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The present and future of chrono-nutrition studies

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The present and future of chrono-nutrition studies

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Editorial: The present and future of chrono-nutrition studies

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Editorial on the Research Topic
The present and future of chrono-nutrition studies

Introduction

The circadian clock plays an important role in the regulation of eating pattern and food absorption/metabolism in mammals. Moreover, nutrient intake modifies the circadian clocks. Such mutual relationship suggests that the timing of food/nutrients intake can act as a key modifiable lifestyle factor for circadian physiology and metabolic health, which has remained to be an active area of investigation in the field of nutrition research. Therefore, "chrono-nutrition" was newly established as an emerging discipline that considers the calorie intake and dietary composition from the perspective of chronobiology. This Research Topic is aimed at collecting papers to understand the recent progress and evidence of chrono-nutrition studies, and to facilitate the development of the global communities of chrono-nutrition research. We appreciated colleagues who submitted manuscripts to this topic, and finally 14 papers were accepted. In this editorial, we summarize those papers and discuss the present and future of the chrono-nutritional research.

Effects of dietary composition on the circadian system

High-fat diet (HFD) induces obesity and is also known to dampen locomotor activity rhythm and gene expression rhythms in the peripheral tissues in mice. To investigate how non-diet induced obesity affects circadian thermoregulation, Herrera-García et al. showed that *Neotomodon alstoni* mouse, naturally obese mouse, develop low amplitude in their daily rhythms of body temperature, of caloric expenditure, and of cold response, suggesting lower aerobic metabolism and thermoregulation. Importantly, functional foods targeting the clock may help to improve circadian function. For instance, caffeine has a potent effect on the circadian clock by changing the period, phase, and amplitude of its rhythmicity (1). Hironao et al. showed that HFD-induced obesity and circadian clock disturbance could be prevented by the black soybean seed coat polyphenol. Circadian clock disturbances are also common

in neurodegenerative disorders. In a mouse model of Huntington's disease, Whittaker et al. demonstrated that ketogenic diet feeding prevented the age-related disturbances of sleep/wake cycle and motor function.

Time-restricted feeding/eating as a confirmed chrono-nutritional treatment

Time-restricted feeding (TRF, one of intermittent fasting) protocol focus on the best interval/window for food intake. TRF is suggested as an efficient strategy to prevent HFD-induced obesity in mice. In humans, meta-analyses has confirmed that time-restricted eating (TRE) by restricting eating time for 8–10 h in a day (2) can control weight and improve metabolic dysfunctions in those who are overweight or obese. Daytime TRE enhances not only mitochondrial function but also day-night rhythmicity of clock gene expressions (3). Circadian clock entrainment induced by TRE and the consequent behavioral changes were revised by Trzeciak and Steel, they summarized the inconsistency of experimental protocols and suggest a series of future food entrainment studies. The strength of TRE and intermittent fasting was evidenced by da Costa Oliveira et al., they show that intermittent fasting improves hypothalamic signaling of leptin, insulin, autophagy, inflammation, and brain-microbiota axis, contributing to weight loss in obesity. TRE is also effective on the blood pressure in humans by meta-analysis (4). Hou et al. demonstrated that TRE in the light phase resulted in reverse dipping of blood pressure in wild-type and diabetic *db/db* mice.

In spite of the benefits of TRE as compared with other intermittent fasting protocols, the adherence of TRE intervention needs to be improved in the future. O'Neal et al. discuss social, family, work, and other lifestyle factors, that hamper the adherence to TRE, especially with short eating windows of 6–10 h. Although in the basic concept of TRE intervention, participants can consume food anytime during the eating window, Guerrero-Vargas et al. demonstrated that distributed food timing during the TRF (i.e., food given every 3 h in 12-h TRF) in rats had more improving effect on the constant light-induced disturbances of locomotor activity rhythm, body temperature rhythm, and estrus cycle, compared with TRF with free access of food during the 12-h window. Taking together, TRE has been identified as a chrono-nutritional intervention protocol for improving health, such as body weight and blood pressure, with sufficient evidence.

Timing of nutrient intake

For decades, how much and what we eat are considered most essential modifiable nutritional factors affecting our wellbeings. However, chrono-nutrition studies are pointing out another important pillar of this paradigm: when we eat. Indeed, meta-analyses have demonstrated that breakfast skipping, and bigger dinner are associated with weight gain (5, 6), indicating that late calorie intake increases the risk for obesity. In this issue, Begemann and Oster investigated how late consumption of one of our favorite snacks—chocolate affects metabolism. They found that rest-phase chocolate consumption in mice leads to a higher body temperature and increased locomotor activity compared to active-phase chocolate consumption, which may contribute to the late-night snack-induced weight gain. Cross-sectional study by Imamura et al. showed that sodium/potassium intake at lunch or lipid intake at dinner influenced the blood pressure, suggesting the importance of timing of nutrient intake for our health. Kim et al. demonstrated that protein intake for breakfast in elderly participants, relative to the daily total protein intake, resulted in higher handgrip strength and muscle mass. An experimental study also concluded that milk protein in the morning rather than in the evening favored an increase of muscle mass in elderly women. The authors also confirmed the timing effect of protein intake on the muscle synthesis using overloading-induced muscle hypertrophy model mice (7). Caba-Flores et al. reviewed the importance of contents and timing of breast milk supplementation to the babies, since the glucocorticoids and melatonin would be the circadian cue for the infant clock. Recent study suggests the importance of the timing of nutrient intake on the gestation length in the pregnant mother (8), thus more research on the chrono-nutrition in pregnancy is needed.

Binge eating disorder vs. circadian disorder

Patients with eating disorders, such as night-eating syndrome, often express evening chronotype with insomnia and overweight. Individuals with binge eating behavior tend to binge during late hours of the day. Moreover, morning bright light therapy is effective for reducing the binge eating behavior, suggesting that a change of the patient's daily rhythms to a morning chronotype can be a successful treatment strategy. One opinion article (Plano et al.) and one systematic scoping review (Romo-Nava et al.) summarized recent findings about the binge eating disorder with the circadian clock and concluded that the circadian system may play a role in the etiology of binge eating behavior. However, further research is needed for understanding a more detailed connection.

Future directions

As mentioned above, novel chrono-nutritional evidence was already confirmed by the meta-analysis which might be ready for the clinical translation. The newly developed chrono-nutritional research should be applied to the health guidelines, such as national dietary reference intakes, in each country. Furthermore, dietary suggestions should be tailored to the individual when considering chronotype, age, gender, social background (e.g., shiftwork), and genetic background. It is fortunate that a growing number of researchers are joining this chrono-nutritional research area. In Japan, the Japan Chrono-Nutrition Society (JCNS) established in 2014. Society members include physicians, public

health nurses, nutritionists, and food companies in addition to nutrition researchers. Around the world, food companies are elaborating products in which the effective timing of intake is suggested. A good example is Brazil, where different products suggest the beneficial effects for sleep or for the activity performance. However, in Japan, based on the Japanese law, products excepting medicine, cannot suggest to the consumers about the timing of intake, which certainly should be changed in the future. In the US, the Dietary Guidelines Advisory Committee appointed by the Departments of Health and Human Services and Agriculture is considering including recommendations on meal timing into the coming *Dietary Guidelines for Americans, 2025–2030*. This highlights the increasing awareness of chrono-nutrition. In Mexico, a country with high levels of overweight and obesity, a growing interest in chrono-nutrition is expressed by nutritionists and food companies. However, more clinical studies are necessary to provide the scientific evidence and benefits for physiology and behavior. Finally, the present collection raises the need to continue the studies on chrono-nutrition and to organize a worldwide chrono-nutrition conference in the future.

Author contributions

All authors wrote and contributed to the article and approved the submitted version.

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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Supplementation of Protein at Breakfast Rather Than at Dinner and Lunch Is Effective on Skeletal Muscle Mass in Older Adults

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Background: The effects of different intake patterns of meal protein on muscle mass have not been clarified. We cross-sectionally and longitudinally examined the effect of different timing of protein intake on sarcopenia-related factors in older adults.

Methods: This cross-sectional study 1 included 219 (male, $n = 69$, female, $n = 150$) elderly subjects aged ≥65 years. Subjects who consumed more protein at breakfast than at dinner were grouped into the morning group (MG, $n = 76$; male, $n = 26$; female, $n = 50$), and those who consumed more protein at dinner than at breakfast were grouped into the evening group (EG, $n = 143$; male, $n = 43$; female, $n = 100$). In cross-sectional study 2-1 (female, $n = 125$), the subjects were classified into four groups according to the number of meals with sufficient protein intake. In cross-sectional studies 2-2 (female, $n = 125$) and 2-3 (female, $n = 27$), the subjects were classified into eight groups and three groups according to whether they had consumed sufficient protein at three meals; sarcopenia-related factors were compared. The intervention study was a placebo-controlled, double-blind, randomized controlled trial that included 40 elderly women with low daily breakfast protein intake. The subjects were divided into four groups: morning protein and placebo intake groups and evening protein and placebo intake groups. Each group consumed the test food (containing 10 g milk protein) or placebo in the morning or evening for 12 weeks. Blood indices and physical function were assessed before and after the intervention.

Results: Comparing all subjects, MG showed significantly higher handgrip strength than did EG ($P < 0.05$). The higher ratio of morning protein intake relative to the total protein intake, the better the muscle mass ($r = 0.452$, $P < 0.05$) and handgrip strength ($r = 0.383$, $P < 0.05$). The intervention study showed an increase in muscle mass with the intake of milk protein in the morning rather than in the evening ($P < 0.05$).

Conclusions: Protein intake at breakfast might have relatively stronger effects on skeletal muscle mass than at lunch and dinner.

Keywords: protein intake timing, protein intake at breakfast, physical function, muscle function, skeletal muscle mass, older adults

INTRODUCTION

As we age, skeletal muscle mass decreases, causing a decline in muscle strength and physical function (1), a condition known as sarcopenia, which increases the risk of impaired physical independence in the elderly (2). Therefore, maintaining or increasing muscle mass could prevent sarcopenia.

Muscle hypertrophy occurs when the rate of muscle protein synthesis (MPS) exceeds that of muscle protein breakdown (MPB), while muscle atrophy occurs when the rate of MPS falls below that of MPB. Since MPS is stimulated by dietary protein, protein intake is important for maintaining and increasing muscle mass (3). In addition, the sensitivity of MPS to stimulation by amino acid intake is reduced in the elderly compared to the young. Therefore, elderly people need to consume more protein (4).

