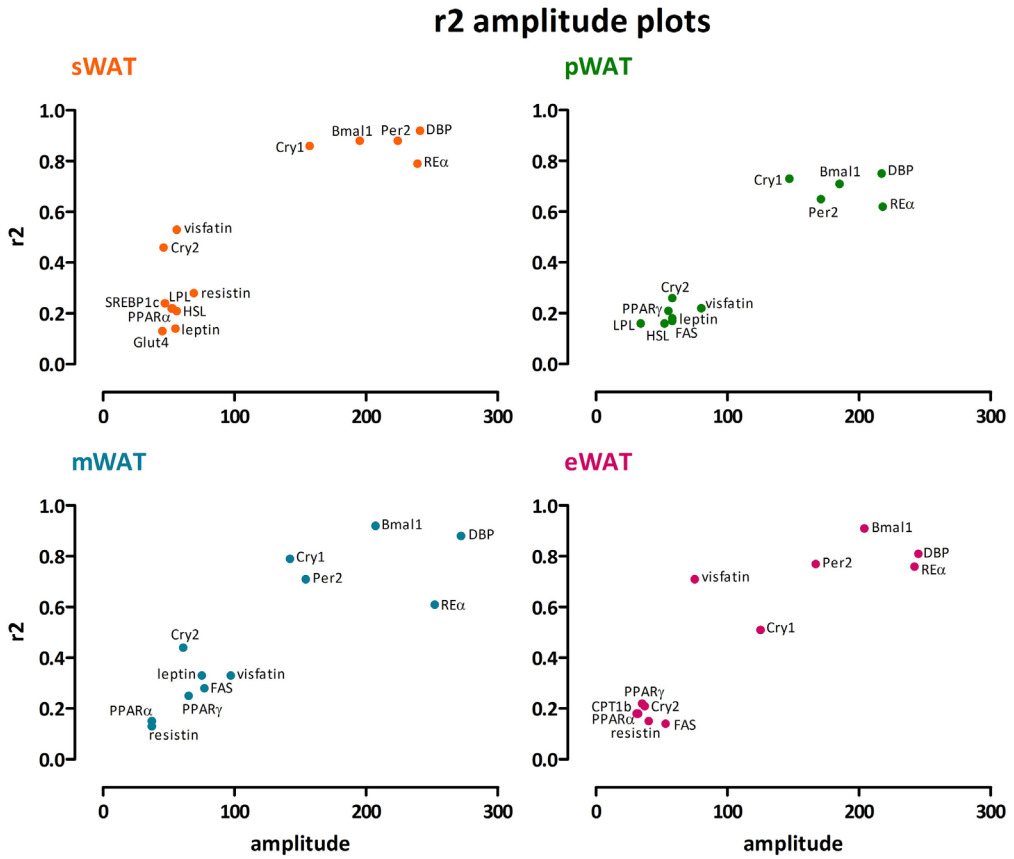

FIGURE 3 | Clock genes show high amplitude together with high r^2, whereas for metabolic genes modest amplitudes go along with low r^2 values. A clear exception is Cry2, which showed much lower amplitude and r^2 values than the other clock genes. We found no distinct differences between subcutaneous and intra-abdominal white adipose tissue (WAT) depots. sWAT, subcutaneous; mWAT, mesenteric; eWAT, epididymal; pWAT, perirenal. Individual expression curves for each gene and WAT depot can be found in Figure S1 in Supplementary Material.

and SREBP1c. In our data, *LPL* showed a 3-h delayed expression in sWAT compared to pWAT. In line with upregulation during the feeding period, we observed peak expression in eWAT when animals are eating. The delayed peak in *LPL* expression in sWAT fits with the hypothesis that intra-abdominal WAT is primarily functional in short term metabolic regulation, and sWAT takes up the lipid overflow for long term energy storage (2). LPL protein concentration also peaks in the active period, but it remains to be determined how the rhythms in mRNA and protein content correspond to activity levels, as most physiological variation in LPL activity appears to be driven by posttranslational mechanisms by extracellular proteins (32).

Leptin concentrations peaked in the middle of the active (dark) phase (**Figure 4**), in line with previous experiments (33). This peak in plasma corresponds with the rhythm in *leptin* mRNA in fat tissue (Figure S1 in Supplementary Material Leptin). We observed the clearest correlation between plasma leptin concentrations and *leptin* mRNA expression in mWAT (Figure S2 in Supplementary Material Leptin correlation). Although we cannot compare absolute mRNA expression levels between depots (due to the number of samples we had to analyze each depot as a separate batch), others have shown that *leptin* mRNA levels are generally much higher in intra-abdominal depots, compared to subcutaneous depots (34). Furthermore, they found plasma leptin levels correlated only with *leptin* expression in mWAT, but not any of the other WAT depots (34), which is in line with the correlations we observed between *leptin* mRNA and plasma leptin concentrations. Another study comparing *leptin* mRNA expression rhythms between WAT depots in rats found expression curves quite similar to our data in mesenteric and perirenal (retroperitoneal) WAT. However, they found epididymal WAT to be rhythmic, in contrast to our dataset. These different observations accentuate the modest amplitude of the leptin expression rhythms; hence conclusions should be drawn with caution. In contrast to rodents, in humans subcutaneous fat tissue is the primary source of circulating leptin levels (35, 36). Therefore, the contribution from subcutaneous leptin mRNA to both plasma leptin levels and their rhythm would be expected to be more important in humans. Indeed *leptin* mRNA is rhythmic in human subcutaneous tissue as well (21). One explanation for this discrepancy between rodents and humans could be a different ratio of subcutaneous versus intra-abdominal fat mass.

A number of other factors in our study may have contributed to variation in gene expression, of both clock and metabolic

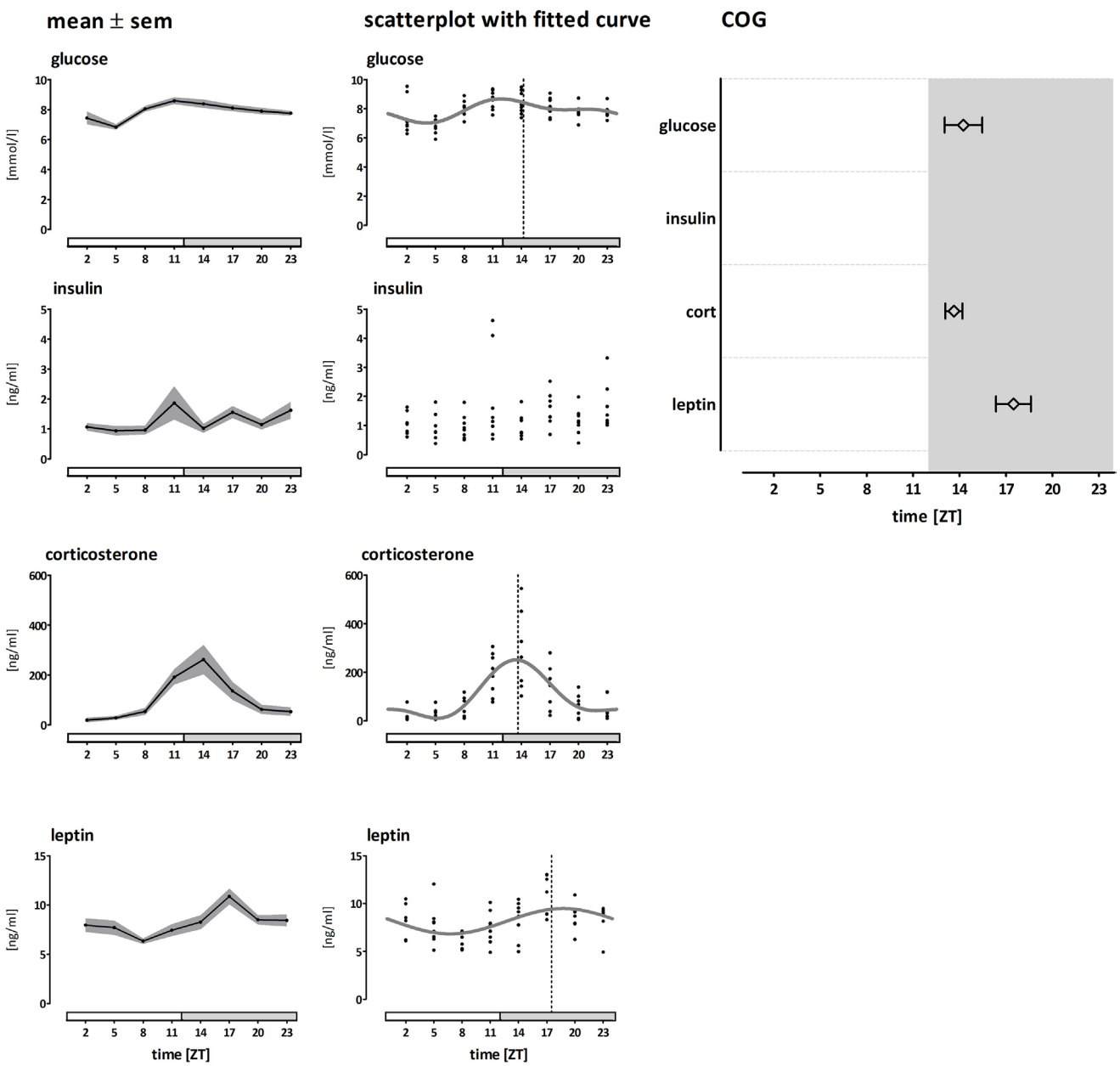

FIGURE 4 | Plasma concentrations of glucose, insulin, corticosterone, and leptin. Left panel shows the mean (±SEM) plasma concentrations. The middle panel shows the individual data points and Circwave curves, the dotted line indicates the Center of Gravity (COG). The right panel shows the COG (±SD). Absence of the Circwave curve and the COG line indicates absence of significant rhythmicity as analyzed by Circwave. Gray bars indicate the dark phase (ZT 12–24).

genes. First, our animals had *ad libitum* access to food, which could have induced small variations in timing of food intake between animals which might have led to less robust rhythms. Second, we have used Circwave to analyze rhythmicity in our data. Circwave recognizes wave forms using Fourier transformation whereby harmonics are added in a step-wise regression like fashion (using *F*-testing). This method is based on the assumption that the rhythms consist of one or more sine waves, and that noise variance is Gaussian (normally) distributed and independent of measurement magnitude. Therefore, it limits the recognition of spiky and saw tooth-shaped wave forms (37). Although the choice for this method might influence the sensitivity with which we were able to recognize rhythms, it will only affect our main conclusion (no rhythmic differences between depots) if there would be major differences in shape of wave between the WAT depots. Looking at the raw data sets (Figure S1 in Supplementary

Material), we may underestimate spiky rhythmicity of insulin or nutrient regulated genes. Nevertheless, alternative methods do not allow for estimation of amplitudes and phases (37), which were our main outcome measures.

We found no evidence that differences in rhythmicity in clock or metabolic genes underlie the functional differences described for the different WAT depots. Alternative explanations for functional differences are differences in pre-adipocyte lineage (2), differences in innervation, or differences in local regulation. Typically, the hypothalamus integrates peripheral signals and ensures energy homeostasis by regulating peripheral energy metabolism *via* humoral pathways and the autonomic nervous system (ANS). Indeed, intra-abdominal and subcutaneous WAT are innervated by separate sets of neurons (38), all the way up to the pre-autonomic neurons in the hypothalamus (39). Subcutaneous (inguinal) WAT gains more adipose cells after denervation compared to intra-abdominal (retroperitoneal) WAT (40). These data indicate that differential innervation can contribute to functional differences between WAT depots, but apparently do not result in differences in rhythmicity. Whether differences in functionality are indeed depending on differences in autonomic activity at the level of WAT still needs to be proven. Moreover, it could well be that ANS mediated differences in WAT functionality only surface during positive or negative energy balance.

Concluding, in contrast to our hypothesis, we did not observe clear differences in (clock) gene expression rhythms between different WAT depots. Moreover, we found only modest rhythmicity in metabolic gene expression rhythms, and no results that could explain differences in metabolic function between the different WAT depots. Therefore, functional differences between WAT depots likely stem from other regulatory levels (i.e., translational) or pathways.

MATERIALS AND METHODS

Animals

Sixty-four male Wistar rats (Harlan, Horst, Netherlands) were kept on a 12/12-h light/dark cycle (lights on at 0700 hours), at a room temperature (20 ± 2°C), with four to six animals per cage. Thirty-two animals were housed in a room with a reversed light/dark cycle. The experiment was carried out in October. After arrival, animals were allowed to adapt to their new environment and the lighting schedule for 3 weeks before the experiment. Food and water were provided *ad libitum*. The experiment was conducted under approval of the Local Animal Welfare Committee.

Experiment

To obtain WAT tissues and plasma, animals were anesthetized with isoflurane and killed by decapitation at a 3-h interval starting at ZT2 (ZT14 for reversed light–dark cycle) and ending at ZT11 (ZT23 for reversed light–dark cycle). At every time point, four animals were obtained from both rooms, thereby spreading the total sampling period over a 48-h period.

Intra-abdominal perirenal (pWAT), epididymal (eWAT), and subcutaneous inguinal (sWAT) white adipose tissues were dissected and snap frozen in liquid nitrogen. Intra-abdominal mesenteric (m)WAT was separated from the gastrointestinal tract and pancreas and snap frozen in liquid nitrogen. Blood was collected in heparinized tubes.

Plasma Analyses

Following decapitation trunk blood was collected and kept on ice in heparinized tubes until centrifugation for 15 min at 3,000 rpm at 4°C. Plasma was transferred to a clean tube and stored at −20°C until use. Plasma glucose was measured using a Biosen apparatus (EKF diagnostics, Cardiff, UK). Plasma insulin, leptin, and corticosterone were measured using a radio immuno assay (Merck Millipore, Billerica, MA, USA).

Gene Expression Analysis

RNA Extraction

Total RNA (tRNA) was extracted from approximately 100 mg of adipose tissue, using the RNeasy lipid kit (Qiagen Benelux, Venlo, Netherlands), with on-column DNAse treatment using RNAse-free DNAse (Qiagen Benelux, Venlo, Netherlands), according to the manufacturer's protocol. tRNA was measured on a Nanodrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA) and diluted to equal concentrations.

cDNA Synthesis

cDNA was synthesized with the Transcriptor First Strand cDNA synthesis kit from Roche (Roche, Almere, Netherlands) using anchored oligo (dT)18 primers and 18 ng tRNA per microliter cDNA. To check for genomic DNA contamination in the extracted RNA, we included several samples for which we replaced reverse transcriptase with PCR grade water (−RT controls). If the fluorescence curve of one of the −RT controls lay within 10 cycles of the cDNA sample with the lowest expression, the PCR assay was rejected because of potential genomic DNA contamination.

RT-qPCR

Gene expression was analyzed by real-time RT-qPCR on a LightCycler 480 system (Roche, Almere, Netherlands), using SybrGreen I Master, primer pairs, PCR grade water and cDNA. All primer pairs were designed intron-spanning if possible, and amplicon size and specificity was checked on electrophoresis gel. If the amplicon size matched and a single band was found, the PCR product was purified using a QIAquick PCR purification kit (Qiagen Benelux, Venlo, Netherlands). The purified PCR product was diluted and used in subsequent PCRs as a positive control combined with melting peak analysis.

LinRegPCR

For each PCR assay, PCR efficiency was checked for all samples individually using LinRegPCR. LinRegPCR software determines baseline fluorescence sets a Window-of-Linearity to calculate PCR efficiency. The starting RNA concentration expressed in arbitrary fluorescence units, is calculated using the mean PCR efficiency per sample, the Cq value per sample and the fluorescence threshold used to determine the Cq (41, 42). Samples that differed more than 0.05 from the efficiency median value were excluded from further analysis.

Normalization

To control for variation in the amount of mRNA input, gene expression levels of the target sequence were normalized to the expression of an endogenous control, hypoxanthine phosphoribosyl transferase (HPRT) gene expression (43).

Several commonly used reference genes show a circadian rhythm in their expression profile (44), and these rhythms may vary between tissues, species, and strains (45). HPRT was chosen as a reference gene because it expressed no, or only very low amplitude rhythms in our samples (data not shown). Additionally, all PCR data are expressed relative to ZT2, to allow comparison between WAT depots.

Genes of Interest

Primer sequences of clock genes *Bmal1, Per2, Cry1*, and *Cry2, RevErbα* and *DBP*, and metabolic genes *SREBP1c, PPARα, PPARγ, FAS, LPL, HSL, CPT1b, Glut4, leptin, visfatin*, and *resistin* have been published previously (27).

Data Analysis and Statistics

For identification of outliers, we used Dixon's *Q* test with two-tailed *Q*-values (46). Samples that were determined outliers were excluded from further analysis (Table S1 in Supplementary Material).

All data (plasma and PCR) are presented as mean ± SEM unless otherwise stated. *p* Values below 0.05 were considered statistically significant.

Variations between time points within one gene in one depot were evaluated by one-way ANOVA and rhythmicity was assessed using Circwave v1.4 (www.hutlab.nl). Circwave software fits one or more fundamental sinusoidal curves through the individual data points and compares this with a horizontal line through the data mean (a constant). If the fitted curve differs significantly from the horizontal line, the data set is considered rhythmic. Circwave provides the following information: number of sines in the fitted curve; data mean, the average of all data points with SD; Centre of Gravity (CoG), representing the general phase of the curve with SD; ANOVA *F* stat, *p*-value and r^2; Circwave *F* stat, *p*-value and r^2. Centre of Gravity (COG) SDs were calculated without assuming the data was circular, as rhythmicity of gene expression was one of the outcome measures.

Centre of gravity data per gene were compared between WAT depots using unpaired two-tailed *t*-test with *F* test. Variances did not differ between WAT depots.

Amplitudes of Circwave curves were calculated as percentages of data mean to enable comparison of amplitudes between data sets [difference between the zenith (highest point) and nadir (lowest point) and divided by the data mean (max − min/mean * 100%)].

ETHICS STATEMENT

All the studies were approved by and performed according to the regulations of the Committee for Animal Experimentation of the Netherlands Institute for Neuroscience (NIN) of the Royal Netherlands Academy of Arts and Sciences (KNAW), Netherlands.

AUTHOR CONTRIBUTIONS

RS, EF, SF, and AK conceived and designed the experiments and wrote the paper. RS, SF, and AK performed the experiments and analyzed the data.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fendo.2018.00206/full#supplementary-material.

FIGURE S1 | Individual expression curves for each gene and white adipose tissue depot.

FIGURE S2 | Correlation between plasma leptin concentrations and leptin mRNA expression in mesenteric WAT.

TABLE S1 | The number of samples after exclusion of outliers for the PCR results at each time point and for each gene investigated.

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MINI REVIEW
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Altered Circadian Rhythms and Breast Cancer: From the Human to the Molecular Level

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Circadian clocks are fundamental, time-tracking systems that allow organisms to adapt to the appropriate time of day and drive many physiological and cellular processes. Altered circadian rhythms can result from night-shift work, chronic jet lag, exposure to bright lights at night, or other conditioning, and have been shown to lead to increased likelihood of cancer, metabolic and cardiovascular diseases, and immune dysregulation. In cases of cancer, worse patient prognoses and drug resistance during treatment have also been observed. Breast, colon, prostate, lung, and ovarian cancers and hepatocellular carcinoma have all been linked in one way or another with altered circadian rhythms. Critical elements at the molecular level of the circadian system have been associated with cancer, but there have been fairly few studies in this regard. In this mini-review, we specifically focus on the role of altered circadian rhythms in breast cancer, providing an overview of studies performed at the epidemiological level through assessments made in animal and cellular models of the disease. We also address the disparities present among studies that take into account the rhythmicity of core clock and other proteins, and those which do not, and offer insights to the use of small molecules for studying the connections between circadian rhythms and cancer. This article will provide the reader with a concise, but thorough account of the research landscape as it pertains to altered circadian rhythms and breast cancer.

Keywords: altered circadian rhythms, shift work, breast cancer, molecular mechanism, hormone pathways, small molecule modulators

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INTRODUCTION

It was first reported in the 1960s that circadian rhythm disruptions can lead to an increased likelihood of mammary tumor development, and that circadian genes may act as tumor suppressors (1). In previous decades, studies have suggested that alterations to circadian rhythms also accelerate breast epithelial stem-cell proliferation, induce mammary-gland development, and increase the formation of spontaneous breast tumors in mammals (2, 3). Disruptions to circadian rhythms in humans have also been associated with the development of several other cancer types, including prostate (4), endometrial (5), colon (6), lung (7), and ovarian cancers (8) and hepatocellular carcinoma (9). In addition, the rhythmic control of cell fate is believed to affect cancer therapies: the efficacy and/or toxicity of radiotherapy and antitumor therapeutics have been shown to be dependent on the timing of dose administration (10, 11). Thus, understanding the link between biological rhythms and cancers can both assist in the development of new treatments, and in optimization of current therapies.

In mammals, the molecular circadian clock can be divided into three components: input pathways, the central pacemaker, and output pathways. The input pathways transmit information from environmental cues (e.g., light) to the central pacemaker, which is located in the suprachiasmatic nucleus (SCN) of the hypothalamus (12). Within the SCN, multiple single-cell circadian oscillators are synchronized to generate daily circadian outputs (13). Output pathways convert the commands from the central pacemaker into circadian oscillations, which regulate physiological and behavioral functions in peripheral organs and tissues (14).

Circadian oscillations are mainly generated through two transcriptional/translational feedback loops (TTFLs) (15). The core loop involves four core clock genes: Circadian Locomotor Output Cycles Kaput (*CLOCK)* (16) and brain and muscle Arnt-like protein 1 (*BMAL1*) (17), which are the activators; and Period (*PER1*, *PER2*, and *PER3*) (18) and Cryptochrome (*CRY1* and *CRY2*) (19), which are the repressors. In the morning, the *CLOCK*:*BMAL1* heterodimer binds to an E-box DNA promoter, activating the transcription of *PER*, *CRY*, and other clock controlled genes. Late in the day, PER and CRY proteins dimerize and translocate from the cytoplasm to the nucleus, where they associate with the *CLOCK*:*BMAL1* complex and suppress its transcriptional activity at the E-box site (20). The suppression of *CLOCK*:*BMAL1* is released through the degradation of PER and CRY by ubiquitin-dependent pathways (21, 22) and casein kinases (CKIδ and CKIε) (23), which also control the timing of PER and CRY's entrance to the nucleus. After PER and CRY are degraded, the cycle begins again with ~24 h periodicity.

The secondary TTFL is mainly driven by transcriptional activation of the retinoid-related orphan receptors (RORs a, b, c) and repression of REV-ERBα/REV-ERBβ (24). To drive the rhythmic oscillation of BMAL1, REV-ERBα binds to the ROR elements in the *BMAL1* promoter, suppressing *BMAL1* transcription. Conversely, RORa and RORb activate *BMAL1* expression (25, 26). The cooperation between the two TTFLs and other kinases and phosphatases, which are critical for regulating period, phase, and amplitude of oscillations, provides robustness against environmental perturbations. This network also helps to maintain accurate circadian timing and adjust phase delays to align with local physiology (27).

EPIDEMIOLOGICAL EVIDENCE OF ALTERED CLOCKS' EFFECTS ON CANCER

Lifestyles have dramatically changed since the invention of the light bulb in 1879. Since then, the daily activities of humans have expanded into the night, including "night-shift" occupations (28). According to the U.S. Bureau of Labor Statistics, in 2016, the majority of the employed population worked in the service industry (80.3%), including health care, social assistance, and transportation, followed by manufacturing (7.9%) (29)—areas with high proportions of shift work. Another report published in 2015 found that about 17–24% of the workforce in United States was assigned to irregular or on-call work schedules, including night and rotating shifts (30). These types of schedules can lead to disruption of the sleep–wake cycle and circadian time organization, in addition to exposure to light at night (LAN) for long periods of time (31, 32). Perturbations to sleep and circadian rhythms can cause metabolic changes (33) and immune suppression (34), which can lead to various health problems, including diabetes (35), obesity (36), and cardiovascular disease (37), in addition to cancer (38). As a result, the International Agency for Research on Cancer has classified "shift-work that involves circadian disruption" as a "potential carcinogenic to humans (Group 2A)" (39).

While debated in some instances, epidemiological studies have provided evidence to support the association between shift work and cancer risk (40, 41). Independent cohort studies of night workers and shift workers have observed increased incidence of breast (42), prostate (4), colon (43), and endometrial epithelial malignancies (44) and non-Hodgkin's lymphoma (45), with risk further increased among individuals who have spent more hours and years working at night (42, 46). A case control study in Western Australia found that there was a 22% increase in breast cancer incidence among those who worked between midnight and 5:00 a.m. (47). Another study in France showed that there was a significant association (OR = 1.95) between breast cancer and women who worked night shifts for more than 4 years before their first full-term pregnancy. At that time their mammary-gland cells were found to be incompletely differentiated, making them more susceptible to circadian disruption effects (48). While it is difficult to eliminate shift work from society, there are some aspects that can be modified, which may decrease the risk of developing adverse health effects. To further understand the contributions of shift work to pathological development, extensive animal and cellular experiments have yielded proposed molecular mechanisms, which will be discussed in Section "Molecular Studies of Circadian Clocks and Breast Cancer."

Jet lag is another environmental factor associated with altered circadian rhythms and higher incidence of cancers (49). Jet lag (or circadian desynchrony) is a sleep disorder arising from the mismatch between internal body clocks and the environmental light/dark cycle. This condition is typically the result of travel through multiple time zones over a short period of time (50). An early study in Finland showed that flight attendants have significantly higher incidence of breast cancer (81.2/100,000) compared with the general female population (57.4/100,000) (51). A later, follow-up assessment strongly suggested that the increased cancer incidence was related to disruption of sleep rhythms, caused by excess exposure of light during normal sleeping hours, resulting in melatonin dysregulation (52). In addition, a recent study published in 2017, which focused on the effect of exposure to LAN in the United States, showed that there was a 14% increased risk of breast cancer in the highest LAN compared with the lowest LAN (53). Similar results were reported in Israel, where there was a 73% higher incidence of breast cancer in communities with the highest LAN than lowest LAN, across 147 communities (54). All of these epidemiological studies have strongly indicated that the disruption of circadian rhythms contributes to cancer risk.

MOLECULAR STUDIES OF CIRCADIAN CLOCKS AND BREAST CANCER

The functions of clock genes in each tissue are unique and show specific oscillation patterns (55). Their expression and regulation play important roles in breast biology. It has been shown that the core clock genes exhibit different mRNA expression patterns during mammary-gland development and differentiation in mice (56). Among 14,070 tested genes in human epithelial cells, 1,029 genes showed rhythmic oscillations during lactation. The expression patterns of these genes can be clustered into two groups, one high in the morning and another in the evening, indicating that the expressions change with a period of 24 h (57). Not only are the expression levels of clock genes variable, but they are affected by different developmental stages of breast tissue, and the extracellular microenvironment (58). Thus, it is posited that disruption of clock genes can affect normal breast biology and induce or affect cancerous development.