Recently, it has been suggested that factors such as the quality and distribution of daily protein intake are more relevant to muscle synthesis than the amount of daily protein intake. In fact, previous studies have shown that consuming enough protein in all three meals is more effective in maintaining and improving muscle mass, strength, and physical function (5). Furthermore, it has been confirmed that genes involved in muscle synthesis and degradation have circadian rhythms, and muscle synthesis may have a diurnal rhythm (6).

Many studies regarding protein intake and sarcopenia-related factors have been conducted on healthy subjects, and few studies have been conducted on obese people, and those requiring support (5). Insulin resistance is common in individuals with obesity. Since insulin can promote MPS, high insulin resistance may inhibit muscle synthesis (7). Protein intake in the morning has been reported to improve insulin sensitivity (8) which could promote MPS. The term "those who require support" is equivalent to frailty; it has been reported that many people with frailty have a bias toward one meal a day with protein (9). Few studies have examined the distribution of protein intake, and none have focused on the effects of differences in the timing of protein intake on sarcopenia-related factors (10, 11). In the present study, we examined the effects of morning or evening protein intake on sarcopenia-related factors in elderly people who were healthy, obese, or requiring support in a cross-sectional study (cross-sectional study 1). Since a protein intake of 0.4 g/kg body weight (BW) per meal is known to be the cut-off value that maximizes the stimulation of MPS (4), we defined a meal with a protein intake exceeding 0.4 g/kg BW as a "sufficient protein intake meal" and examined the effects of different protein intake patterns (i.e., different timing of the three meals with sufficient protein intake) on sarcopenia-related factors in elderly women (cross-sectional study 2). Finally, we aimed to examine the effect of 10 g milk protein supplementation in the morning or evening on sarcopenia-related factors in elderly women with inadequate morning protein intake, which is common in Japan (intervention study).

METHODS

Cross-Sectional Study

Participants

Two hundred and nineteen adults aged 65 years or older were included in the study (72.5 ± 0.4 years, mean age ± standard error [SE]). The subjects were healthy, obese, or required support; the study was conducted from August 2017 to February 2019 in Tokyo and Hokkaido (Japan). Subjects who required support in this study were those able to live on their own but needing partial assistance in their daily activities and have been certified by the nursing care insurance system in Japan. Some of the data used in this study were collected earlier for a previous study on the effects of morning or evening protein intake on the physical functions of the elderly ($N = 60$) (12).

Subjects without regular exercise habits were included in this study. Regular exercise habit was defined as continuous physical activity for at least 30 min per session, at least three times per week, as defined by a previous study (13). Subjects who were habitual smokers or had smoked within the past three years, and those who had suffered from cardiovascular disease in the past and/or had a pacemaker were excluded from this study. All subjects completed a questionnaire on dietary intake, lifestyle habits, health, and medication status prior to study enrollment.

All subjects were fully briefed on the outline and safety of the study, and written consent to participate was obtained. The study protocol conformed to the Helsinki Declaration and was approved by the ethics committee for humans at Waseda University (approval numbers: 2017-231, 2018-031, 2018-137).

Study Protocol

In cross-sectional study 1, we examined the effect of different timings of protein intake on sarcopenia-related factors in all subjects. All subjects were divided into two groups: those who consumed more protein at breakfast than at dinner were grouped into the morning group (MG) and those who consumed more protein at dinner than at breakfast were grouped into the evening group (EG) (**Figure 1**).

In cross-sectional study 2, 125 healthy or obese female subjects (71.1 ± 0.4 years, mean age ± SE) were examined for the effects of different patterns of protein intake (depending on whether or not they had sufficient protein intake at breakfast, lunch, and dinner) on sarcopenia-related factors. In the cross-sectional study 2-1, the subjects were classified into four groups according to the number of meals with sufficient protein intake (**Figure 1**): 0 meals, 1 meal, 2 meals, and 3 meals. In the cross-sectional study 2-2, the protein intake patterns were classified into eight patterns according to whether the protein intake during the three meals (breakfast, lunch, and dinner) was sufficient (**Figure 1**; **Supplementary Figure 1**). In other words, those who had zero meals with sufficient protein intake were classified into the 0 meal group, and those who had sufficient protein intake at only one meal were classified into the 1 meal B (breakfast), 1 meal L (lunch), and 1 meal D (Dinner) groups. Those with sufficient protein intake at only two meals (breakfast and lunch) were

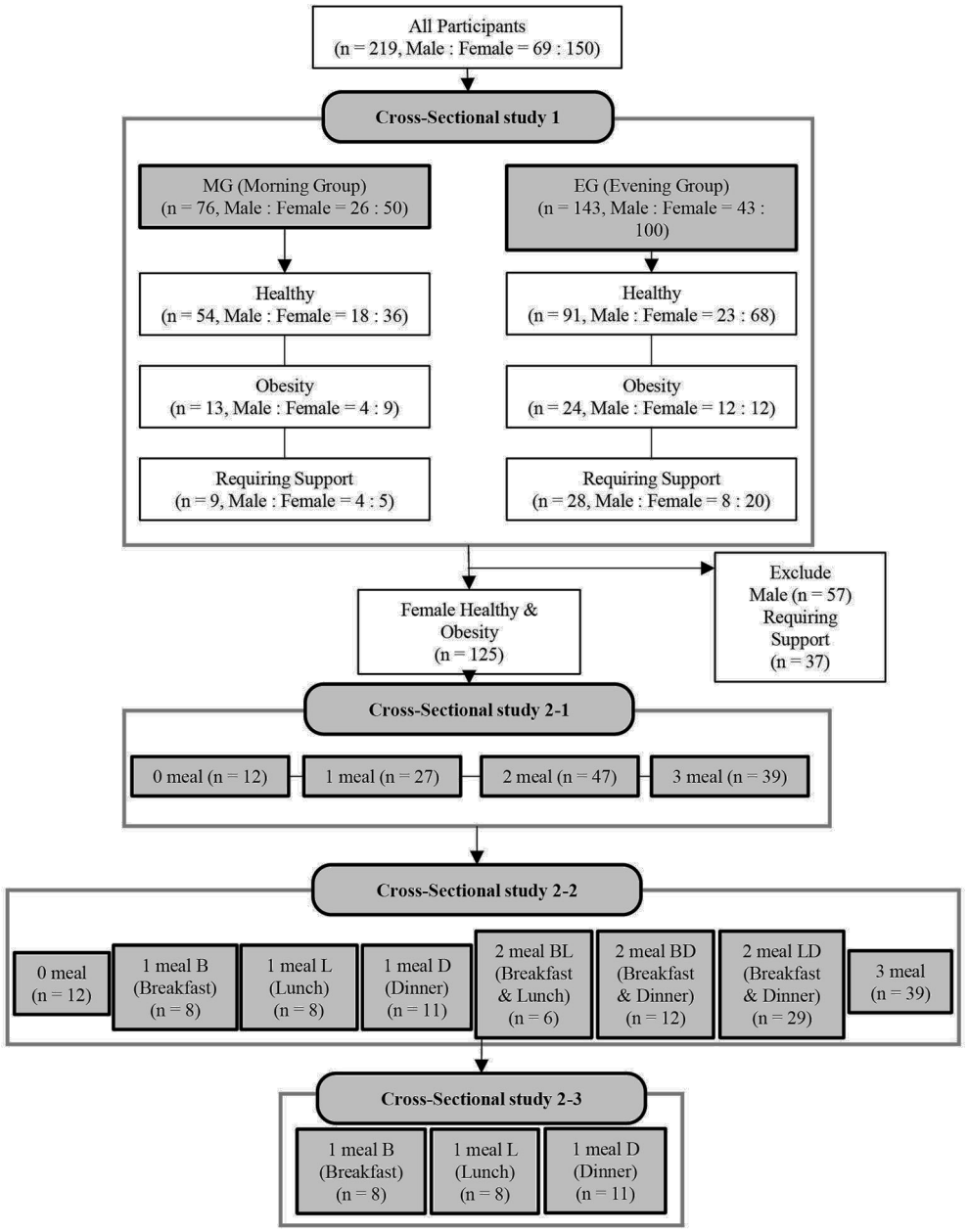

FIGURE 1 | Flow diagram showing the design of the cross-sectional study. B, breakfast; L, lunch; D, dinner.

classified into the 2 meals BL group, those with sufficient protein intake at only two meals (breakfast and dinner) into the 2 meals BD group, those with sufficient protein intake at only two meals (lunch and dinner) into the 2 meals LD group, and those with all three meals into the 3 meals group. In the cross-sectional study 2-3, we compared three groups (1 meal B, 1 meal L, and 1 meal D) in which protein intake was sufficient at only one meal (**Figure 1**).

Measurements

Anthropometry

BW was measured to the nearest 0.1 kg using a digital balance (Inbody 230, Inbody Inc., Tokyo, Japan) and height was measured to the nearest 0.1 cm using a wall-mounted stadiometer (YS-OA, As One Corp., Japan). Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters.

Muscle Mass, Muscle Strength, and Functional Test

Muscle mass was measured by direct segmental multi-frequency (20 kHz to 100 kHz) bioimpedance analysis (InBody230, InBody Co., Ltd., Tokyo, Japan). The skeletal muscle index (SMI) was calculated as the skeletal muscle mass (kg) divided by the square of height (m). Muscle strength was measured using a digital hand dynamometer (T.K.K.5401, Takei Scientific Instruments Co., Ltd., Niigata, Japan). The handgrip strength of the dominant hand was measured twice while standing, and the mean of these measurements was used for analysis. Gait speed test was also evaluated. The subjects walked a 5 m straight course at a normal speed. The walking time for the mid 3m of the course was measured using a digital gait speed measuring instrument (YW, Yagami Co., Ltd., Tokyo, Japan), and the gait speed (m/s) was calculated by dividing the distance in meters by the time in seconds.

Physical Activity Assessment

All participants were asked to wear a triaxial accelerometer (Active style Pro HJA-750C; Omron Corp., Kyoto, Japan) for a week. They always wore the accelerometer each day from morning until night, except during shower and bedtimes. In this study, we selected 4 weekdays and one holiday in which the wearer wore the device for at least 10 h (600 min) and averaged the data to calculate the daily physical activity. We used moderate-to-vigorous physical activity (MVPA) and the number of steps required for evaluation. All minute recordings that were $\geq$3 METs (metabolic equivalents) were classified as MVPA (14).