Breast cancer is heterogeneous and can be classified into subtypes based on histology, tumor grade, lymph node status, and the presence of specific biomarkers (59). The three markers generally used in characterization are estrogen receptor (ER), human epidermal growth factor receptor 2 (HER2), and progesterone receptor (PR) (60, 61). Based on marker status, breast cancer can be grouped into at least four subtypes: luminal A (ER^+, $PR^{+/-}$, $HER2^-$), luminal B (ER^+, $PR^{+/-}$, $HER2^+$), HER2 (ER^-, PR^-, $HER2^+$), and Basal (ER^-, PR^-, $HER2^-$) (62, 63). Basal tumors are typically difficult to treat and have poor prognoses. Because they lack ER, PR, and HER2, they are sometimes referred to as "triple-negative."

The disruption of nuclear hormone levels and signaling has also been posited to alter circadian rhythms, drawing another connection between rhythms and breast cancer (64). The estrogen receptor-α (ERα) signaling pathway (65) has been linked to the disruption of PER2 in breast cancer (**Figure 1**) (66, 67). It is known that *PER2* is a direct transcriptional target of ERα and its expression is inducible by 17 β-estradiol (E2) simulation (64, 68). In normal human breast epithelial cells, both *ER*α and *PER2* show rhythmic oscillations. The ubiquitous presence or absence of clock proteins has been predominantly used to investigate the relationship between circadian rhythms and breast cancer development (**Table 1**) (69–71). Knockdown of either *PER2* or *ER*α results in aberrant circadian oscillations of *ER*α, *PER2*, *BMAL1*, and *RARA* (another direct ERα target gene) and affects breast acinus structures (66). It was first reported in 2007 that suppression of *PER2* leads to ERα stabilization, and conversely, overexpression of *PER2* in breast cancer cells significantly inhibited cell growth and promoted apoptosis (64, 72). This work was corroborated by showing that complete loss of *PER2* mRNA oscillations occurred only in ERα-positive breast cancer cells, while ERα-negative breast cancer cells retained partially rhythmic oscillations (66, 67). In mice, downregulation of *PER2* enhanced breast tumor growth, leading to further enhancement of amplitude and phase delay (70). All of these studies have suggested that the expression of clock genes may be disrupted by hormone levels and their signaling circuits (**Figure 1**) (73, 74). In addition, genome-wide DNA methylation profiling has shown that PER1 is significantly hypomethylated in ER^+/PR^+ breast cancer tissues (75). A separate study also showed that PER1, 2, and 3 exhibited deviant protein expressions in 55 resected breast cancer tissue sections, when compared with adjacent non-cancerous tissue samples. These fluctuations may be the result of methylation of the *PER* promoter (76). However, the detailed mechanisms of how hormone signaling affects circadian clocks and *vice versa* are still unclear.

BMAL1 has also been proposed to act as a tumor suppressor. In separate studies performed in lung cancer and glioma cells, knockdown of *BMAL1* promoted cancer cell proliferation, invasion, and tumor growth, while its overexpression reduced cellular invasiveness (71, 79). Effects occurred in a p53-independent manner (*p53* expression was decreased in all *BMAL1* knockdowns) and were accompanied by activation of the phosphoinositide 3-kinase (PI3K)–Akt–MMP-2 signaling pathway (79). While these studies used other cancer models to study the role of *BMAL1*, the findings are likely relevant to breast cancer. *p53* mutations in breast cancer are relatively frequent (~20%) (85, 87), and the PI3K/Akt pathway is commonly affected (~70%) (88). However, the same study found that p21 (a p53 target protein) and c-myc exhibited different expression levels in various *BMAL1*-knockdown colon cancer cells, indicating that the relationships among BMAL1, p21, and c-myc are probably cell-type specific (71).

By contrast, CLOCK has been indicated as a tumor driver. Healthy breast patient tissues showed lower CLOCK expression than breast tumor tissues (77, 78). Knockdown of *CLOCK* resulted in attenuation of breast cancer proliferation (77) and downregulation of several cancer-associated genes, including ones related to breast tumor progression and metastasis initiation, such as *CCL5*, *BDKRB2*, and *SP100* (78). Furthermore, increased methylation in the promoter region of *CLOCK* has been associated with decreased breast cancer risk (78). While these studies provide valuable insight to the involvement of clock proteins in breast cancer development, most of these experiments do not account for the dynamic nature of circadian rhythms, and the fact that they may be altered but not abolished with human behavior and disease.

More recently, a number of *in vitro* studies have investigated clock gene expression profiles in a time-dependent manner in various breast cancer cell lines, including: MCF7 and T47D (luminal A subtype); HCC-1954 (HER2 positive subtype); MCF10A and MDA-MB-231 (basal-like subtype), and others (67, 80–83). Intrinsic circadian oscillations in cultured cells can be entrained through treatment with high concentrations of serum to serum starved cells (89), or by chemical induction of signaling pathways, such as protein kinase A (*via* forskolin) (90) or the glucocorticoid receptor (*via* dexamethasone) (91). After entrainment, the expression patterns of clock genes, including *BMAL1*, *CLOCK*, *PER1*, *PER2*, *CRY1*, and *CRY2*, have largely been analyzed through quantitative real-time PCR, with conflicting results. While some studies revealed rhythmic gene expression in all breast cancer cell lines (67, 82), others did not (80, 83). Major factors contributing to the discrepancies were likely non-uniform cell culture and synchronization methods (i.e., varied serum depletion times before serum shock), which

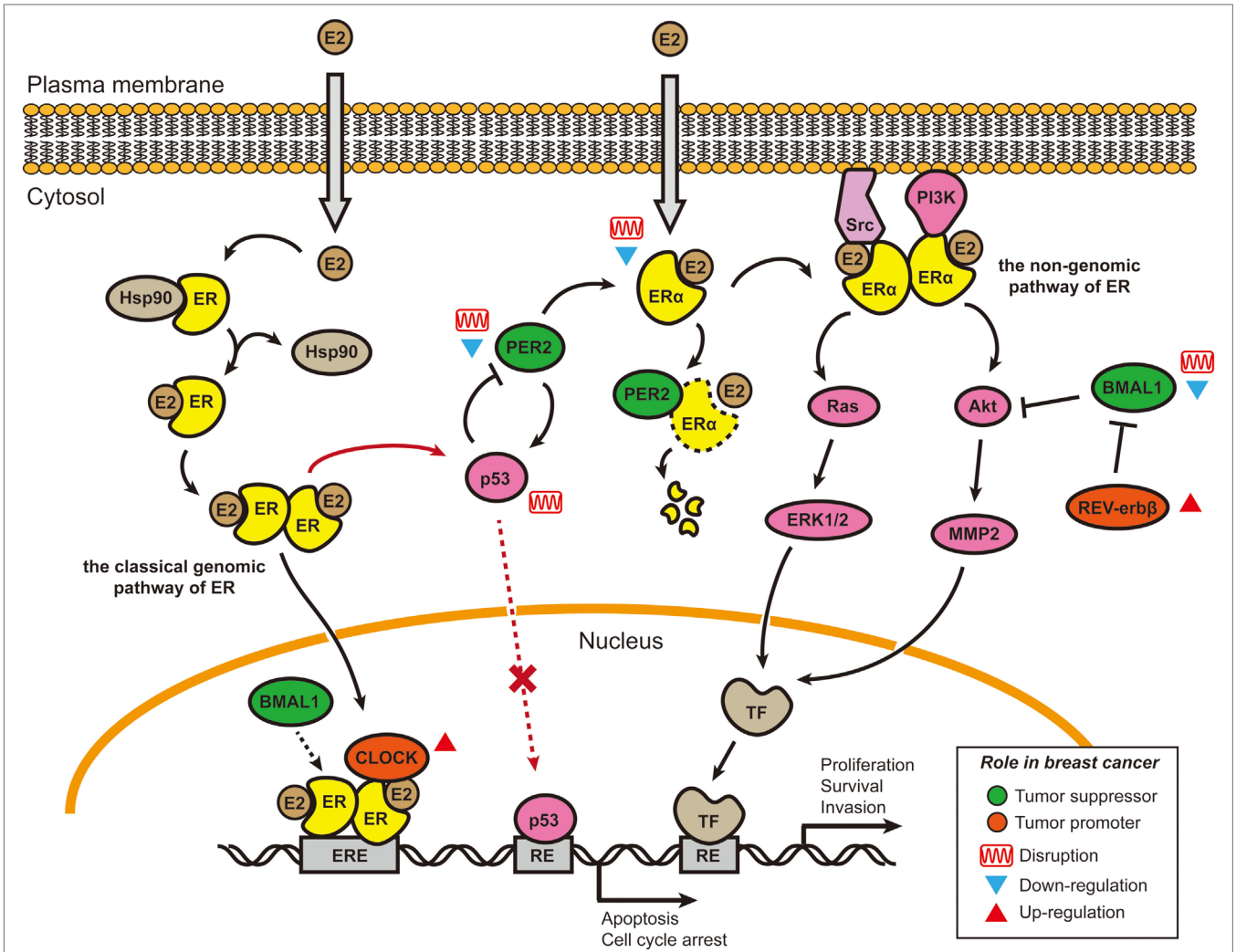

FIGURE 1 | Cross talk between E2–estrogen receptor-α (ERα) signaling pathways and circadian rhythms in breast cancer. Two of the four estrogen signaling pathways involve E2 stimulation and are shown here (65). In the classical genomic pathway, E2-bound estrogen receptor (ER) (either ERα or ERβ) dimerizes, changes conformation, translocates to the nucleus, and binds to the estrogen response elements (EREs). After binding to the EREs, the E2–ER complex recruits other co-activators, including Circadian Locomotor Output Cycles Kaput (CLOCK) and possibly brain and muscle Arnt-like protein 1 (BMAL1) (74), to initiate the transcription of target genes. CLOCK overexpression in breast tumors and promotion of tumor cell proliferation may be caused by co-activation with E2–ER complexes (77, 78). In the non-genomic pathway, E2–ERα complexes accumulate near the membrane and then recruit protein kinases [Src and phosphoinositide 3-kinase (PI3K)] to activate signaling cascades (Akt and Ras/MAPK). BMAL1 has been shown to suppress the Akt/MMP2 pathway and further inhibit cancer cell invasion (79). BMAL1 can suppress cancers, and its expression is downregulated or disrupted in various breast cancer cell lines (67, 80–83). By contrast, REV-ERBβ (a repressor in the secondary transcriptional/translational feedback loop) is generally overexpressed in breast tumor samples; its protective function can allow cancer cells to develop chemotherapy resistance (84). *PER2* is a direct ERα target gene and can bind to ERα and cause its degradation. In ERα-positive breast cancer cells, both PER2 and ERα lose their circadian oscillations, the underlying mechanism of which is not well understood. The cancer suppressor p53 can directly bind to the *PER2* promoter and inhibit its transcriptional activity (85). E2–ER complexes can block the induction of proapoptotic p53 target genes by binding to p53 protein in ER-positive breast cancer cells, thus helping cancer cells avoid apoptosis (73). Re-introduction of PER2 into the ER-positive breast cancer cells can induce p53 expression (72). Abbreviations: TF, transcriptional factor; RE, response element; E2, 17 β-estradiol.

may affect dampening rates over time, due to loss of synchronicity. In addition, the short-term data collection (typically ≤48 h) and insufficient numbers of data points (generally ≥4 h intervals) utilized in these studies are generally insufficient to yield good statistical curve fittings (92, 93), contributing to inaccurate analysis of rhythmic oscillations. However, within each study, it is apparent that oscillations of clock proteins vary across different breast cancer cell models. Application of luciferase reporters and fluorescent proteins (e.g., GFP) can provide better resolution for long-term tracking of circadian oscillations in synchronized cells (14). However, cancer cells can be heterogeneous even in culture conditions (94). Future work should focus on real-time analysis at the single-cell level to reveal how circadian rhythms are involved, disrupted, and deviate from one another in breast cancer. Furthermore, posttranscriptional and -translational modifications to core circadian clock components should also

TABLE 1 | Roles of clock genes in breast cancer development.

Circadian genes	Experimental approaches	Phenotype	Possible mechanism	Reference
CLOCK	Immunohistochemical assay(s) and qRT-PCR	Overexpressed in breast cancer cells; low expression in healthy breast tissue	Increased methylation in *CLOCK* promoter decreases breast cancer risk	(77, 78)
	Knockdown(s)	Reduced cell proliferation; downregulation of cancer-associated genes (CCL5, BDKRB2, and SP100)	E2–estrogen receptor (ER) pathway may couple to the circadian machinery due to presence of estrogen response element in the *CLOCK* promoter	(77, 78)
BMAL1	qRT-PCR	Disrupted mRNA expressions in breast cancer cells	Not clear	(67, 80–83)
	Knockdown(s)	Promoted cancer cell proliferation and invasion *in vitro* and tumor growth *in vivo*	Antagonized *Bcl-w* oncogene, which can activate phosphoinositide 3-kinase (PI3K)/Akt/MMP2 pathway; effects on *p53* and *c-myc* are cell-type specific	(71, 79)
PER1, 2, and 3	Immunohistochemical assay(s) and qRT-PCR	Downregulated in ER-positive breast cancer cells	Methylation in *PER* promoter in ER^+/PR^+ breast cancer tissues	(70, 75, 76)
	Knockdown(s)	Aberrant circadian oscillation of other clock genes; enhanced tumor growth *in vivo*; changed the structure of breast acinus	Coupling with E2–ER pathway and p53 pathway	(66)
	Overexpression	Significantly inhibited cell growth and promoted apoptosis	Inhibit the activation of ER and p53 target genes	(64, 72)
CRY1 and 2	qRT-PCR	Disrupted mRNA expressions in breast cancer cells	Not clear	(67, 80)
REV-ERBα	RNAi screen	Co-expression in *ERBB2*-positive breast tumors ($HER2^+$ subtype)	Upregulating several genes that are involved in *de novo* fatty acid synthesis, which further enhance the energy production for survival	(86)
REV-ERBβ	Overexpression	Protect tumor cells against chemotherapy	Not clear	(84)

be taken into consideration (95), since many malignant transformations occur posttranscriptionally.

CIRCADIAN CHRONOTHERAPY AND CANCER TREATMENT

Nearly, all metabolic functions are regulated in a circadian manner: food intake, digestion, detoxification, breakdown, and storage of sugars and fats (96–98). When organs are exposed to xenobiotics (e.g., drugs or environmental toxicants), they undergo classical absorption, distribution, metabolism, and elimination processes, which are all regulated by circadian clocks (11). Hence, accounting for circadian rhythms in the development of treatments and dosing regimens has the potential to improve disease outcomes. Two recent studies reported the effects of chemotherapy on circadian rhythms in patients with metastatic colorectal cancer (99, 100). It was found that chemotherapy-induced disruption was observed in approximately 50% of the patients and was correlated with shortened overall survival rate. Eliminating this perturbation has been suggested to reduce toxicity and enhance efficacy of chemotherapy.

Recently, compounds that specifically target clock components and/or modulate its oscillations have received a great deal of attention (101). There are many advantages to the usage of small molecules in studies of circadian-related diseases: (1) they can help us to better understand the molecular circadian network; (2) they can serve as lead structures for developing drugs; and (3) unlike genetic approaches, which can result in immutable modifications, small molecules can be used in reversible, time- and dose-dependent manners (102, 103). One common example is the amelioration of jet lag *via* use of the hormone melatonin (104, 105). A double-blind trial showed that melatonin can significantly reduce jet lag and sleep disturbance in an international cabin crew (106). Small molecules can also be used to modify circadian rhythm periods to minimize chronodisruption resulting from shift work. Since the entrained phase is associated with circadian period, the period modification should change the preferred phase of behavior (107). Amplitude enhancement has also been shown to combat metabolic syndromes (108), reverse age-related effects (109), and protect against psychiatric diseases (110).

Small molecules have been used to elucidate the connections between circadian rhythms and breast cancer, for example the role of *REV-ERBs* in the $HER2^+$ subtype (111). The *NR1D1* (REV-ERBα coding gene) is connected to *ERBB2* (HER2 coding gene) in the 17q12 amplicon, resulting in their co-expression in *ERBB2*-positive breast tumors (86). The same study suggested that REV-ERBα serves as a survival factor for $HER2^+$ breast cancer cells. However, more recent work has shown disagreements. By activating REV-ERBs *via* the synthetic agonist SR9011, decreased cell proliferation was observed in various breast cancer cells, independent of their ER or HER2 status (112). Another study found that dual inhibition of REV-ERBβ and autophagy by ARN5187, a novel REV-ERBβ ligand, can induce cytotoxicity in breast cancer cells (84). It was also shown that REV-ERBβ was dominantly expressed in breast tumor samples, while REV-ERBα was the predominant form in normal tissues.

Overexpressed REV-ERBβ appeared to result in protection that made tumor cells resistant to chloroquine, a clinically relevant lysosomotropic agent suppressing autophagy. With ARN5187 treatment, REV-ERB-mediated transcription was inhibited. Grimaldi et al. suggested that this compound has the potential to serve as an anticancer agent (84). Although clock modulators alone may not be sufficient to induce anticancer effects, combined treatment with well-established anticancer drugs should enhance their potency and efficacy, and reduce toxicity of the drugs. Characterization of disrupted circadian patterns in various types of cancer can provide clues for the application of clock modulators in combination with anticancer drugs to achieve the best possible therapeutic results.

CONCLUSION

Circadian rhythms are essential to the regulation of many physiological and behavioral functions in mammals. Their disruption has been linked to development of many health problems, including breast cancer. This is supported by epidemiological evidence, assessing both shift workers and people exposed to chronic jet lag. The status of core circadian clock components has also been evaluated in cancerous versus healthy tissues, and the significance of these components has been investigated *via* overexpression or deletions. While more recent studies have addressed changes in oscillations across cancer types, investigations at higher resolutions are required to facilitate more thorough analysis. From the work reviewed here, it is clear that circadian rhythms and proto-oncogenes/signaling pathways (e.g., *PI3KCA*, *p53*, or E2–ER) can both affect one another. However, the molecular mechanisms behind these associations are not well understood, and currently very few studies exist that examine the effects of altered rhythms on oncogenic pathways. Future work should also take advantage of existing technologies (including high-resolution confocal microscopy) (113) to track and analyze dynamic circadian oscillations at the single-cell level. While posttranscriptional and -translational modifications are also critical elements of the puzzle, real-time monitoring of these processes remains difficult to achieve. By increasing knowledge of the molecular mechanisms associated with disrupted clocks in cancer, new therapeutics and adjuvants can be developed with enhanced efficacy against the disease.

AUTHOR CONTRIBUTIONS

H-HL wrote this article and generated the graphic and table, with content and editorial revisions from MF.

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Food-Anticipatory Behavior in Neonatal Rabbits and Rodents: An Update on the Role of Clock Genes

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In mammals, the suprachiasmatic nucleus (SCN), the master circadian clock, is mainly synchronized to the environmental light/dark cycle. SCN oscillations are maintained by a molecular clockwork in which certain genes, Period 1–2, Cry1–2, Bmal1, and Clock, are rhythmically expressed. Disruption of these genes leads to a malfunctioning clockwork and behavioral and physiological rhythms are altered. In addition to synchronization of circadian rhythms by light, when subjects are exposed to food for a few hours daily, behavioral and physiological rhythms are entrained to anticipate mealtime, even in the absence of the SCN. The presence of anticipatory rhythms synchronized by food suggests the existence of an SCN-independent circadian pacemaker that might be dependent on clock genes. Interestingly, rabbit pups, unable to perceive light, suckle milk once a day, which entrains behavioral rhythms to anticipate nursing time. Mutations of clock genes, singly or in combination, affect diverse rhythms in brain activity and physiological processes, but anticipatory behavior and physiology to feeding time remains attenuated or unaffected. It had been suggested that compensatory upregulation of paralogs or subtypes genes, or even non-transcriptional mechanisms, are able to maintain circadian oscillations entrained to mealtime. In the present mini-review, we evaluate the current state of the role played by clock genes in meal anticipation and provide evidence for rabbit pups as a natural model of food-anticipatory circadian behavior.

Keywords: circadian rhythms, clock gene mutant, restricted feeding, food entrainment, corticosterone, PER1 protein

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INTRODUCTION

The suprachiasmatic nucleus (SCN), located in the ventral forebrain lateral to the third ventricle, is the master circadian pacemaker necessary for the control of endogenous physiological and behavioral rhythms in mammals (1). At the cellular level, a group of genes, known as clock genes, are necessary to generate and sustain circadian rhythms controlled by the SCN. This clock mechanism is a transcription–translation autoregulatory feedback loop with the positive arm comprised of *Clock* and *Bmal1* genes and their proteins. CLOCK and BMAL1 proteins form heterodimers that bind to E-box enhancer elements in the promoter region of the Period (*Per1–2*) and Cryptochrome (*Cry1–2*) genes to activate their transcription. In turn, PER and CRY proteins constitute the negative arm of the loop. CLOCK and *Bmal1* also activate the transcription of retinoic orphan receptor α, β, and REV-ERBα,β,γ, which form an auxiliary loop driving rhythmic Bmal1 transcription with activating and repressing actions, respectively.

In mammals, light is the main entraining signal for circadian rhythms. However, food also can be a synchronizer. When rats are fed one meal per day, within a few days they display "food-anticipatory activity" [FAA (2–4)], with arousal and an increase in locomotor behavior occurring some hours before mealtime. August Forel was the first to describe this phenomenon around one century ago, showing that bees anticipate the time of morning meals (4). In rats, in addition to increase in locomotor behavior, there is also an increase in serum levels of corticosterone and core body temperature before mealtime (5).

Food-anticipatory activity exhibits circadian properties such as limits of entrainment close to 24 h, transient cycles following phase shifts and persistence during fasting conditions [Rev (3)]. Following the discovery of the SCN as the locus of the master circadian clock, whether the SCN also served as the neural substrate of FAA was explored. Surprisingly, the anticipatory increase in locomotor activity, core body temperature, and corticosterone in food-entrained rats was not abolished by lesions of the SCN (5). This and subsequent experiments led to a search for the existence of a food-entrainable oscillator (3) distinct from the SCN light-entrainable oscillator. Many neural loci and glands were examined as potential sites regulating FAA, including the adrenal gland, several brain regions in the hypothalamus (i.e., ventromedial, dorsomedial and paraventricular nuclei, lateral preoptic area), the parabrachial nuclei, olfactory bulb (OB), hippocampal formation, cerebellum, amygdala and nucleus accumbens, among others [Rev (6, 7)]. In all cases, lesions or excision failed to abolish FAA. These studies suggested that FAA lies in a specific, unknown locus or, perhaps more likely, consists of an organized, distributed system of interacting structures both at the central and peripheral levels (7). In the present overview, we provide evidence for a role of clock genes in FAA, providing a new strategy to explore this phenomenon.

CIRCADIAN GENES RELEVANT TO FOOD ANTICIPATION

Clock genes serve as key elements for the generation of circadian oscillations in the SCN. When one of these elements is rendered non-functional, alterations in behavioral and physiological rhythms appear. Because FAA is under the control of a circadian mechanism, it is logical to hypothesize that clock gene mutations might also affect FAA (**Table 1**).

One of the first studies pointing to a role for clock genes in FAA comes from studies of *ClockΔ19* gene mutant mice (8). The circadian locomotor behavior of these mice is arrhythmic when animals are exposed to constant darkness (DD) conditions (22, 23). When exposed to restricted-feeding schedules, *ClockΔ19* mice show strong FAA and its persistence during food deprivation indicates that the *Clock* gene is not necessary for FAA. While this study suggested that *Clock* is not essential for FAA, it was later shown that the *Npas2* gene can compensate for the loss of *Clock*, acting as a positive transcription factor in the SCN to maintain circadian oscillations (24). This finding suggested that *Npas2* might be implicated in the regulation of FAA. Indeed, *Npas2* mutant mice exposed to temporally restricted feeding show altered FAA, requiring two or three more days to develop FAA relative to WT animals (9). Thus, *Npas2* appears to be an important gene in the regulation of FAA. However, FAA does not disappear entirely in *Npas2* mutant mice, indicating that other genes contribute to the maintenance of FAA.

TABLE 1 | Summary of the effects of clock genes mutations in circadian locomotor activity rhythms and food-anticipatory activity (FAA).

Clock gene	Circadian alteration (locomotion)	FAA (locomotion, wheel-running)	Reference
ClockΔ19	Arrhythmic in DD	Normal	Pitts et al. (8)
Npas2	Normal	Delayed	Dudley et al. (9)
Bmal1	Arrhythmic in DD	Normal	Bunger et al. (10) and Pendergast et al. (11)
Bmal1 (brain-specific)	Shortened period in DD	Attenuated	Mieda and Sakurai (12)
Per1	Shortened period in DD	Normal	Zheng et al. (13) and Feillet et al. (14)
Per2^{Brdm1}	Arrhythmic in DD	Absent/normal	Zheng et al. (15), Feillet et al. (14), and Pendergast et al. (16)
Per2 (liver-specific)	Normal in DD	Absent	Chavan et al. (17)
Cry1–2	Arrhythmic in DD	Attenuated	van der Horst et al. (18) and Iijima et al. (19)
Rev-erbα	Shortened period in DD	Attenuated	Preitner et al. (20) and Delezie et al. (21)

As mentioned previously, the positive loop of the clockwork also includes *Bmal1*, a gene that is rhythmically expressed in the SCN and other peripheral oscillators (25). Global mutations of *Bmal1* lead to arrhythmic behavior when animals are in DD conditions (10), while FAA is normal in these animals (11). However, in one study, *Bmal1* deletions confined to the dorsomedial hypothalamus eliminated FAA (26). The reason for this discrepancy is not readily clear; because methods and protocols to measure locomotor activity differ between studies, this conclusion remains to be confirmed (26, 27). Importantly, in another study using mice with a nervous system-specific deletion of *Bmal1*, excluding the SCN clock, it was demonstrated that FAA is strongly affected, suggesting the necessity of *Bmal1* in an extra-SCN brain locus for FAA (12). Further confirming a role for *Bmal1* in FAA, in *Rev-erbα*-mutant mice exposed to restricted-feeding schedules, FAA was negatively affected (21). *Rev-erbα* is a transcription factor with a repressor activity on *Bmal1* (20).