Chronotype Assessment

To determine the chronotype of the subjects (morningness to eveningness), a lifestyle survey was conducted using the Morningness-Eveningness Questionnaire (MEQ) (15). The scores ranged from 16 to 86 points. The participants were divided into the following three chronotype groups: morningness (score 59–86), intermediate (score 42–58), or eveningness (score 16–41).

Dietary Assessment

The Food Frequency Questionnaire (FFQ) was used to assess the dietary and nutritional intake of the subjects. It consists of questions on 29 food groups and 10 cooking methods. Most FFQs for Japanese are highly effective in estimating nutrients (16). Average daily energy intake is depicted as kilocalories per day (kcal/day). The total dietary fiber quantity was described as grams per day (g/day). In addition, protein-related items such as animal products (meat, eggs, milk, and fish), protein-rich vegetables (beans and soybeans), and dairy products were assessed for protein intake at each meal (breakfast, lunch, and dinner) and recorded.

Intervention Study

Participants

Forty healthy elderly women aged $\geq$65 years (69.5 $\pm$ 0.7 years, mean age $\pm$ SE) who consented to participate in the present study and whose daily breakfast protein intake did not meet 0.4 g/kg BW were included in the intervention study. This study included elderly women without regular exercise habits and the inclusion criteria were as follows: (1) no antioxidant, anti-obesity, or anti-diabetes supplements use; (2) no medical diagnosis of diabetes, dyslipidemia, or sleep apnea syndrome; (3) no hypertension (systolic blood pressure: >140 mmHg, diastolic blood pressure: >90 mmHg). Therefore, it was only intended for subjects who had been confirmed to have no medication use, disease history, or smoking habits at the initial recruitment stage.

This study was conducted in Tokyo (Japan) from August 2017 to February 2019. The participants were those who had no special lifestyle changes during the intervention period and did not have a regular exercise habit.

This study was approved by the Ethics Committee of Waseda University (approval no. 2017-231) and was conducted in accordance with the guidelines established in the Declaration of Helsinki. The human trial of the present study was registered at https://upload.umin.ac.jp/cgi-open-bin/ctr_e/ctr_view.cgi?recptno=R000032737 as UMIN000028612.

Study Design

A placebo-controlled, double-blind, randomized controlled trial was conducted. Forty subjects were randomly assigned to one of the following four groups: the morning protein group (MPRO, $n = 10$), morning placebo group (MPLA, $n = 11$), evening protein group (EPRO, $n = 9$), and evening placebo group (EPLA, $n = 10$) (**Figure 2**). The random assignment of subjects was performed by someone other than the researchers. It was done by using a sequence generated by combining the RAND and RANK functions of Microsoft Excel. During the intervention period of 12 weeks, subjects in the MPRO and MPLA groups consumed milk protein or placebo in the morning (6:00–10:00 a.m.), and subjects in the EPRO and EPLA groups consumed milk protein or placebo in the evening (6:00–10:00 p.m.). The test food was consumed at home. We asked the participants to keep a diary to record their intake of the test foods and we checked their intake rates. The intake rate of the test foods was above 80% for all participants.

Protein Content

Meiji Co., Ltd provided both the test food (containing milk protein) and the control food (placebo) used in the present study. We asked Meiji Co., Ltd, the organization that provided the test foods, to have the participants and the person in charge of the experiment pack the test foods in such a way that they could not be identified, and then mail them to each subject. Disclosure regarding the test foods was made after the experiment was completed. As summarized in **Supplementary Table 1**, milk protein contained 10 g/meal total protein, while placebo contained 0 g total protein. Each test food was also matched and adjusted based on appearance and flavor such that they could not be distinguished. The participants dissolved the milk protein or placebo in 150 ml or more of water and consumed it at the specified time. Milk protein and placebo were available in two flavors (plain and matcha) and were selected according to the subject's preference.

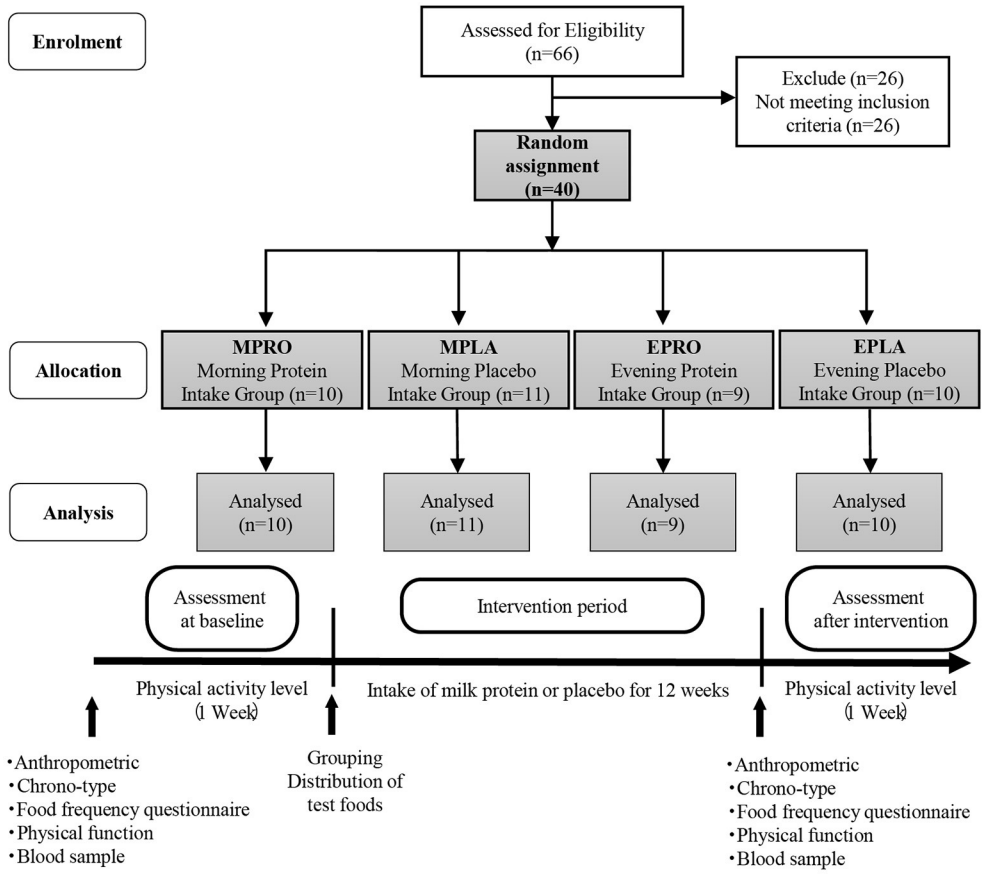

FIGURE 2 | Flow diagram showing the design of the intervention study and protocol.

Anthropometry, Physical Activity Level, Chronotype, Energy Intake, Muscle Mass, and Physical Function

Anthropometry, physical activity levels, chronotype, and energy intake were assessed before and after the intervention using the same methods as in the cross-sectional studies. In addition to the items investigated in the cross-sectional study, the subjects participating in the intervention study underwent measurements of appendicular skeletal muscle mass and appendicular skeletal muscle index (ASMI), physical fitness tests (Balance test, Time Up and Go (TUG), and "Sit to Stand" Test), taken before and after the intervention.

ASMI was calculated as appendicular skeletal muscle mass (kg) divided by the square of height (m). A one-leg stand test was conducted to evaluate the balance ability of the participants. The participants were instructed to stand on one leg on a flat surface for as long as possible. The arms were held by the body with the eyes opened. If the participant was able to stand on one leg for more than 120 s, the measurement was stopped at 120 s. The measurement was performed twice, and a better record was used for the analysis. The sit-to-stand test was performed five times to assess lower extremity muscle strength. When measuring the time taken to change from a sitting to a standing position and vice versa, participants were instructed to stand up from sitting five times as quickly as possible without using their arms for support. The total duration was recorded in seconds. The TUG test was used to assess the mobility and balance of the subjects. The time (s) it took for the subject to stand up from a sitting position in a chair, walk to 3 m, and then return to the original chair position was measured.

Blood Sample

Venous blood samples were collected before and after the intervention. The participants were required to refrain from any strenuous exercise for at least a day before the collection of the blood sample and fast for at least 12 h overnight. The participants' fasting blood was collected at 10–11 am the following day. After collection, blood for serum analysis was allowed to stand for 30 min at room temperature, whereas blood for plasma analysis was centrifuged at 3,500 rpm for 10 min. After centrifugation, serum and plasma samples were extracted from the respective blood collection tubes and stored at −80°C until the day of the assay. Plasma insulin, glucose, and serum growth hormone (GH) were analyzed by Kotobiken Medical Laboratories, Inc. (Tokyo, Japan).

Statistical Analysis

The Predictive Analytics Software for Windows (SPSS Japan Inc. Tokyo, Japan) was used for data analysis. The total sample size was calculated to be able to detect a medium effect. Total sample size of 128 (Cross-sectional study) and 48 (Intervention study) were required to have ∼80% power to detect large effects at a significance level of 0.05 (G*Power, version 3.1.9.2, Universitat Kiel, Germany). The normal or non-normal distributions of the data were analyzed using the Shapiro–Wilk test. In cross-sectional study 1, the mean values between the MG and EG groups were compared, and data that were normative and equally distributed were evaluated using the unpaired *t*-test. Data that were not confirmed to be normative or equally distributed were evaluated using Mann-Whitney's U test. In cross-sectional studies 2 and 3, data with normality and equal variance were evaluated using one-way analysis of variance (ANOVA), while data without normality or equal variance were evaluated using the Kruskal-Wallis test. Pearson's product-moment correlation coefficient was used to examine the relationship between the rate of protein intake in each meal and physical function.

The intervention study used a two-way ANOVA to compare the rate of change in physical function before and after the intervention among the groups. When normality and equality of variance were not confirmed, the Wilcoxon's *t*-test was used for the corresponding data, while the Mann-Whitney U test was used for the non-corresponding data. Statistical significance was set at $P < 0.05$. Additionally, $P < 0.1$ was considered a statistically significant tendency.