With regard to the negative arm of the clockwork, double *Cry* gene mutations (*Cry1–2*) lead to arrhythmic behavior in mice held under DD (18) and FAA is markedly reduced (19). *Per* genes (1–2), also important components of the negative loop, are essential in the control of circadian rhythmicity. These genes are expressed rhythmically in diverse brain structures and peripheral organs [liver, heart, and lung (13, 15, 28)]. Whereas *Per1*$^{-/-}$ mutants show normal FAA, FAA is absent in *Per2*^{Brdm1}-mutant mice (14). However, in a more recent examination of the same *Per2* mutant mice, FAA was not altered (16). Thus, the effects of global mutations of *Per2* on FAA remain to be clarified. Interestingly, when *Per2* is knock down specifically in the liver, FAA is totally eliminated and can be rescued by viral overexpression of liver

Per2 (17). This study indicates that FAA is not only dependent upon the brain but that it also requires normal *Per2* expression in the liver for its manifestation, confirming that *Per2* is likely an important component of the molecular mechanisms of FAA (**Table 1**).

Most studies of FAA examine rodents under a schedule of food restriction. However, most animals in their natural environments do no experience food restriction on a circadian schedule. In contrast, rabbit pups are fed for brief periods on a circadian schedule in nature and the lab. Thus, in the present contribution, we present evidence that supports notion that the rabbit pup constitutes a natural model of food entrainment.

FAA IN THE RABBIT

Rabbit pups are born altricial, they have no fur, their eyelids and outer ears are sealed, and they remain in the maternal burrow in darkness for the first 2 weeks of their life (29). Behavioral studies (29, 30) confirm that shortly after parturition the mother leaves the nest and returns every day with a circadian periodicity to nurse pups whether they are maintained in continuous light or in light–dark conditions (31, 32). Although parturition occurs throughout the day, the time of nursing is rapidly established on lactation day 1 and then nursing occurs every 24 h at around the same hour every night, 03:52 h across lactation days 1–15 (33, 34).

LOCOMOTOR BEHAVIOR

Although pups are not entrained by the light–dark cycle (their eyes do not open until postnatal day 10) (35), they receive periodic time cues through feeding. Every day at around the same time they ingest up to 35% of their body weight in milk (36) in around 5 min (31, 32). Hence food, in this case milk, seems to be a potent zeitgeber for rabbit pups. To explore in detail behavioral, physiological, and neural consequences of timed feeding, we scheduled nursing at two different hours, at 10:00 a.m. and at 02:00 a.m. (i.e., during the day and during the night, respectively) from postnatal (PD) 1. At PD3 (02:00 a.m. group) and PD4 (10:00 a.m. group), despite their altricial condition, pups show a significant increase in locomotor behavior 2 h before the mother's arrival. Immediately after suckling, locomotor behavior decreases and pups remain inactive and huddled in the nest. Moreover, this locomotor increase persists for 2 days in nurse-deprived pups at the same hour of the last nursing (37).

CORTICOSTERONE

In contrast to neonatal rodents which are in a stress hyporesponsive period (38), we found that 7- to 9-day-old rabbits exhibit rhythmic secretion of corticosterone with higher plasma levels at the time of nursing, reaching a nadir 12 h later and increasing again in advance of the next nursing bout (39). Peak levels of corticosterone shift in parallel with the nursing schedule either during the day or the night and persist during fasting conditions (40, 41), indicating entrainment by time of nursing. In adult rodents this hormone reaches a peak at the time of food presentation (5, 42). See **Figure 1**.

CORE BODY TEMPERATURE

Rabbit pups maintained in constant dim light exhibit a 24-h rhythm of core body temperature with a significant anticipatory rise of 0.4–0.6°C around 3 h before daily nursing. This increase is followed by a secondary postprandial rise, followed within 1–3 h by a temperature drop. Moreover, during a 48-h fast, the anticipatory rise in temperature persists, while the postprandial increase in temperature does not (45, 47). These results indicate that the anticipatory increase is endogenous and entrained by the timing of nursing, whereas the postprandial increase is induced by food ingestion. In **Figure 1**, we present a comparison of daily rhythms of locomotor activity, corticosterone release and body temperature in relation to FAA in adult rodents and rabbit pups. In these species, there are changes in FOS protein, clock genes, and PER1 protein in some brain structures described further below.

CLOCK GENES AND FAA IN THE RABBIT PUP

Olfactory Bulb

Postnatal day 7 pups receive temporal time cues through the brief daily visit by their mother and ingestion of a meal once a day. To successfully ingest milk, pups depend on their OB to detect the emission of a mammary pheromone (48) and to grasp the nipple; anosmic pups are unable to suckle milk and will die of starvation (49). At PD7, rhythms of the clock genes *Per1*, *Bmal1*, and *Cry1* are already established in the OB, whereas a clear rhythm is not detected until PD45 in the SCN (50). The earlier maturation of the clockwork in the OB is consistent with the dependence on suckling at this age. Analysis of PER1 protein in the OB has been explored to determine the pattern of rhythms in this protein relative to the timing of suckling. At PD7, neonatal rabbits express robust rhythms of PER1 in layers of both main and accessory OBs that shift in parallel to the timing of suckling (i.e., either during the day or during the night). Moreover, PER1 expression persists during fasting conditions. Additionally, significant increases in FOS protein were detected at the time of suckling (i.e., during FAA), suggesting that the OB has a clock mechanism that anticipates nursing (51). This finding is consistent with previous work showing that the OB has an SCN-independent circadian pacemaker (52).

A milk/nipple stimulus appears to be important for OB oscillations. In this regard, the role of a mammary pheromone has been explored as an entraining signal (50); however, its importance remains unclear as the pheromone was applied at concentrations far beyond the effective concentration to elicit the oral nipple grasping response (53). Additionally, food has been explored as the entraining signal. In neonatal rabbits, the intragastric infusion of milk formula at PD7 once during the day or during the night without any maternal contact entrained rhythms of locomotor behavior and CORT, with peak values at the time of FAA. The milk stimulus also entrained rhythms of PER1 in hypothalamic nuclei (see below). These rhythms shift in parallel to the timing of milk formula infusion, demonstrating that food, in this case milk, is sufficient to entrain behavioral, physiological, and neural

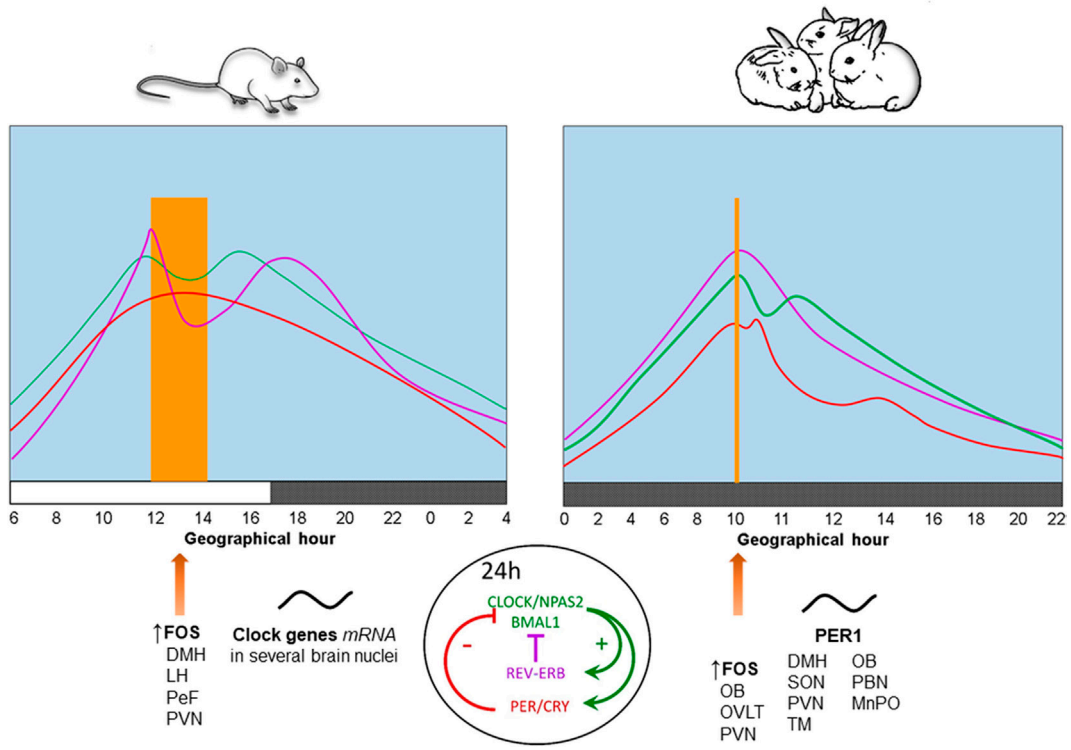

FIGURE 1 | Physiological, behavioral, and neural changes associated with food-anticipatory activity and the molecular clockwork. Daily rhythms of locomotor activity (), corticosterone (), and body temperature () increase in anticipation of the time of feeding in both species. The molecular clock is comprised of two principal feedback loops for the expression of clock genes. In the positive loop (green) the proteins CLOCK/NPAS2 and BMAL1 act on the transcription sites of *Per*, *Cry*, and *Rev-erbα* genes to induce their mRNA expression. Once the final proteins of PER and CRY (negative loop; red) are produced, these have the ability to repress their own transcription *via* an inhibitory action on the *Clock-Npas2/Bmal1* dimer. The REV-ERBα protein is a transcriptional repressor for the *Bmal1* gene driving rhythmic *Bmal1*. FOS protein expression and rhythms of clock genes and proteins in several brain nuclei synchronize to mealtime. DMH, dorsomedial hypothalamic nucleus; LH, lateral hypothalamus; MnPO, median preoptic nucleus; OB, olfactory bulb; OVLT, organum vasculosum of lamina terminalis; PeF, perifornical nucleus; paraventricular nucleus; SON, supraoptic nucleus; TM, tuberomammillary nucleus. Vertical bar and big arrow, feeding time. Figure derived from data previously published by: Angeles-Castellanos et al. (43), Caba et al. (37), Escobar et al. (44), Honma et al. (42), Jilge et al. (45), Mistlberger (7), Morgado et al. (40, 41, 46), and Rovirosa et al. (39).

parameters in the neonatal rabbit (46), similar to what is seen in nursed pups. In contrast, the mammary pheromone is likely necessary for nipple detection, but not FAA.

Suprachiasmatic Nucleus

In the SCN, there is a rhythm of PER1 in nursed and fasted pups fed either during the day or the night from PD1 (37). More importantly, there is a shift in PER1 peak expression of 2.5 h between day and night nursed pups, suggesting an entraining effect of timed nursing on the pup's SCN. A larger shift of *Per1*, *Per2*, and *Bmal1* rhythms was demonstrated by shifting the time of nursing from PD4–PD7 (54). However, in this same study, there was a spontaneous advance in *Per1* of around 7 h from PD3 to PD9 in pups nursed at the same time since birth. Therefore, it is not clear if the influence of ontogenetic development of the SCN on the shift in clock genes is mediated by the timing of nursing. Although retinal projections are present in the SCN at birth, the nucleus is immature in its response to a light pulse until PD12 (55). Despite methodological differences, it is possible that the pups' SCN is sensitive to non-photic cues. The effect of food restriction on the SCN has been reported in adult rats and may be involved in the neural mechanism of food entraining (56), although, as already mentioned, this nucleus is not essential for FAA.

Other Brain Structures

In the dorsomedial hypothalamic nucleus (DMH) there is a complete phase shift of PER1 in parallel to a change in the time of nursing that persists in fasted pups (37). These results agree with publications in rodents (57, 58), indicating that the DMH might play an important role in food entrainment, although not as the unique brain structure regulating FAA (17). PER1 has been also analyzed in the median preoptic nucleus (MnPO), organum vasculosum of lamina terminalis, and medial preoptic area (59). However, a robust rhythm of PER1 is only detected in the MnPO at the time of FAA, a rhythm that persists during fasting. To our knowledge, there are no reports regarding a role of the MnPO in FAA in rodents, pointing to a need for further exploration. In the brainstem the dorsal vagal complex (DVC) and parabrachial nucleus (PBN) express PER1 in neonatal rabbits. Whereas the DVC shows rhythms related to food ingestion, the PER1 rhythm

was entrained by milk intake in the PBN, a rhythm that persists during fasting (60). It is possible that changes in PER1 are due to food ingestion as the paraventricular, supraoptic, and tuberomammillar nuclei shows PER1 rhythms that shift in parallel to the timing of intragastric milk formula infusion (46).

METABOLIC AND HORMONAL SIGNALS AND THE REWARD SYSTEM

Metabolic fuels such as glycogen and free fatty acids follow a rhythm associated with the full and empty stomach to maintain stable glucose levels; those levels are maintained even in fasting conditions (40, 41). Interestingly, the orexigenic hormone, ghrelin, which acts on the arcuate nucleus, also follows a rhythm with peak levels 12 h after the last nursing, likely participating in triggering the next FAA episode (40, 46). Indeed, in rats under restricted feeding, plasma ghrelin levels peak before mealtime (61) and, in combination with leptin, modulates the reward circuitry by acting on dopaminergic neurons in the ventral tegmental area to reinforce FAA (62, 63).

CONCLUSION

Food-anticipatory activity is the expression of a circadian phenomenon in different species, usually studied in adult subjects. Here, we demonstrate that the neonatal rabbit circadian system is an ideal natural model to study the brain and molecular mechanism of FAA. FAA depends, in part, on some clock genes expressed in a circadian network of brain structures, oscillating in synchrony, and coordinated by the SCN. Combining information on brain clock gene expression in rabbit pups with mouse models of clock gene mutations for the study of FAA will help increase understanding of the molecular mechanisms implicated in food anticipation in the wild.