RESULTS

Cross-Sectional Study 1

Characteristics of Participants and Energy Intake

We compared the characteristics of all participants and within the different subject categories of the MG and EG. There was no significant difference in physical characteristics between the groups for all subjects, obese subjects, and those requiring support. However, age was significantly higher in healthy MG, especially in healthy women of the MG, compared to those in the EG ($P < 0.05$) (**Supplementary Table 2**).

There were no significant differences in energy intake and total protein intake between the groups (**Supplementary Table 3**).

Comparison of Sarcopenia-Related Factors

Comparing all subjects ($n = 219$; MG = 69, EG = 150), MG showed significantly higher handgrip strength than did EG ($P < 0.05$). Correcting handgrip strength for each subject's weight reduced the significance of the MG's higher handgrip strength ($P = 0.054$). In all healthy subjects ($n = 145$; MG = 54, EG = 91), SMI and handgrip strength of the MG were significantly higher than those of the EG ($P < 0.05$, respectively), and the MG tended to have higher muscle mass than the EG ($P = 0.071$). In contrast, there were no statistically significant differences between groups among all obese subjects ($n = 37$; MG = 13, EG = 24) and all those requiring assistance ($n = 37$; MG = 9, EG = 28).

Considering the influence of sex differences, we also examined the results separately for men and women. In all men (healthy, obese, and those requiring support), there were no statistically significant differences in sarcopenia-related factors between the groups. On the other hand, healthy female subjects ($n = 104$; MG = 36, EG = 68) showed significantly higher values or a trend toward higher values for SMI, handgrip strength, and handgrip strength (weight-corrected) in the MG than in the EG ($P < 0.01$, $P < 0.05$, $P = 0.069$). In addition, in women with obesity ($n = 21$; MG = 9, EG = 12), handgrip strength and handgrip strength (weight-corrected) were significantly higher in the MG than in the EG ($P < 0.05$, $P < 0.001$) (**Table 1**).

Effect of Number of Melas With Adequate Protein Intake on Sarcopenia-Related Factors (Cross-Sectional Study 2-1)

Subjects with fewer meals of adequate protein intake had significantly higher values for BW, BMI, percentage fat, and fat mass (**Supplementary Table 4**). The results of the comparison of sarcopenia-related factors according to the number of meals meeting 0.4 g/kg BW are shown in **Figures 3A–F**. The higher the number of meals with adequate protein intake, the significantly higher the muscle mass (weight-corrected) (0 meal group vs. 1 meal group, 2 meals group, 3 meals group: $P < 0.05$, $P < 0.05$, $P < 0.01$, respectively) and handgrip strength (weight-corrected) (0 meal group vs. 1 meal group, 3 meals group: $P < 0.05$, $P < 0.01$, respectively; 2 meals group vs. 3 meals group; $P < 0.01$) (**Figures 3B,E**). In terms of gait speed, those who had more meals with sufficient protein intake tended to walk faster (0 meal group vs. 3 meals group; $P = 0.053$, 1 meal group vs. 3 meals group; $P = 0.076$) (**Figure 3F**).

Effects of Eight Different Protein Intake Patterns on Sarcopenia-Related Factors (Cross-Sectional Study 2-2)

Protein intake patterns were classified into eight groups, according to whether the protein intake during the three meals was sufficient or not, and the subjects belonging to each intake pattern were divided into eight groups for comparison (**Supplementary Figure 1**).

The results of the comparison of sarcopenia-related factors by protein intake patterns are shown in **Figures 4A–F**. There were no significant differences in muscle mass (weight-corrected) or gait speed between the groups (**Figures 4B,F**). However, handgrip strength (weight-corrected) was significantly higher in the 1 meal B group than in the 0 meal, 1 meal D, 2 meals BL, and 2 meals LD groups (one meal group vs. 0 meal, one meal D, and two meals BL groups; $P < 0.05$, respectively; 1 meal group vs. 2 meals LD group; $P < 0.01$). In addition, handgrip strength (weight-corrected) was significantly higher or tended to be higher in the 3 meals group than in the 0 meal group, 1 meal D group, 2 meals BL group, or 2 meals LD group (3 meals group vs. 0 meal group, 2 meals LD group; $P < 0.01$, respectively; 3 meals group vs. 1 meal D group, 2 meals BL group; $P = 0.059$, $P = 0.053$) (**Figure 4E**).

TABLE 1 | Comparison of sarcopenia-related factors between MG and EG (cross-sectional study 1).

All	All participants		Healthy ($n = 145$)		Obesity ($n = 37$)		Participants($n = 37$) requiring support ($n = 37$)	
	MG ($n = 76$)	EG ($n = 143$)	MG ($n = 54$)	EG ($n = 91$)	MG ($n = 13$)	EG ($n = 24$)	MG ($n = 9$)	EG ($n = 28$)
Muscle mass (kg)	21.35 ± 0.49	20.75 ± 0.35	21.13 ± 0.58	20.04 ± 0.38	22.72 ± 1.25	24.25 ± 0.96	20.71 ± 1.42	20.05 ± 0.78
Muscle mass (kg/BW)	0.38 ± 0.01	0.37 ± 0.00	0.39 ± 0.01	0.38 ± 0.00	0.34 ± 0.01	0.36 ± 0.01	0.35 ± 0.02	0.34 ± 0.01
SMI (kg/m^2)	8.53 ± 0.12	8.39 ± 0.09	8.35 ± 0.12$^{\#}$	8.05 ± 0.09	9.08 ± 0.31	9.53 ± 0.19	8.84 ± 0.35	8.52 ± 0.21
Hand grip (kg)	25.88 ± 0.86$^{\#}$	24.10 ± 0.65	26.17 ± 1.03$^{\#}$	23.65 ± 0.73	27.03 ± 1.76	28.40 ± 1.79	22.46 ± 2.80	21.84 ± 1.52
Hand grip (kg/BW)	0.46 ± 0.01	0.42 ± 0.01	0.48 ± 0.02$^{\#}$	0.44 ± 0.01	0.41 ± 0.02	0.42 ± 0.02	0.38 ± 0.04	0.37 ± 0.02
Gait speed (m/s)	1.42 ± 0.03	1.35 ± 0.02	1.48 ± 0.03	1.44 ± 0.02	1.40 ± 0.05	1.39 ± 0.04	1.07 ± 0.07	1.03 ± 0.05
Male	**MG ($n = 26$)**	**EG ($n = 43$)**	**MG ($n = 18$)**	**EG ($n = 23$)**	**MG ($n = 4$)**	**EG ($n = 12$)**	**MG ($n = 4$)**	**EG ($n = 8$)**
Muscle mass (kg)	25.72 ± 0.66	25.76 ± 0.53	25.44 ± 0.83	25.06 ± 0.64	28.18 ± 1.29	27.87 ± 1.12	24.50 ± 1.47	24.61 ± 1.10
Muscle mass (kg/BW)	0.42 ± 0.01	0.40 ± 0.00	0.43 ± 0.01	0.42 ± 0.01	0.38 ± 0.01	0.39 ± 0.01	0.39 ± 0.01	0.39 ± 0.01
SMI (kg/m^2)	9.34 ± 0.19	9.55 ± 0.13	9.10 ± 0.20	9.25 ± 0.13	10.26 ± 0.57	10.27 ± 0.20	9.52 ± 0.53	9.32 ± 0.41
Hand grip (kg)	32.92 ± 1.42	33.36 ± 0.91	33.65 ± 1.65	33.05 ± 1.18	34.58 ± 2.28	36.32 ± 1.19	27.96 ± 4.98	29.84 ± 2.65
Hand grip (kg/BW)	0.54 ± 0.03	0.52 ± 0.01	0.57 ± 0.03	0.55 ± 0.02	0.47 ± 0.02	0.51 ± 0.02	0.44 ± 0.07	0.47 ± 0.04
Gait speed (m/s)	1.41 ± 0.05	1.34 ± 0.04	1.46 ± 0.07	1.43 ± 0.05	1.36 ± 0.11	1.34 ± 0.06	1.22 ± 0.10	1.08 ± 0.09
Female	**MG ($n = 50$)**	**EG ($n = 100$)**	**MG ($n = 36$)**	**EG ($n = 68$)**	**MG ($n = 9$)**	**EG ($n = 12$)**	**MG ($n = 5$)**	**EG ($n = 20$)**
Muscle mass (kg)	19.08 ± 0.37	18.59 ± 0.21	18.97 ± 0.45	18.34 ± 0.21	20.3 ± 0.84	20.63 ± 0.46	17.68 ± 0.87	18.23 ± 0.66
Muscle mass (kg/BW)	0.36 ± 0.01	0.35 ± 0.00	0.37 ± 0.00	0.36 ± 0.00	0.33 ± 0.01	0.33 ± 0.01	0.32 ± 0.02	0.33 ± 0.01
SMI (kg/m^2)	8.11 ± 0.10	7.90 ± 0.07	7.97 ± 0.12**	7.65 ± 0.06	8.56 ± 0.22	8.79 ± 0.10	8.30 ± 0.34	8.20 ± 0.20
Hand grip (kg)	22.21 ± 0.63**	20.11 ± 0.41	22.43 ± 0.76*	20.48 ± 0.45	23.67 ± 1.13*	20.49 ± 0.80	18.06 ± 1.55	18.64 ± 1.30
Hand grip (kg/BW)	0.42 ± 0.01*	0.38 ± 0.01	0.44 ± 0.01	0.41 ± 0.01	0.38 ± 0.02**	0.33 ± 0.01	0.34 ± 0.05	0.33 ± 0.02
Gait speed (m/s)	1.43 ± 0.03	1.36 ± 0.03	1.50 ± 0.03	1.44 ± 0.02	1.43 ± 0.05	1.43 ± 0.06	0.98 ± 0.07	1.01 ± 0.06

*Values are expressed as mean and standard errors. *$P < 0.05$, **$P < 0.01$ compared to EG (t-test). $^{\#}P < 0.05$ compared to EG (Mann-Whitney). MG, Morning Group; EG, Evening Group; BW, Body Weight; SMI, Skeletal Muscle Index.*

Handgrip strength (weight-corrected) was lower in the group that consumed sufficient protein at both breakfast and lunch than in the group that consumed sufficient protein at only one meal in the morning. If we consider the importance of protein intake in the morning, we can assume that the comparison of the two groups would yield similar results. However, the results of this study differ from our hypotheses.