AUTHOR CONTRIBUTIONS

MC and JM contributed to the writing of the manuscript and approved the final version.

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Circadian Regulation of Glutamate Transporters

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L-glutamate is the major excitatory amino acid in the mammalian central nervous system (CNS). This neurotransmitter is essential for higher brain functions such as learning, cognition and memory. A tight regulation of extra-synaptic glutamate levels is needed to prevent a neurotoxic insult. Glutamate removal from the synaptic cleft is carried out by a family of sodium-dependent high-affinity transporters, collectively known as excitatory amino acid transporters. Dysfunction of glutamate transporters is generally involved in acute neuronal injury and neurodegenerative diseases, so characterizing and understanding the mechanisms that lead to the development of these disorders is an important goal in the design of novel treatments for the neurodegenerative diseases. Increasing evidence indicates glutamate transporters are controlled by the circadian system in direct and indirect manners, so in this contribution we focus on the mechanisms of circadian regulation (transcriptional, translational, post-translational and post-transcriptional regulation) of glutamate transport in neuronal and glial cells, and their consequence in brain function.

Keywords: circadian rhythms, clock genes, EAATs, glutamate transporters, neurodegenerative disorders

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CIRCADIAN BIOLOGICAL CLOCK

Life has adapted to 24-h rhythms, better known as circadian rhythms (1). Consequently, a large number of organisms have circadian clocks that anticipate daytime and establish endogenous 24-h rhythms, which organize their physiology and behavior (2, 3). These endogenous rhythms are synchronized with the environment through external signals, the so-called *zeitgebers* ("time giver" in German), being the light the principal time cue (4).

Intracellularly, the mechanisms involved in circadian regulation are transcription-translation feedback loops of a group of genes denominated *clock genes* (5–7). In mammals, Brain muscle arnt-like 1 (BMAL1) and Circadian locomotor output cycles kaput (CLOCK) complexes control the periodic expression of *Cryptochrome 1* and *2* (*Cry1* and *2*), and *Period 1* and *2* (*Per1* and *2*), whose protein products inhibit BMAL1 and CLOCK, as well as their own transcription (5–8). These circadian transcription factors regulate thousands of clock-controlled genes, which orchestrate diverse physiological, metabolic and behavioral functions, resulting in a synchronized organism (3). Most tissues and cell types in the body possess a molecular clock (peripheral clocks) synchronized by the principal pacemaker located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus (2, 3, 9). Approximately, around 2–30% of each tissue's transcriptome is rhythmically synthesized (10, 11).

In mammals, the SCN receives direct photic input from photosensitive retinal ganglion cells via the retinohypothalamic tract (RTH) (12, 13). This tract mainly uses glutamate (Glu) as its neurotransmitter; however, pituitary adenylate cyclase-activating peptide (PACAP) and substance

P are two peptide co-transmitters that also participate in retino-hypothalamic transmission (14–16). Interestingly, it has been shown that both of these co-transmitters regulate Glu neurotransmission, although the mechanism by which it is carried out remains unknown (15, 17–19). *In vivo* and *in vitro* studies have identified both metabotropic and ionotropic Glu receptors in the SCN (20–22), although it has been demonstrated that specific distribution and abundance of each Glu receptor subunit differs in this structure resulting in different effects of Glu on SCN neurons (21).

GLUTAMATE

Glutamate (Glu), the main excitatory neurotransmitter in the mammalian central nervous system (CNS), activates two subtypes of Glu receptors: ionotropic (iGluRs) and metabotropic (mGluRs) (23–25). The first group refers to a family of ligand-gated ion channels that have been classified by means of their pharmacological properties into: N-methyl-D-aspartate (NMDA), and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and kainate (KA) receptors (24). The second subtype of Glu receptors belongs to class C of G-protein-coupled receptors, and its classification is based on the homology of their sequences, pharmacology, and signal transduction mechanisms (23, 25). It includes group I (mGluR1 and mGluR5), group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, mGluR7, and mGluR8) (23, 25). Both subtypes of Glu receptors are widely expressed on pre- and post-synaptic terminals as well as on astrocytes that surround synapses (23, 26, 27).

Glu concentration in the synaptic cleft is in the low millimolar range (28, 29). However, after periods of intense glutamatergic activity, an excessive extracellular Glu concentration leads to an overstimulation of Glu receptors resulting in neuronal death, a phenomenon known as excitotoxicity, which is involved in neurodegenerative diseases (26, 30). In this context, Glu uptake from the extracellular space plays an essential role in the prevention of excitotoxic insults (28). A family of Na^+-dependent high affinity Glu transporters carries out the Glu removal from the synaptic space. The excitatory amino acid transporters (EAATs) comprise five different Glu transporters: Glu/aspartate transporter (GLAST), Glu transporter 1 (GLT1), excitatory amino acid carrier 1 (EAAC1), excitatory amino acid transporter 4 (EAAT4), and excitatory amino acid transporter 5 (EAAT5) or EAAT 1-5 according to rodent and human nomenclature, respectively (28, 31–36). These transporters display a 50–60% amino acid sequence similarity, although different pharmacological and molecular properties, structure, and expression patterns are present for each subtype (28, 37). Within the CNS, Glu transporters have differential cell expression (glial or neuronal) (31, 36, 38–40). GLAST and GLT1 are found predominantly in the astrocytic plasma membrane (38–40), whereas EAAC1/EAAT4/EAAT5 are neuronal transporters mainly localized in hippocampal neurons, Purkinje cells, and rod photoreceptor and bipolar cells of the retina, respectively (31, 35, 36, 38, 41). However, GLT1 expression in neurons (28, 42–44), as well as EAAC1 and EAAT4 immunoreactivity in cortical and spinal cord astrocytes have also been reported (45, 46). GLAST and GLT1 carry out ∼80–90% of the Glu uptake in the brain (28), and decreased expression and/or malfunction of these Glu transporters are related to several neurodegenerative disorders like Parkinson's, Huntington's and Alzheimer's diseases (47–49).

GENERAL CHARACTERISTICS OF GLUTAMATE TRANSPORTERS IN NEURODEGENERATIVE DISEASES

Through an antisense approach, it has been demonstrated that Glu transporters malfunction is involved in neurodegeneration in normal animals (47). Subsequently, Tanaka and colleagues reported, in mice lacking GLT1, a decrease of transport activity, lethal seizures and increased susceptibility to neurotoxicity (48). Years later, several research groups have demonstrated the role of Glu transporters in various neurodegenerative diseases. For example, Alzheimer's disease (AD) patients and animal models display a dramatic decrease in Glu transporters protein expression and in Glu uptake that is not correlated to its mRNA levels, demonstrating that other levels of regulation are present (50–54). In addition, Scott and coworkers described that GLT1 mRNA alternative splicing controls Glu uptake both in disease and in normal conditions (55). Moreover, glial Glu transporters have aberrant expression in distinct types of neurons (56, 57).

In the case of Parkinson's disease (PD), as with AD, there is also a decrease in Glu uptake; in PD, Glu transporters have an unusual trafficking between membrane and cytoplasm leading a decrease in Glu transporters at the plasma membrane (58). This phenomena relies in Glu transporters' ubiquitination by the E3 ubiquitin ligase Nedd4-2 (neuronal precursor cell expressed developmentally down-regulated 4–2) (58).

Likewise, Glu transporters have a critical role in Huntington's disease (HD), in which the expression of these transporters is diminished, the symptoms of HD worsen (59). In this sense, it has been demonstrated that aberrant huntingtin reduces GLT1 activity, either by dysfunction of the transporter itself or a transcriptional down-regulation, aggravating excitotoxicity (59, 60).

It is well-known that Glu transporters are regulated at different levels, at the transcriptional translational and post translational levels through modifications of transporter protein, as well as by the transporter targeting and trafficking (61–64). Nevertheless, there is compelling evidence demonstrating that Glu transporters are regulated in a circadian fashion.

CIRCADIAN REGULATION OF GLUTAMATE TRANSPORTERS

Transcriptional, Translational, and Post-translational Regulation

Until today, it has been demonstrated that in SCN both *Glast* mRNA and protein levels present a diurnal rhythm in 12/12 h light-dark conditions (65). According to these results, it has been proven that in the *Per2* mutant mice, GLAST protein is

arrhythmic, highlighting the presence of a circadian regulation (65). Subsequently, using a cortical astrocytes culture from *Npas2* and *Clock* mutant mice, it was reported a decrease in *Glast* mRNA and protein levels, implying that glial Glu uptake is modulated via clock genes expression: *Per2*, *Clock,* and *Npas2* (66, 67). CLOCK and NPAS2 proteins are involved in *Glast* transcription or in *Glast* mRNA translation and/or stability (28), while PER2 modulates GLAST and by these means Glu uptake. In this sense, modifications in NPAS2 and/or CLOCK diminish PER2 levels and Glu uptake (66). More recently, it has been reported that glial Glu uptake within the SCN is modulated in a diurnal fashion (high levels of uptake during the light phase) but it does not exhibit circadian fluctuations (68). Leone and colleagues also report that Glu uptake activity does not change in constant darkness (68). It is important to mention that the possibility that Glu uptake is regulated by circadian clock *in vivo* cannot be ruled out. In line with these results, another research group also reported that Glu uptake in SCN is increased during the circadian day (22). Brancaccio and coworkers demonstrated that astrocytes modulate circadian timekeeping in SCN through glutamatergic signaling, and identified the presence of self-sustained circadian oscillations of Glu extracellular levels (22). The authors suggest that, in the light phase, Glu uptake is mediated by EAATs, including GLAST, GLT-1, and EAAC1 (22). These results could indicate that both Glu release and uptake are regulated in a circadian fashion.

It is reasonable to suggest that when there is a lack of GLAST transporter, compensation via upregulation of GLT1 is favored (65). For instance, in the *Per2* mutant mice it has been determined a shift in GLT1 protein maximal expression, from zeitgeber time 6 (ZT6, in control mice) to ZT18 (65), indicating that GLT1 protein is regulated by circadian clock. It is important to mention that shift in maximal expression of the GLT1 transporter correlates with ZT in which there is a downregulation of GLAST (65), suggesting that total uptake of Glu could be modulated by clock.

Through the use of *in situ* hybridization techniques in SCN, supraoptic nuclei, cingulate cortex and reticular thalamus of rats in constant darkness, it was found that *Eaac1* mRNA expression was rhythmic only in the SCN (69). Circadian expression of this transporter is associated with GABAergic activity regulation in the SCN, due an increased demand of GABA synthesis and release, immediately preceded by an increase in *Eaac1* mRNA expression (69). Increase in the expression of this transporter contributes to the neuronal clearance of Glu, which in fact is a precursor of GABA. Within the SCN, 95% of neurons are GABAergic (70), and together with astrocytes regulate circadian timekeeping through glutamatergic signaling (22), suggesting an important role of Glu transporters in the internal timekeeping system. In contrast, Kinoshita and colleagues could not find any a circadian-mediated *Eaac1* mRNA expression neither in serum-shocked SH-SY5Y cells and mouse mesencephalon by qRT-PCR (71). Taking together, these results suggest that temporal changes in *Eaac1* mRNA might be controlled by circadian clock in a tissue-dependent fashion. In addition, Kinoshita and collaborators also described that EAAC1 protein expression exhibits a diurnal variation in a 12/12 h light/dark cycle in mouse mesencephalon (71).

Post-transcriptional Regulation (Circadian MicroRNAs)

In recent years, the proposal for a novel circadian regulatory system has been gaining ground. MicroRNAs (miRNAs) are a good example of a system that can rapidly respond to external stimuli since it is activated without changes in transcription and/or translation (71). In this context, miRNAs have revealed to be a key factor in the regulation of several circadian components (72–75). It has also been proved that peripheral oscillators exert circadian regulation over miRNAs expression (73–78). Increasing evidence indicates that miRNAs controlled by the circadian clock, regulate Glu transporters. Thus, miRNA-124 increases GLAST expression (79), while miRNA-142-3p and miRNA-155-5p decrease it (80, 81). Moreover, it has been demonstrated that miRNA-124 and miRNA-181a positively regulate GLT1 (82, 83), while miRNA-107 inhibits GLT1 expression (84). Specifically, EAAC1 rhythm is negatively controlled by miRNA-96-5p (71), miRNA-26a-5p (85) and miRNA-101b (86). This former miRNA also negatively regulates to EAAC1 protein (86). However, no evidence shows that miRNAs can target EAAT4 and EAAT5.

FUTURE DIRECTIONS

In the last two decades, several research groups have examined the different signaling pathways that modulate glial Glu transporters expression (GLAST and GLT1). Scarce information about EAAC1, EAAT4, and EAAT5 transporters is available. Particularly, EAAC1 has a much less evolutionarily conserved sequence in the 5′ noncoding region compared to GLAST and GLT-1, hindering the identification of *cis*- and *trans*-elements involved in its transcriptional regulation. Specifically, the circadian regulation of Glu transporters is an emerging theme that promises to be an indispensable tool in the preventing and/or treatment of diseases related to alterations in glutamatergic system. Future research should be directed to study of molecular mechanisms involved in circadian modulation of these transporters.