Comparison of Three Groups With Sufficient Protein Intake at One Meal (Cross-Sectional Study 2-3)

To examine the effects of protein intake at breakfast, lunch, and dinner in more detail, we compared sarcopenia-related factors in three groups that had sufficient protein intake at only one meal. There were no significant differences in physical characteristics or energy intake between the groups (**Supplementary Table 5**).

In terms of sarcopenia-related factors, handgrip strength (weight-corrected) was higher in the 1 meal B group than in the 1 meal L and 1 meal D groups ($P = 0.080$ and $P < 0.001$, respectively) (**Figure 5A**). Additionally, handgrip strength was higher in the 1 meal B group than in the 1 meal D group ($P < 0.001$) (**Table 2**). Furthermore, the correlation between each item and the ratio of breakfast protein intake to total protein intake was examined. Muscle mass, SMI, and handgrip strength were positively correlated with the proportion of breakfast protein intake (muscle mass, $r = 0.452$; SMI, $r = 0.442$; handgrip strength, $r = 0.383$; $P < 0.05$, respectively) (**Figures 5B–D**). However, there was no statistically significant association between each item and the ratio of lunch and dinner protein intake to total protein intake.

Effect of Milk Protein Supplementation in The Morning or Evening on Sarcopenia-Related Factors in Elderly Women With Routinely Inadequate Morning Protein Intake (Intervention Study)

There were no significant differences between characteristics of participants, and pre-intervention energy intake in each group (**Supplementary Tables 6, 7**).

The results of the comparison of the rate of change of sarcopenia-related factors by intervention are shown in **Figures 6A–K**. In muscle mass (weight-corrected), ASMM, and ASMI, the rate of change in the MPRO group significantly exceeded or tended to exceed the rate of change in the EPRO group (muscle mass [weight-corrected]; $P = 0.066$, ASMM, and ASMI; $P < 0.05$, respectively) (**Figures 6B,D,E**). In addition,

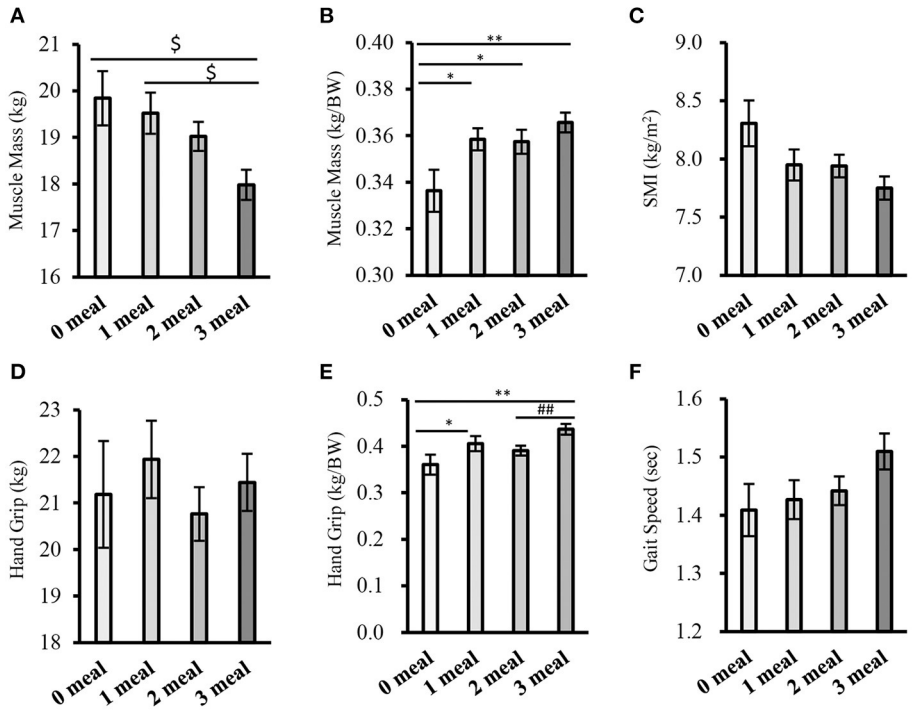

FIGURE 3 | Comparison of sarcopenia-related factors by the number of meals meeting 0.4g/kg BW (cross-sectional study 2-1). **(A)** Muscle mass, **(B)** Muscle mass (corrected for body weight), **(C)** SMI, **(D)** Handgrip strength, **(E)** Handgrip strength (weight-corrected), **(F)** Gait speed. Values are expressed as mean and standard error. $^{\$}P < 0.05$ compared to 3 meals (one-way ANOVA), $^{*}P < 0.05$, $^{**}P < 0.01$ compared to 0 meal (Mann-Whitney), $^{\#\#}P < 0.01$, compared to 3 meals (Mann-Whitney), BW, Body Weight; SMI, Skeletal Muscle Index.

muscle mass, SMI, and ASMI tended to increase in the MPRO group compared to the pre-intervention (muscle mass (pre: 18.62 ± 0.66 kg, post: 19.22 ± 0.64 kg; $P = 0.059$), SMI (pre: 7.78 ± 0.22 kg/m^2, post: 7.94 ± 0.22 kg/m2; $P = 0.057$), ASMI (pre: 5.69 ± 0.17 kg/m^2, post: 5.85 ± 0.16 kg/m^2; $P = 0.061$). In the EPRO group, ASMI showed a decreasing trend after the intervention compared with before the intervention (pre: 5.51 ± 0.07 kg/m^2, post: 5.45 ± 0.10 kg/m^2; $P = 0.084$).

In contrast, the rate of change in the EPLA group was significantly lower than that in the MPLA group in handgrip strength and handgrip strength (weight-corrected) ($P < 0.01$, respectively) (**Figures 6F,G**). In addition, in the within-group comparison, handgrip strength and handgrip strength (weight-corrected) decreased in the EPLA group after the intervention compared to before the intervention (handgrip strength (pre: 21.15 ± 1.25 kg, post: 19.03 ± 1.33 kg; $P = 0.059$), handgrip strength (weight-corrected) (pre: 0.41 ± 0.03 kg/BW, post: 0.36 ± 0.02 kg/BW; $P < 0.05$).

DISCUSSIONS

The present study was a cross-sectional and interventional investigation of the effects of different protein intake patterns on sarcopenia-related factors. The main results showed that adequate protein intake in the morning is important for maintaining muscle mass and strength, with the effect being more pronounced in women. In the intervention study, only morning milk protein intake over 12 weeks resulted in increased muscle mass. These results suggest that morning protein intake is also effective in improving muscle mass and strength.

Relationship Between Pattern and Timing of Protein Intake and Sarcopenia-Related Factors

Sarcopenia-related factors, such as muscle mass, grip strength, and physical function, may be influenced by the MEQ scores and physical activity. Morningness has been shown to have higher daily physical activity than eveningness (17) and increased physical activity may result in higher muscle mass (18). However, in the present study, sarcopenia-related factors in healthy and obese subjects were not associated with the MEQ score or physical activity. Therefore, the higher muscle mass and strength in the MG are due to protein intake, rather than the effects of chronotype or physical activity level.

However, the higher muscle mass and strength in the MG may not be explained solely by the higher protein intake in the morning than in the evening. It has been reported that an even intake of protein at all three meals, rather than a bias toward the evening meal, is associated with higher muscle mass and strength (10, 19). In the present study, the MG was more likely to consume equal amounts of protein at breakfast, lunch,