CONCLUSION

Optimal functioning and precise regulation of Glu removal from the synaptic cleft is critical to prevent an excitotoxic insult and thus avoid several neurodegenerative pathologies. To date, compelling evidence suggests that Glu transporters could be regulated in a circadian fashion (**Figure 1**). It is clear that desynchronization or aberrant functioning of circadian system results in significant health consequences. In this sense, disruptions in the circadian regulation of Glu transporters is likely to be involved in neurological disorders like Parkinson, Huntington and Alzheimer diseases. Therefore, a better understanding of the molecular mechanisms that participate in the circadian regulation of EAATs might prove important for the proper development of therapeutic strategies aimed to prevent and/or treat pathologies related to excitotoxicity.

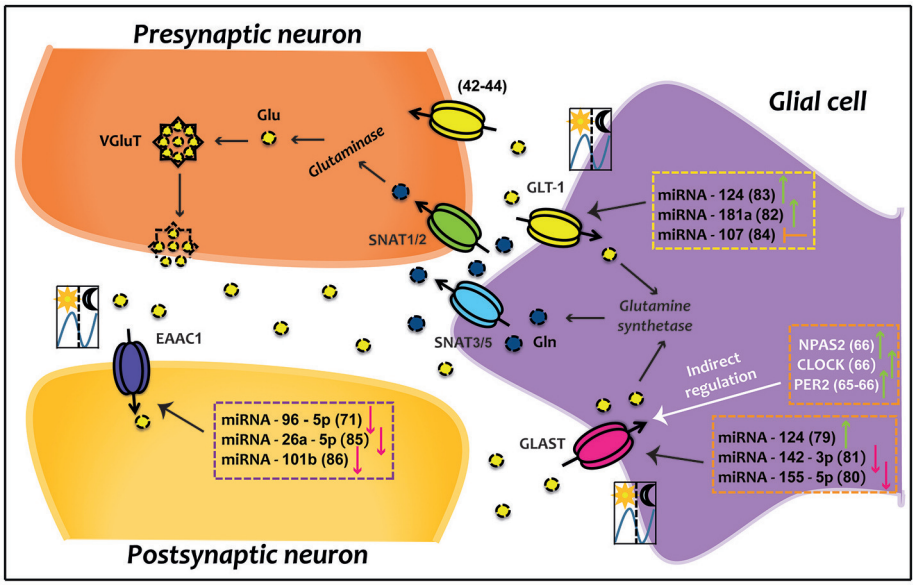

FIGURE 1 | Direct and indirect circadian regulation of EAATs. Glutamatergic synapse which is composed of presynaptic neuron, postsynaptic neuron and glial cell compartment are represented. Some clock genes indirectly up-regulate GLAST; while several miRNAs directly down- or up-regulate GLAST, GLT-1, and EAAC1. Green arrows represent up-regulation, red arrows indicate down-regulation, and orange arrow denotes inhibition. The illustration of day/night indicates that transporter present a circadian rhythm in 12/12 h light/dark conditions. Numbers in parentheses refer to cited publications. CLOCK, circadian locomotor output cycles kaput; EAAC1, excitatory amino acid carrier 1; GLAST, glutamate aspartate transporter; Gln, glutamine; GLT-1, glutamate transporter 1; Glu, glutamate; NPAS2, neuronal PAS domain-containing protein 2; PER2, period 2; SNATs, sodium-coupled neutral amino acid transporters; VGluT, vesicular glutamate transporter.

AUTHOR CONTRIBUTIONS

DC-C gathered the relevant information, wrote the manuscript, as well as elaborated the figure. AO revised and edited the final version of the manuscript.

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Genetic Factors Affecting Seasonality, Mood, and the Circadian Clock

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In healthy humans, seasonality has been documented in psychological variables, chronotype, sleep, feeding, metabolic and autonomic function, thermoregulation, neurotransmission, and hormonal response to stimulation, thus representing a relevant factor to account for, especially when considering the individual susceptibility to disease. Mood is largely recognized as one of the central aspects of human behavior influenced by seasonal variations. This historical notion, already mentioned in ancient medical reports, has been recently confirmed by fMRI findings, which showed that seasonality in human cognitive brain functions may influence affective control with annual variations. Thus, seasonality plays a major role in mood disorders, affecting psychopathology, and representing the behavioral correlate of a heightened sensitivity to factors influencing circannual rhythms in patients. Although the genetic basis of seasonality and seasonal affective disorder (SAD) has not been established so far, there is growing evidence that factors affecting the biological clock, such as gene polymorphisms of the core clock machinery and seasonal changes of the light-dark cycle, exert a marked influence on the behavior of patients affected by mood disorders. Here we review recent findings about the effects of individual gene variants on seasonality, mood, and psychopathological characteristics.

Keywords: seasonality, mood disorders, clock genes, circadian rhythm, seasonal affective disorder

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INTRODUCTION

Seasonality is a central aspect of environmental variability, which has strongly influenced life on Earth by driving the development of biodiversity among living organisms and the evolution of extreme physiological adaptations and behaviors, such as migration and hibernation. In most species, periodic variations of environmental conditions, particularly those related to the light-dark cycle and depending on latitude, season, and time of day, require that internal timing mechanisms induce the adaption of behavioral or physiological functions to such changes (1).

Biological rhythms with an approximate 24-h period, close to the daily light-dark cycle, are known as circadian rhythms and defined by three fundamental properties: persistence of an ∼24-h rhythm, entrainability, and temperature compensation (2). The observation that these endogenous processes are also present among organisms such as cyanobacteria, which represent one of the earliest and most primitive species, suggests that circadian rhythms implicated a clear evolutionary advantage (1).

CLOCK GENES AND MOOD REGULATION

At the cellular level, circadian rhythms are generated by a core molecular clock consisting of multiple transcriptional/translational feedback loops (3). The transcription factors circadian locomotor output cycles kaput (CLOCK) and brain and muscle Arnt-like (ARNTL), or neuronal pas domain protein 2 (NPAS2) proteins, dimerize and initiate the expression of the clock proteins PERIOD (PER1, PER2, PER3), and CRYPTOCHROME (CRY1, CRY2). With rising accumulation, PER1-3 and CRY1/2 inhibit CLOCK:ARNTL (or CLOCK:NPAS2) activity and therefore block their own expression (3). An additional feedback loop is generated by CLOCK:ARNTL (or CLOCK:NPAS2) mediated transcription of REV-ERB and RORs, which in turn also regulate ARNTL transcription (see **Figure 1**).

As recently reviewed by Albrecht, there is already solid scientific evidence showing that the above-mentioned proteins "not only self-promote their own temporally fluctuating transcription, but also regulate the transcription of a large number of clock-controlled genes (CCGs) and/or modulate key molecular pathways via protein–protein interactions, such as the monoaminergic system, the HPA axis or neurogenic pathways" [(4), p. 1]. Several cellular processes in the brain are under the control of the circadian clock, including "differentiation, growth, motility and apoptosis, immune functions and neuroinflammation, neurogenesis, and neuroplasticity" [(5), p. 236]. A desynchronization of the circadian gene network and disruption of its downstream mechanisms has therefore widespread potential implications for a vast array of physiological processes.

Hampp et al. demonstrated that the functional triade of *PER2, ARNTL,* and *NPAS2* and their encoded proteins, directly regulate the activation of the monoamine oxidase A gene *(Maoa).* In fact, the transcription and activity of the MAOA enzyme in the mesolimbic neurons is decreased in mice carrying a genetic deletion of the *Per2* gene, causing an increase of the dopamine levels and an altered neuronal activity in the striatum, as well as behavioral changes (6, 7).

Dopamine is an important neurotransmitter in the reward system, and its levels in the nucleus accumbens show a circadian rhythmicity (6, 8). Considering that many other brain areas of the reward system, including the ventral tegmental area, prefrontal cortex, and amygdala, are also involved in both mood regulation and clock genes expression, this suggests that the entire reward circuit may be under the influence of the circadian clock, via dopamine metabolism (5).

Cryptochromes (CRY2 and CRY1) are key components of the molecular clock, which drive several functions of the circadian pacemaker (9) and are necessary for the development of intercellular networks in the suprachiasmatic nucleus (10). CRY2 and CRY1 proteins are functionally repressors of the transcription-translation loops, and inhibitors of the cyclic adenosine monophosphate signal pathway (11–14). Due to these important molecular properties at the circadian clock level, it has been suggested that CRY2 and CRY1 may play a major role in the metabolism of glucose and lipids (15, 16) and contribute to mood regulation on daily basis, as well as to seasonal variations in mood and behavior (17).

Finally, *PER3* is one of the most robustly rhythmic genes in humans and animals, playing a significant role in the temporal organization of peripheral tissues and being associated with diurnal preference, mental disorders, non-visual responses to light, as well as brain and cognitive responses to sleep loss/circadian misalignment (18). Some genetic variants are supposed to interfere with the stabilizing effect of PER3 on PERIOD1/2 proteins, which play critical roles in circadian timing. These findings suggest that *PER3* may represent an important element of the missing molecular linkage between sleep and mood regulation by adapting these processes to seasonal changes (19).

CLOCK GENES IN MOOD DISORDERS

Several human population genetic studies have identified specific single nucleotide polymorphisms (SNPs) or variable number of tandem repeats (VNTR, see **Supplementary Table 1**) of different circadian clock genes that are associated with mood disorders (20, 21). These associations remain controversial, since most findings could either not be replicated or hold up to correction for multiple testing (22). From a pathophysiological point of view, recent experimental work, and mathematical models suggest that changes in period length and/or decreased amplitude of the circadian oscillation may depend on the impact of specific polymorphisms on the overall function in terms of structure and stability of a given clock protein (23). By as of yet poorly understood processes, the resulting functional changes of the clock-machinery and misalignment between clock-regulated functions and the environment can influence core psychopathological features of mood disorders, including the timing of onset and recurrence of illness episodes, individual symptomatology, and response to treatments (5).

Depressive Disorder

In depressive disorder (DD) (7), two *TIMELESS* polymorphisms have been found to be associated with excessive daytime fatigue among women, as well as a two-way interaction of *TIMELESS* and *ARNTL (rs1868049)* with early-morning awakening among men (24). Lavebratt et al. demonstrated that *RORA, PER2,* and *NPAS2* are associated with DD and the onset of depression within 3 years independently from financial strain (25). Both an increased or decreased *PER3* transcriptional activity may implicate a higher risk for MDD. In particular, Shi and colleagues identified a missense mutation in *hPER3 (hPER3-P856A)*, which slightly lengthens the circadian period and is related to MDD in females, by likely driving changes in clock-controlled genes as opposed to SCN timing. Moreover, the authors describe other sex-dependent associations of common polymorphisms with a *CLOCK* variant protective of MDD in males and *NPAS2* polymorphisms with association of MDD especially in females (26). *NPAS2* and *CRY1* were also linked with DD in a study by Soria et al. (27), with the latter finding replicated by Hua et al. (28), who, instead, did not find any association of *CRY2*