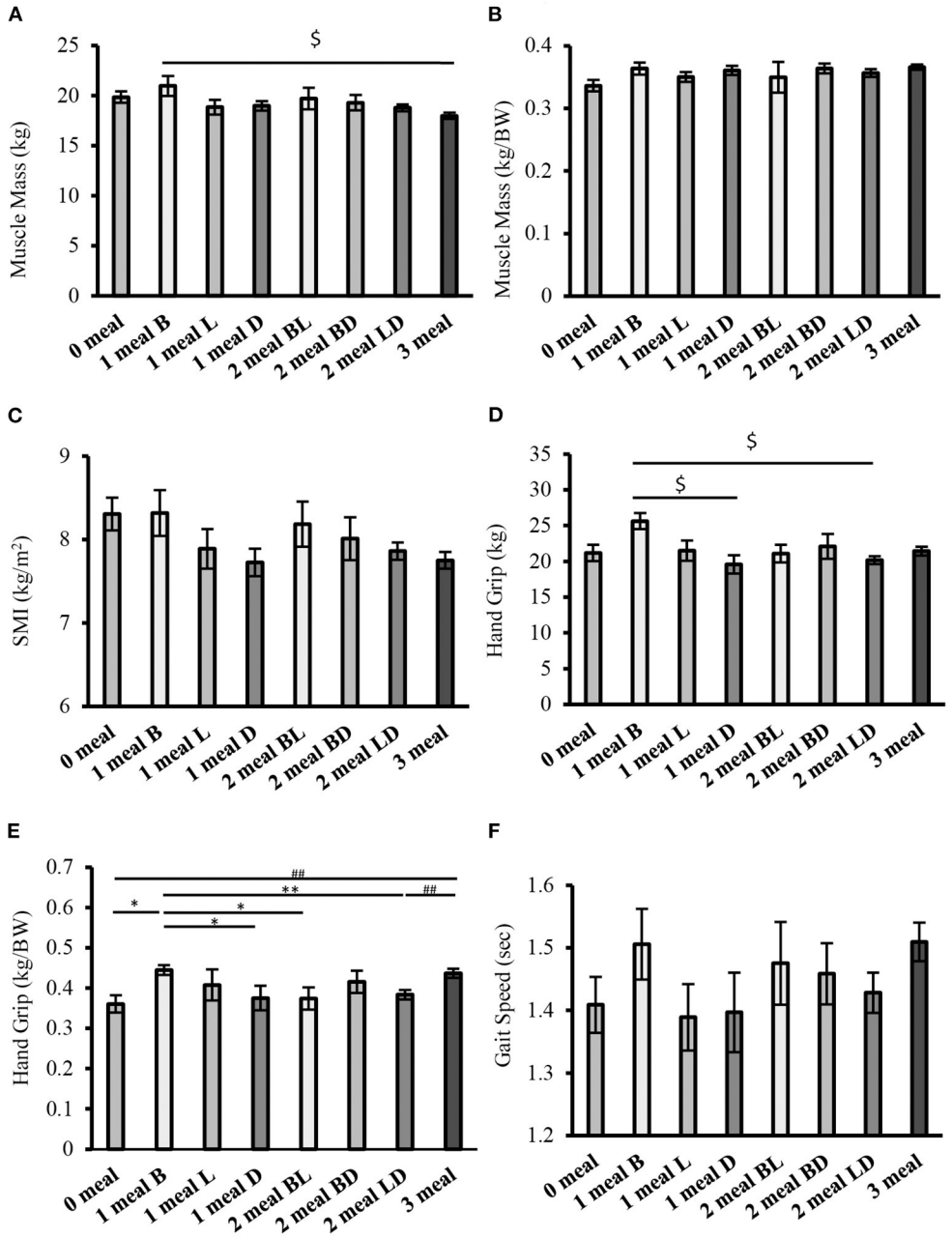

FIGURE 4 | Comparison of sarcopenia-related factors by protein intake pattern (cross-sectional study 2-2). **(A)** Muscle mass, **(B)** Muscle mass (weight-corrected), **(C)** SMI, **(D)** Handgrip strength, **(E)** Handgrip strength (weight-corrected), **(F)** Gait speed. Values are expressed as mean and standard error. $^{\$}P < 0.05$ compared to 1 meal B (one-way ANOVA), $^{*}P < 0.05$, $^{**}P < 0.01$ compared to 1 meal B (Mann-Whitney), $^{\#\#}P < 0.01$, compared to 3 meals (Mann-Whitney), BW, Body Weight; SMI, Skeletal Muscle Index; B, Breakfast, L, Lunch; D, Dinner; BL, Breakfast and Lunch; BD, Breakfast and Dinner; LD, Lunch and Dinner.

and dinner, and consequently had better values for sarcopenia-related factors. In fact, when we compared the protein intake of healthy elderly people ($n = 145$; MG: $n = 54$, EG: $n = 91$) divided by their BW (**Supplementary Table 3**), we found that the protein intake of MG exceeded 0.4 g/kg BW at three meals, while that of EG was below 0.4 g/kg BW in the morning. Therefore, to examine the effects of the different timing of protein intake on sarcopenia-related factors in more detail, we included the pattern of evening protein intake below 0.4 g/kg BW. Therefore, in cross-sectional study 2, we examined different patterns of dietary protein intake above 0.4 g/kg BW/meal.

In cross-sectional study 2-1, we examined how differences in the number of meals with more than 0.4 g/kg BW of protein affect

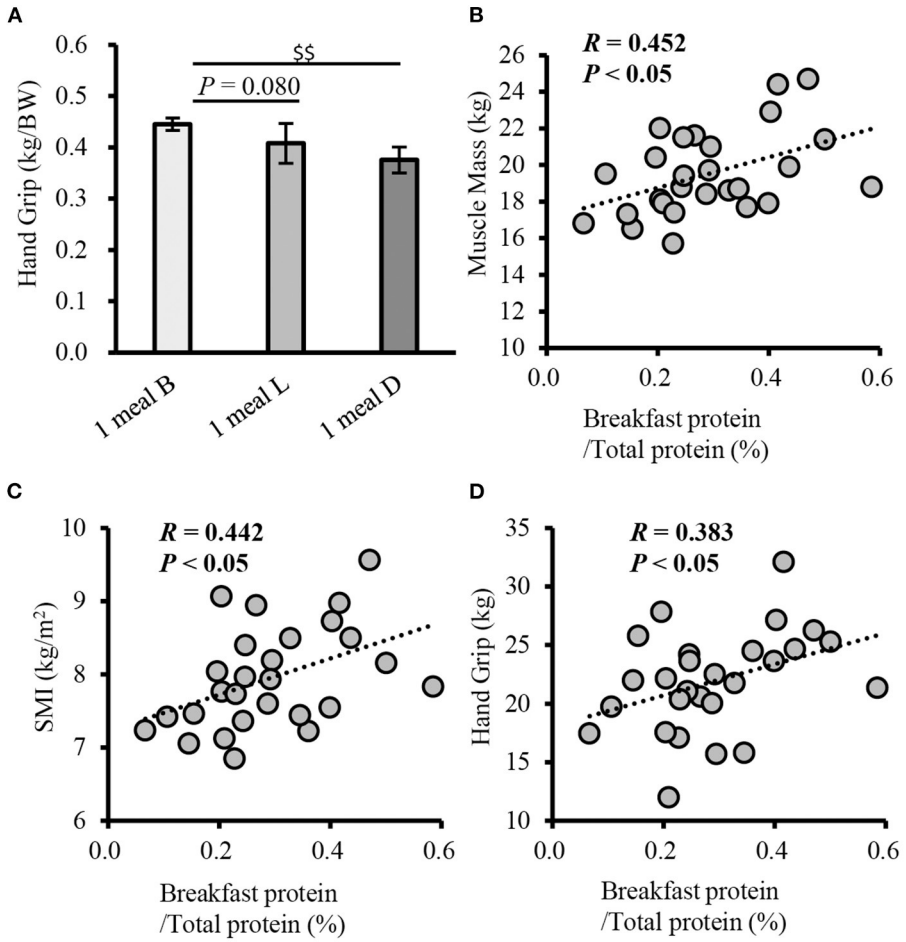

FIGURE 5 | Comparison of handgrip strength between patterns with only one meal of adequate protein intake, and correlation of the rate of breakfast protein intake with physical function (cross-sectional study 2-3). **(A)** Comparison of grip strength between patterns with only one meal of adequate protein intake, Correlation between muscle mass **(B)**, SMI **(C)**, handgrip strength **(D)**, and the rate of breakfast protein intake. Values are expressed as mean and standard error. $^{\$\$}P < 0.01$, compared to 1 meal D (one-way ANOVA). Pearson's product-moment correlation coefficient. SMI, skeletal muscle index; B, breakfast; L, lunch; D, inner.

sarcopenia-related factors. The results showed that the greater the number of meals with sufficient protein intake, the higher the muscle mass (weight-corrected) and grip strength (weight-corrected), and the faster the walking speed. These results are in agreement with those of previous studies (20). Since protein intake of 0.4 g/kg BW or more stimulates MPS, the more often protein is consumed above 0.4 g/kg BW, the more often MPS is stimulated. Therefore, the rate of MPS may have exceeded the rate of MPB more frequently in those who consumed sufficient protein, resulting in higher values of muscle mass and strength.

In cross-sectional study 2-2, we examined the effects of eight different patterns of protein intake timing, for the three meals, on sarcopenia-related factors. In cross-sectional study 2-3, we examined the effects of different timings of breakfast, lunch, and dinner on sarcopenia-related factors in people who consume enough protein in only one meal. The results showed that adequate protein intake at only one breakfast meal was more important for maintaining muscle strength than at only one lunch meal or one dinner meal. Furthermore, it was suggested that the effect of sufficient protein intake at only one breakfast meal on muscle function was equivalent to that of sufficient protein intake at all three meals.

It has been reported that 3.4% of genes expressed in the skeletal muscle exhibit circadian rhythms (684 genes are found in fast-twitch muscle and 1,359 in slow-twitch muscle) (21, 22). The detailed mechanism by which morning protein intake results in higher muscle mass and strength is unknown. However, previous studies have shown that protein synthesis in the skeletal muscle of mice is high during the early active phase (corresponding to the morning in humans), and expression of *Atrogin-1* and *MuRF-1*, genes involved in muscle degradation, is high from the late active phase to the early inactive phase (corresponding to nighttime in humans) (21, 23). These findings suggest that dietary proteins may be more available for muscle synthesis in the morning and less available in the evening. In fact, our recent

TABLE 2 | Comparison of sarcopenia-related factors between patterns of adequate protein intake with only one meal (Cross-sectional study 2-3).

	1 meal B ($n = 8$)	1 meal L ($n = 8$)	1 meal D ($n = 11$)
Muscle Mass (kg)	20.96 ± 1.00	18.84 ± 0.74	18.96 ± 0.51
Muscle mass (kg/BW)	8.32 ± 0.27	7.89 ± 0.24	7.72 ± 0.18
SMI (kg/m^2)	0.36 ± 0.01	0.35 ± 0.01	0.36 ± 0.01
Hand grip (kg)	25.62 ± 1.11$^{\$\$}$	21.50 ± 1.42	19.57 ± 1.10
Hand grip (kg/BW)	0.45 ± 0.01	0.41 ± 0.04	0.38 ± 0.03
Gait speed (m/s)	1.51 ± 0.06	1.39 ±0.05	1.40 ± 0.06

Values are expressed as mean and standard errors. $^{\$\$}P < 0.01$ *compared to 1 meal D (One-Way ANOVA). BW, Body Weight; SMI, Skeletal Muscle Index; B, Breakfast; L, Lunch; D, Dinner.*

study in rodents showed that mice consuming more protein at breakfast gained more muscle mass than mice that consumed more protein at dinner or consumed equal amounts in the morning and evening (12). Therefore, it is thought that a diet with enough protein in the morning could stimulate MPS to the maximum extent for muscle synthesis utilization because the amount of protein ingested was commensurate with the variation in muscle synthesis utilization of dietary protein. Thus, muscle mass and strength were maintained or increased and showed high values. In addition, when protein was consumed evenly in all three meals, the amount of protein at dinner was excessive, but the MPS for muscle synthesis utilization could be stimulated to the maximum extent, so that muscle mass and strength were maintained or increased to the same extent as when enough protein was consumed in the morning. Therefore, morning protein intake is more effective than evening protein intake in maintaining or increasing muscle mass and strength, and the effect is the same as when sufficient protein is consumed at all three meals.

Differences in Protein Intake Balance and Sarcopenia-Related Factors Among Gender

This cross-sectional study showed that high protein intake in the morning maintained higher muscle mass and strength in healthy and obese elderly women. However, the same results were not obtained for men as for women. There are two possible reasons for this finding.

First, it is possible that women are more dependent on dietary protein for muscle synthesis than men. In a previous study, it was shown that the rate of muscle mass loss was lower with higher daily protein intake in elderly women, but there was no association between daily protein intake and muscle mass in elderly men (24). Furthermore, it has been reported that protein intake is associated with maintenance of grip strength and physical performance in women (25). It has also been reported that women have a higher MPS rate and a higher rate of myofibrillar synthesis of dietary protein than men, suggesting that women are more dependent on dietary protein for muscle synthesis than men (26). Therefore, it is possible that the muscle mass and strength of the elderly women in the MG were higher than those of the EG because they were more susceptible to the effects of differences in timing (differences in the efficiency of protein intake). In addition, testosterone secretion related to MPS is 10 times higher in men than in women, and muscle mass and strength are known to be higher in men than in women at all ages, despite the decrease in testosterone secretion with aging (27, 28). Therefore, men may have been more influenced by other factors, such as testosterone, in muscle synthesis than women, and may have been less affected by the timing of protein intake.

Second, the diurnal variation in muscle function-related genes may differ between men and women. Sex differences in the diurnal variation of muscle function-related genes have not yet been clarified. However, in the present study, the effect of the different timing of protein intake on muscle mass and strength was determined only in females. Therefore, there may be sex differences in the diurnal variation in muscle function-related genes. In other words, there may be a small diurnal variation in males and a large diurnal variation in females. In contrast, it has been reported that the expression of *MyoD*, a gene involved in muscle differentiation, has a diurnal rhythm controlled by clock genes and is greatly affected by diet (29, 30). In the present study, it is unclear how the difference in the timing of protein intake between morning and evening affected the expression of *MyoD*, but it is possible that morning protein intake in elderly women suppressed the decrease in muscle regeneration, which was weakened by the decrease in female hormone secretion via *MyoD*. However, it is possible that the morning protein intake in elderly women suppressed the decrease in muscle regeneration capacity, which was weakened by decreased female hormone secretion through *MyoD*.

Differences in Protein Intake Balance and Sarcopenia-Related Factors in Obese People and Those Requiring Support

In cross-sectional study 1, we examined the association between different timing of protein intake and sarcopenia-related factors in obese people and people requiring support as well as healthy people. In obese subjects, as well as in healthy subjects, it was shown that women who consumed more protein in the morning had higher muscle strength. It was also shown that muscle strength was lower when the percentage of protein intake in the evening was high. This may be because the morning protein intake improved insulin resistance, which is worsened in obese people (8), and suppresses the decrease in MPS velocity.

On the other hand, among those requiring support, there was no difference in sarcopenia-related factors among subjects of both sexes who showed a balance of protein intake in the morning or evening. However, it was shown that muscle mass and strength were higher in subjects who needed support in the morning type. In the subjects requiring support in this study, we were not able to examine the effect of physical activity on muscle mass and strength because we were not able to sufficiently measure physical activity meters. However, it has been shown that the amount of daily physical activity is higher in morning-type people (17), and it is possible that the muscle

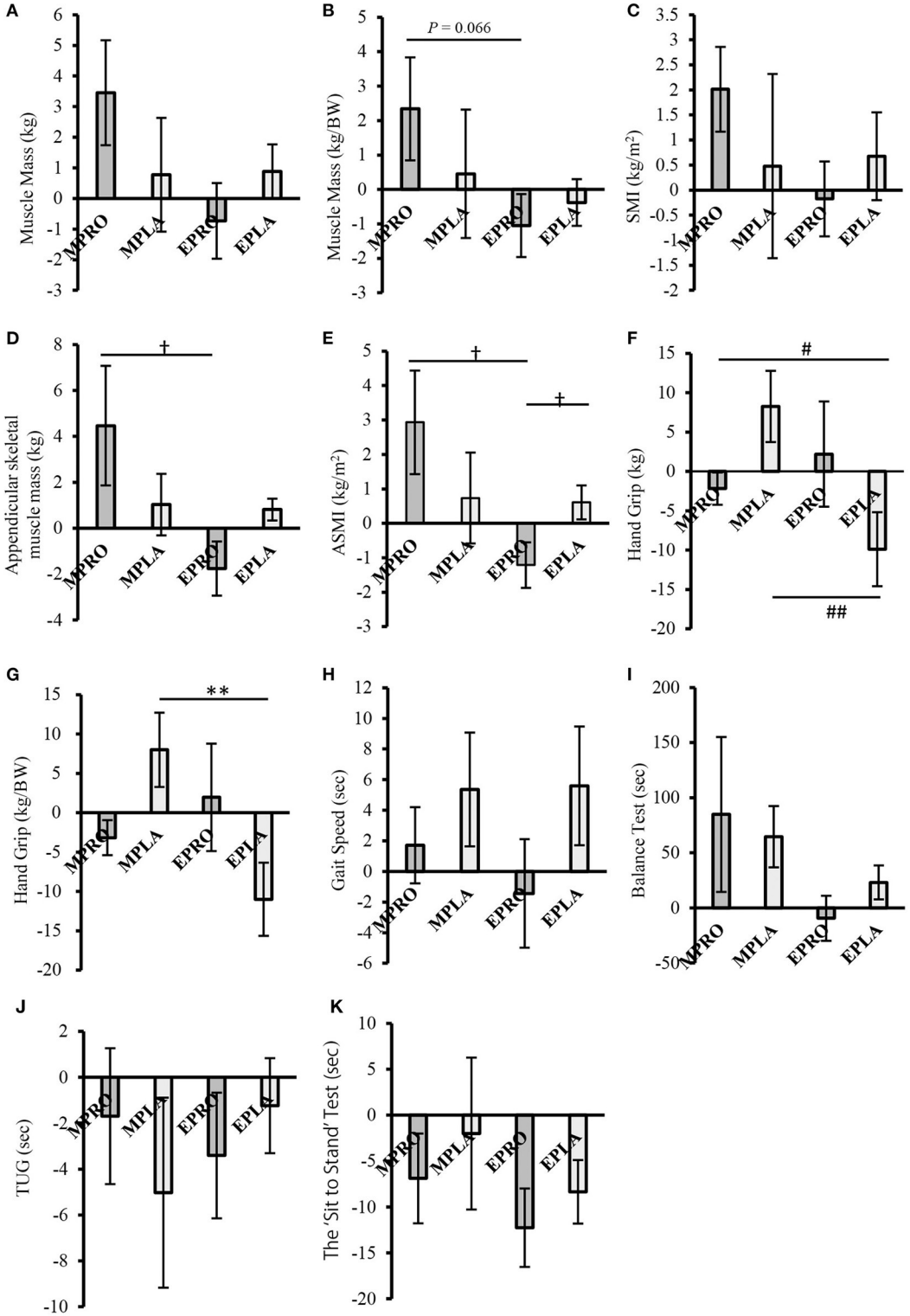

FIGURE 6 | Comparison of the rate of change of sarcopenia-related factors by intervention (intervention study) **(A)** Muscle mass, **(B)** Muscle mass (weight-corrected), **(C)** SMI, **(D)** Appendicular skeletal muscle mass, **(E)** ASMI, **(F)** Handgrip strength, **(G)** Handgrip strength (weight-corrected), **(H)** Gait speed, **(I)** One-leg stand test, **(J)** TUG, **(K)** The "Sit to Stand" Test. Values are expressed as the mean ± standard error. $^{\dagger}P < 0.05$, compared to EPRO (Mann-Whitney). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared to EPLA (Mann-Whitney). $^{**}P < 0.01$ compared to EPRO (Two-Way ANOVA). MPRO, morning protein intake group; MPLA, morning placebo intake group; EPRO, evening protein intake group; EPLA, evening placebo intake group; BW, body weight; SMI, skeletal muscle index; ASMI, appendicular skeletal muscle index; TUG, time up and go.

mass and strength of the people requiring support showed higher values because the amount of physical activity was higher in morning-type people. Since people who require support have some difficulties in their daily activities, their physical functions may be lower than those of healthy or obese people. Therefore, it may be more important to increase the amount of physical activity than the balance of protein intake to maintain muscle mass, strength, and physical function.

Different Timing of Milk Protein Intake and Sarcopenia-Related Factors

In this intervention study, the intake of milk protein in the morning rather than in the evening increased appendicular skeletal muscle mass and ASMI. Two factors may have contributed to this result.

The first factor is the increase in the number of meals with adequate protein intake. It is known that dietary protein has a dose-dependent effect on MPS stimulation (31). Therefore, supplementation of milk protein at breakfast (MPRO group), when protein intake is inadequate, increases protein intake in the morning and enhances MPS stimulation in a dose-dependent manner. Therefore, in the MPRO group, the frequency of MPS stimulation during the day increased, and appendicular skeletal muscle mass hypertrophy was thought to have occurred. In contrast, when milk protein was supplemented in the evening (EPRO group), the frequency of MPS stimulation did not increase as much as in the MPRO group because the protein was not supplemented in the morning when protein intake was insufficient and MPS stimulation occurred only in the afternoon or evening. Furthermore, because protein MPS stimulation has a dose-dependent effect, there is a threshold for this effect (4, 32), it is possible that the MPS stimulation of protein at dinner was already at the threshold and the MPS stimulation of milk protein supplemented in the evening was ineffective in those who had already consumed sufficient protein at dinner.

Second, based on the results of cross-sectional studies 2-2 and 2-3, it is possible that muscle mass was increased by consuming protein at the time of the morning. As shown in the results of cross-sectional study 1, the additional intake of milk protein in the morning may have increased appendicular skeletal muscle mass because the amount of protein consumed was commensurate with the variation in muscle synthesis utilization of dietary protein, which efficiently stimulated the MPS. In contrast, in the group that consumed additional milk protein in the evening, the protein required for the muscle synthesis utilization of dietary protein remained insufficient in the morning, and the MPS could not be stimulated to the maximum extent for muscle synthesis utilization, resulting in MPB being triggered, and a decrease in appendicular skeletal muscle mass may have been observed.

There was a significant difference in handgrip strength between the placebo groups; those who consumed the placebo in the morning showed an increase in handgrip strength compared to those who consumed the placebo in the evening. The reason for this difference is not clear, but it may be due to the higher carbohydrate content of the control food (placebo) compared to the test food. In previous studies, breakfast carbohydrate intake was reported to improve exercise performance (33). Therefore, the group that received the placebo in the morning may have increased their morning carbohydrate intake, leading to improved exercise performance.

In contrast, there was no statistically significant difference between MPRO and EPRO. However, there was a slight increase in the rate of change of handgrip strength before and after the intervention only in EPRO. In general, sports performance, such as muscle strength and flexibility, is associated with diurnal variation in body temperature, which has been reported to be greatest in the evening (34). However, it was not possible to clarify the effect on muscle strength in the timing of protein intake in the intervention trials of this study. On the other hand, the cross-sectional study in the present study showed that MG had higher handgrip strength compared to EG. The duration of the intervention study in this study was 12 weeks, which may have been insufficient to examine the changes in muscle strength. Therefore, it is necessary to clarify the effect of the timing of protein intake on muscle strength by examining a longer period of time in the future.