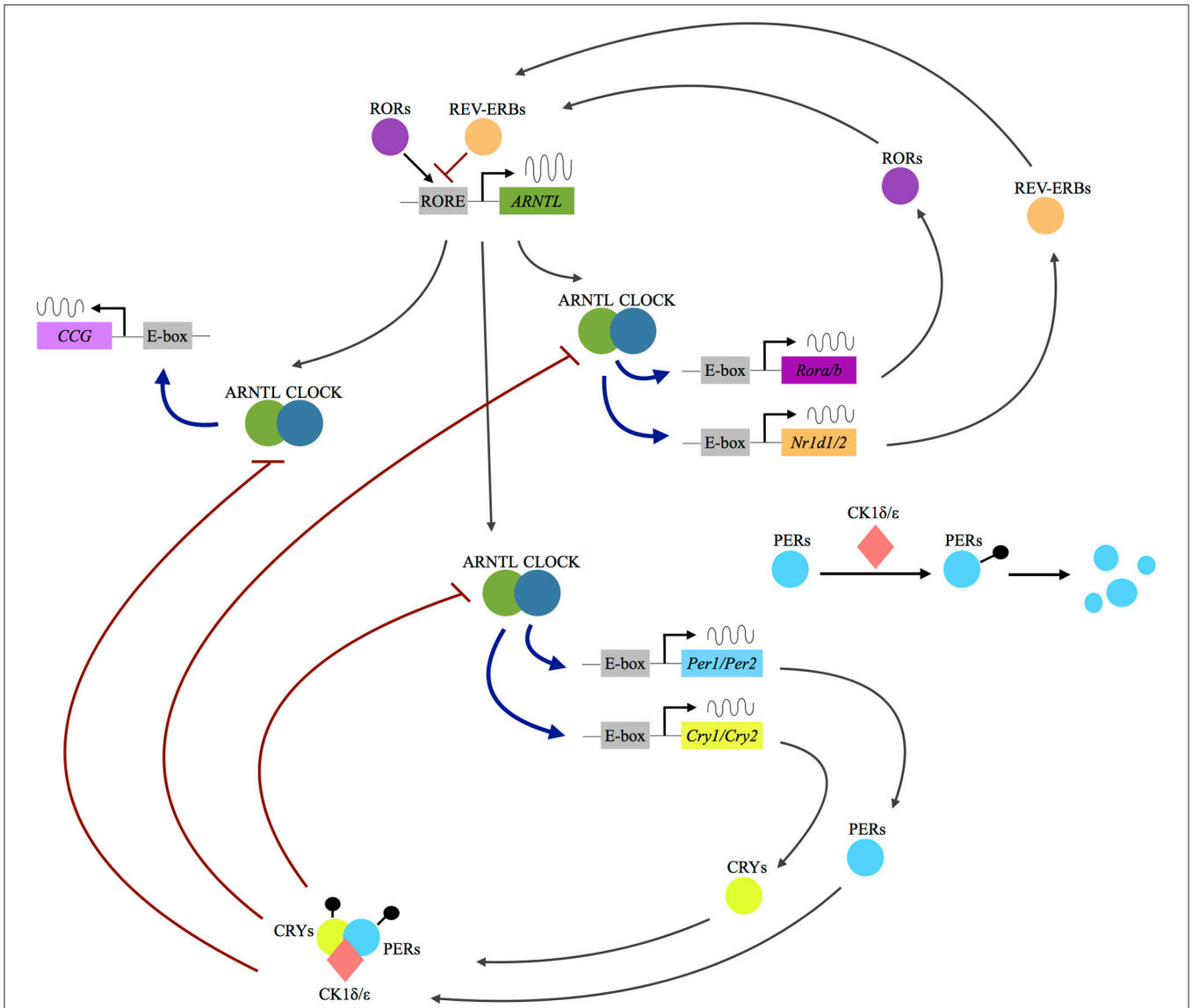

FIGURE 1 | Molecular mechanisms of the circadian clockwork**.** Following the dimerization of the transcription factors circadian locomotor output cycles kaput (CLOCK) and brain and muscle Arnt-like (ARNTL) or neuronal pas domain protein 2 (NPAS2) proteins, the expression of the clock proteins period (PER1, PER2, PER3) and cryptochrome (CRY1, CRY2) is initiated. The PER and CRY proteins interact with the serine/threonine kinases casein kinase 1 δ/ε, (CK1 δ/ε) and form a complex allowing nuclear translocation. In the nucleus they act as inhibitors of CLOCK:ARNTL (or CLOCK:NPAS2) activity and therefore block their own expression. An additional feedback loop is generated by CLOCK:ARNTL (or CLOCK:NPAS2) mediated transcription of REV-ERB and rar-related orphan receptor A/B (RORA/B), which in turn also regulate ARNTL transcription. Up to 10% of the human genome is under the influence of the molecular clock (clock-controlled genes, CCG). RORE: ROR response element.

(rs10838524) with major depressive disorder (MDD). However, Kovanen et al. suggested that CRY2 and the protein kinase C delta binding protein (PRKCDBP, or CAVIN3) variants may represent risk factors for MDD (29). Finally, the best association between a SNP and MDD based on genome-wide association studies has been found for *NR1D1* (30).

Bipolar Disorder

The observation that patients with bipolar disorder (BD) show alterations in circadian rhythms, and recurrent fluctuations of mood and sleep disturbances (31) has suggested a possible dysfunction of the biological clock in the pathogenesis of BD (32). Moreover, since heritability in BD is estimated to be as high as 85% (33), an increasing interest in identifying genetic risk factors has supported different association studies looking at the link between BD and some core clock genes (7).

Significant SNPs associations with bipolar 1 disorder were found for *TIMELESS* and *ARNTL* (34), as well as for *NPAS2*, *RORB 9*, and *CRY2* (35). Gonzalez et al. performed a family-based association study of circadian genes and BD in a Latino

population, reporting nominal associations between SNPs of *CSNK1E, ARNTL, CSNK1D, CLOCK*, as well as statistically significant associations between *CSNK1E* and *ARNTL* haplotypes and BD, with either increased susceptibility or protective effect against the development of the disorder respectively (36). Shi et al. demonstrated the three-way interaction of *BHLHE40*, *TMEM165 (transmembrane protein 165),* and *CSNK1E* with bipolar disorder (37), while McGrath et al. focusing their analysis on the *RORA* and *RORB* genes, found that 4 *RORB* SNPs were associated with bipolar 1 disorder (38). Etain et al. indicated a significant association of *TIMELESS* and of *RORA* with BD (39), while Lee et al. found *CLOCK 3111T/C* to have significant allelic and genotypic associations with the disease (40). *GSK3beta* was associated with bipolar type 2 disorder in women (41). General associations of *NR1D1* (42) and of *VIP* (27) with BD were also reported. In genome-wide association studies, the associations of *ARNTL, GSK3beta, RORB,* and *CRY 2* gene variants with BD have gained further support (30, 43).

Circadian Genes and Phenotypic Characteristics in Bipolar Disorder

Genetic polymorphisms influencing clock genes functions have shown major effects on the phenotypic clinical features of disease (44). A SNP in *CLOCK* gene, which is known to influence diurnal preference in healthy subjects (45), also impacts on bipolar patients, leading to worsening of insomnia, higher evening activity and delayed sleep onset. Carriers of the allelic C variant also showed a higher episode recurrence rate and different neuropsychological performance (46–48), while the G allele of the same polymorphism has been linked with symptoms of appetite disturbances in females (49). A correlation with violent suicide attempts was shown for other SNPs in *CLOCK* and *TIMELESS,* while the latter is also associated with the lifetime number of suicide attempts and a positive family history of suicide (50). A VNTR of *PER3* gene was shown to influence the general age of onset, as well as a postpartum depressive onset of the disorder (51, 52). *PER3* was also linked to an increased preference for the evening hours in daily activity among BD patients (42). Maciukiewicz et al. observed further associations between SNPs of *ARNTL* variants with sleep, appetite and depressive dimensions in BD (49).

A functional SNP in the promoter region of the *GSK3beta* gene *(nt −171 to +29*), which also shows a general association to impulsivity and suicide risk among patients with bipolar disease, was found to influence the age at onset of BD, as well as the response to treatment with antidepressant, lithium salts and chronotherapeutics (53–55). This polymorphism was recently shown to also influence white matter microstructure of bipolar patients under ongoing lithium treatment (56) and gray matter volumes in areas critical for the generation and control of affect implicated in BD pathophysiology (57).

Other polymorphisms influencing treatment response, such as the mood stabilizer effect of lithium salts (variant in the promoter of *NR1D1*) and a general association with positive treatment response (*CRY1)* have been described (58). Finally, Sjöholm et al. identified two risk haplotypes and one protective haplotype in the *CRY2* gene associated with rapid cycling in BD (59) (see **Supplementary Table 1**).

GENETICS OF SEASONALITY AND SEASONAL AFFECTIVE DISORDER

The interplay between mood variations and seasonal rhythms in humans has received renewed interest since the diagnosis of Seasonal Affective Disorder (SAD) was proposed by Rosenthal in 1984, as "a condition characterized by recurrent depressive episodes that occur annually at the same time each year" [(60), p. 72]. The observation that many adults experience a "subsyndromal SAD", with milder vegetative symptoms in the fall/winter months (61, 62), suggested that "seasonality may be a dimensional process rather than a discrete syndrome" [(63), p. 315].

Serotonergic Genes

Although the genetic basis of seasonality and SAD has not yet been completely identified, several studies suggest that both conditions have an inherited component (64–66). From a pathophysiological point of view, the typical symptoms of SAD, such as overeating, carbohydrate craving, weight gain, and oversleeping, point to a dysfunction of the serotonergic system (66). Moreover, the serotonin level in the human hypothalamus shows seasonal variations, with a general decrease during the winter season (67). The serotonin hypothesis is also supported by the large therapeutic evidence that selective serotonin reuptake inhibitors (SSRIs) and bright light therapy are effective in winter SAD (68–71), with reversion of this effect by rapid tryptophan depletion (70, 72).

Therefore, the first pioneer genetic studies focused on the molecular components of the serotonergic system (73). Rosenthal et al. showed that the short (s), as opposed to the long (l), allele of the 5-HT transporter linked polymorphism *(5-HTTLPR)* contributes to the trait of seasonality and is a risk factor for SAD (74). First reports showing an association of this variant with general susceptibility and several features of the clinical course among patients with SAD (75–77) could not be corroborated by a meta-analysis by Johansson et al., but the authors concluded that the polymorphism may have an effect on seasonal behavioral traits (78, 79).

Recent Positron Emission Tomography (PET) studies showed a significantly higher activity of serotonin transporter binding potential in several brain regions, during fall and winter, compared to spring and summer, in healthy volunteers (80, 81). Furthermore, "the first [11C]DASB PET longitudinal study investigating whole-brain seasonal 5-HTT fluctuations in both patients with SAD and in healthy individuals reported that a whole-brain seasonal change in 5-HTT predicted symptom severity in patients with SAD, an effect primarily driven by females with the short *5-HTTLPR* genotype (S' carriers)" [(82), p. 2], (83). These findings were later confirmed by other groups (83, 84).

The serotonin *5-HT2A* receptor gene has also been proposed as major candidate gene in association studies of seasonality

and SAD (85, 86). In particular, it has been suggested that "downregulation of 5-HT2A receptors may underlie the therapeutic effects of SSRIs" [(64), p. 656], (87) and the effectiveness of light therapy in the treatment of SAD has also been linked to an alteration of the sensitivity of 5-HT2A receptors (76). Moreover, specific sequence polymorphisms in the coding region of the serotonin *5-HT2A* receptor gene have been found to be associated with the clinical features and course of depressive disorder or directly with seasonality and SAD (64, 86, 88–90).

Circadian Genes

Apart from an extensive connection between SAD and the serotoninergic system, genes of the core clock family have also been implicated in the disease. After a first report of a SNP in *NPAS2* being linked to SAD (91), Partonen et al. found further SNPs of *PER2*, *ARNTL,* and *NPAS2* to be associated with seasonality and SAD (92, 93).

Kim et al. also reported an association of *NPAS2* and *ARNTL,* especially with the metabolic components of seasonality (body weight and appetite). In addition, they found increased seasonal variations of mood and behavior among individuals carrying a *CLOCK* polymorphism previously implicated in bipolar disorder (40, 46–48, 94). These recent findings are in contrast with a previous work from the same group, showing that the same SNP of *CLOCK* is not associated with seasonal fluctuations in a sample of Korean college students (95).

Furthermore, another recent investigation highlighted the impact of two rare genetic variants of the *PERIOD3 gene (PER3)* on a circadian phenotype and a seasonal mood trait, which may be especially critical under conditions of short photoperiod (e.g., during the winter season) (19).

Other Genetic Findings

Environmental light detection in humans is mediated by melanopsin containing intrinsically photosensitive retinal ganglion cells (ipRGCs), which are located in the inner retina (96–98). Some polymorphisms of the melanopsin gene may be linked to a greater sensitivity to light, thus determining functional variations in ipRGC activity. During shortened photoperiods (e.g., during the winter months) this may contribute to inter-individual differences in sleep and alertness (99, 100). A missense variant *(P10L)* in the melanopsin *(OPN4)* gene, which has also been found in SAD patients, has been proposed to contribute to changes in melanopsin sensitivity (99). Reduced retinal light sensitivity, especially during the winter months, as a pathophysiological hypothesis of SAD (101–103) recently gained first supporting evidence. A study by Roecklein et al. found a reduced post-illumination pupil response (PIPR) in SAD patients, compared with controls, in winter but not in summer (104).

A study by Delavest et al. investigating the *rs2072621* polymorphism of the X-linked *GPR50 gene,* a member of the G protein-coupled melatonin receptor subfamily, found an association with SAD in females, thus providing the first potential gender-specific molecular link between the hormone melatonin and SAD (105).

Yang et al. studied the relationship between *ST8SIA2* and *NCAM1*, two genes forming the polysialic acid neural cell adhesion molecule (NCAM) complex in the SCN, and circadian preferences, as well as seasonality, in healthy adult Korean subjects. The association of 8 SNPs of *ST8SIA2* and 2 SNPs of *NCAM1* with seasonality remained significant after correction for multiple testing (106).

Another study by Nam et al. found that the *GNB3* (G-protein β3 subunit) *C825T* polymorphism, which is associated with various medical conditions (107, 108) and psychiatric disorders, including recurrent winter depression or SAD (109, 110), also plays a role in seasonal variations in mood, body weight, energy level, and appetite, particularly in females.

CONCLUSIONS

Gene polymorphisms of the core clock machinery and seasonal changes of the light-dark cycle substantially impact on the behavior of patients with mood disorders. The relationship between biological clock and behavior suggests a specific sensibility of these patients to psychobiological factors that can modify the circadian timing system, such as environmental synchronizers (light phase and seasonal photoperiod changes), and conditions directly perturbing the clock (sleep deprivation, or phase advance/delay). These factors can trigger or worsen the severity of mood disorders, but also be successfully exploited to treat manic and depressive episodes (111).

Current models of circadian homeostasis suggest that the hierarchical control exerted by the SCN on circadian rhythms of behavior, physiological functions, and on peripheral clocks (112), interacts with homeostatic mechanisms that also contribute to these phenomena. In rodents, a similar dependence of behavior on clock gene mutations occurs in the absence of other regulators of circadian rhythmicity, such as melatonin, and is abolished when these homeostatic components are restored (113). Therefore, we suggest that the high sensitivity of mood-disordered patients to clock gene variants is underpinned by a deficit in homeostatic mechanisms regulating the circadian timing system. Recent discoveries in humans of yet unknown circulating substances affecting the circadian phenotype and overcoming the timing of the clock gene machinery (114, 115), lead to hypothesize that a systematic investigation of these mechanisms will shed new light on the nature of circadian disruption in mood disorders.

AUTHOR CONTRIBUTIONS

Both authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, writing, or revision of the manuscript. In particular: CG conceived, designed and drafted the manuscript. FB drafted and critically reviewed the manuscript. CG and FB approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2018.00481/full#supplementary-material

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