Timing of Intake of Different Milk Proteins and Blood Indices

In a 12-week intervention study, differences in the timing of milk protein intake did not affect blood indices (insulin, glucose, and growth hormone) (**Supplementary Table 8**). Insulin and growth hormones are known to promote MPS (7, 35). Insulin promotes MPS by activating the mammalian target of rapamycin (mTOR) signal transduction pathway (7). On the other hand, in the elderly, insulin resistance increases with age, and the ability to synthesize MPS is reduced (36). In addition, GH replacement therapy has been reported to significantly increase skeletal muscle mass in healthy elderly men (37).

The lack of changes in these blood indices in the present study suggests that the intake of milk protein at different intake timings did not affect these blood indices. In addition, the increase in appendicular skeletal muscle mass in the MPRO group did not improve insulin resistance or the hormonal environment. Therefore, it is possible that the 12-week period of milk protein intake was too short, and that a longer intervention should be conducted in the future. In addition, the blood samples in this intervention study were taken in the fasting state, and we did not compare the blood indices in the morning and evening after protein intake before and after the intervention. Considering that skeletal muscle protein synthesis in the elderly is resistant to the anabolic effects of insulin and that this is an important factor in the development of sarcopenia (36), it is necessary to examine the changes in blood indices after protein intake in more detail. Furthermore, metabolome analysis showed that most postprandial metabolites related to the glycolytic system, tricarboxylic acid cycle, and amino acids were elevated in the morning compared to the evening, indicating that postprandial metabolic responses are higher in the morning (38). Therefore,

a more detailed study including metabolites may help clarify the differences in metabolic responses related to skeletal muscle protein synthesis in the different protein intake balance between morning and evening.

Limitations of Study

This study had several limitations. The study was conducted on elderly subjects. However, elderly people are less sensitive to the stimulation of muscle protein synthesis by amino acids than younger people (39). Since leucine has been shown to significantly inhibit activation of the mTOR signaling pathway (40), differences in protein intake may have different effects on muscle function in young adults. Therefore, further studies on young adults are required.

Cross-sectional study 2-2 showed that handgrip strength (weight-corrected) was lower in the group that consumed protein only at two meals, in the morning and at lunch, than in the group that consumed enough protein only at one meal in the morning. If it is important to consume protein in the morning, it is expected that muscle strength would show similar values when the group consumed enough protein at only one meal and when the group consumed enough protein at two meals: breakfast and lunch. This may be due to the small number of participants, as there were only six participants who consumed enough protein in two meals: breakfast and lunch. Therefore, there is a need to increase the number of elderly people with inadequate protein intake patterns in the future for a more detailed study.

Previous studies have not reached a consensus on the differences between men and women in muscle protein synthesis pathways, such as mTOR activity, due to diet and exercise, and much remains unclear (41, 42). In addition, there is still insufficient data to define whether the protein requirements of elderly men and women differ. Therefore, further investigation on the differences between men and women, including the expression rhythms of muscle function-related genes, is needed.

Calcium was a component of the test food. Several studies have reported no association between calcium intake and muscle mass or function (43–45). However, in adults over 50 years of age, low blood calcium levels may lead to decreased muscle mass. Particularly, a low calcium intake has been shown to be a possible predictor of muscle loss in women (46). Therefore, the effect of calcium intake on muscle mass and function, in this study, is undeniable. However, since the calcium intake of each protein group was similar, it is likely that the biological effects were comparable. Furthermore, more detailed studies are needed in the future to clarify the effects of calcium intake on muscle mass and muscle function.

CONCLUSION

In conclusion, the current study suggests that adequate protein intake in the morning may be effective in maintaining and increasing muscle mass and strength, and this effect is particularly pronounced in older women. Furthermore, the results demonstrated that morning milk protein supplementation may lead to increased muscle mass in older women with inadequate morning protein intake.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee for Humans at Waseda University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

H-KK, HC, and SS: conceptualization. HC and H-KK: data curation. H-KK and HC: formal analysis. SS, SH, and MS: funding acquisition. H-KK, HC, MF, JO, MO, and TN: investigation. H-KK, HC, and SS: methodology. H-KK and SS: project administration. SS and H-KK: resources. SS: supervision. H-KK and HC: visualization. H-KK, HC, MT, and SS: writing—original draft. TN, MT, and SS: writing—review and editing. All authors read and approved the final 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/fnut.2021.797004/full#supplementary-material

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Association Between Na, K, and Lipid Intake in Each Meal and Blood Pressure

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Cardiovascular diseases (CVDs) are one of the leading causes of death worldwide, and one of the most significant risk factors for CVDs is high blood pressure. Blood pressure is associated with various nutrients, such as sodium, potassium, and cholesterol. However, research focusing on the timing of intake of these nutrients and blood pressure has not been conducted. In this study, we used dietary data and a questionnaire asking about the sleep, physical activity, and blood pressure, collected from the food-log app "Asken" (total $N = 2{,}402$), to investigate the relationship between the dietary data of nutrient intake in the breakfast, lunch, and dinner and blood pressure. Daily total intake of various nutrients such as sodium, sodium-to-potassium ratio, total energy, lipid, carbohydrate, and saturated fat showed a significant association with blood pressure depending on the meal timing. From multiple regression analysis, eliminating the confounding factors, lunch sodium-to-potassium ratio, dinner energy, lipid, cholesterol, saturated fat, and alcohol intake were positively associated with blood pressure, whereas breakfast protein and lunch fiber intake showed a negative association with blood pressure. Our results suggest that nutrient intake timing is also an important factor in the prevention of high blood pressure. Our study provides possibilities to prevent hypertension by changing the timing of nutrient intake, especially sodium, together with potassium and lipids. However, because our research was limited to food-log app users, broader research regarding the general population needs to be conducted.

Keywords: blood pressure, dietary pattern, sodium, potassium, lipid, chrono-nutrition

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INTRODUCTION

Cardiovascular diseases (CVDs) are one of the leading causes of death worldwide, and one of the most significant risk factors for CVD is high blood pressure (1, 2). Blood pressure exhibits a circadian rhythm, rising from morning to afternoon and dipping at night (3). Mammals are under the control of this circadian rhythm, a rhythm of ∼24 h, and the circadian clock mechanism plays an important role in physiological functions such as sleep/wakefulness, hormone secretion, and metabolism (4–6). In organs such as the kidney, peripheral clocks generate physiological rhythms (7). The circadian rhythm of blood pressure is driven by a complex molecular network of clock genes, and alterations in blood pressure rhythm from genetic manipulation of various clock genes

have been reported in rodent studies (8). Mice lacking one of the core clock genes, Cryptochrome-1 and Cryptochrome-2, show salt-sensitive hypertension due to abnormally high synthesis of the mineral corticoid aldosterone, indicating a potential link between disturbances in the circadian rhythm and hypertension (9). Individuals with disrupted clocks, such as shift workers, showed higher blood pressure and a higher prevalence of CVDs (10). In patients with hypertension, loss of the day-night rhythm of blood pressure has been reported (11).

The blood pressure is well-controlled by urine and sodium excretion through the Na/K reuptake mechanism located in the renal tubule (12). Excess sodium intake and insufficient potassium intake have been shown to result in high blood pressure (13, 14). To observe the joint effects of sodium intake and potassium intake, the use of the sodium-to-potassium ratio (Na/K ratio) has been proposed in various studies. The Na/K ratio has been reported to show a stronger association with blood pressure than with sodium or potassium alone (15). Other nutrients have also been reported to be associated with hypertension. High cholesterol levels are known to cause arterial stiffness and arteriosclerosis, and systolic pressure is influenced by arterial stiffness (16). In addition, the benefits of dietary fiber intake on blood pressure have been reported (17). Reducing alcohol consumption lowers the blood pressure in a dose-dependent manner (18). Therefore, it is important to focus on various nutrients to prevent high blood pressure.

From transcriptome and metabolome analyses of human blood and tissue samples, it has been reported that circadian rhythms also exist in food digestion, absorption, and metabolism (8, 19). "Chrono-nutrition" is the study focusing on the intake timing of nutrients. There have been reports showing that nutrients show more health benefits depending on the intake timing (20). Catechin suppressed the elevation of postprandial glucose more effectively when taken in the evening than in the morning (21). Continuous beginning of the active phase administration of sesamin and episesamin improved lipid metabolism compared to administration at the end of the active phase (22). Morning intake of fish oil, which is abundant in docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), suppressed serum triglyceride levels and decreased serum total saturated fatty acids and serum n-6 polyunsaturated fatty acids (23). Thus, it is important to consider not only what to eat but also when.

As previously stated, the association between blood pressure and the circadian clock has been suggested in various studies. Furthermore, the metabolism and excretion of nutrients associated with blood pressure exhibit a circadian rhythm. The excretion of sodium and potassium by the kidneys is controlled by the circadian timing system (24). Cholesterol metabolism also exhibits a 24-h rhythm (25). The influence of eating patterns and meal timings on blood pressure has also been suggested. Consuming meals irregularly has been suggested to be adversely associated with cardiometabolic risk, including blood pressure (26). Later lunch compared to the conventional Australian mealtime pattern showed higher blood pressure in women (27). Compared to breakfast eaters, elevated blood pressure was observed among female chronic breakfast skipping groups (28). Reversed feeding completely reversed the blood pressure rhythm in mice (29). These finding suggest that considering the prevention of high blood pressure from a "chrono-nutrition" point of view may be important.

According to the National Nutrition Survey in Japan (NNSJ) in 2019, compared to the estimated average requirement, the average intake of sodium and saturated fat is excessive, while that of potassium and dietary fiber is insufficient (30). Proposing the intake timing would be an effective way to approach the discrepancy between the actual and recommended intake of these nutrients. However, research focusing on blood pressure and the timing of intake of various nutrients has not been conducted. In this study, we aimed to investigate the relationship between dietary data of nutrient intake in three meals and blood pressure among ∼2,400 users of "Asken," a mobile health application for dietary management.

MATERIALS AND METHODS

Study Participants and Mobile Health App "Asken"

Dietary data and questionnaire answers were collected through a popular food log and food coaching app, "Asken." Based on the Dietary Intake Standards for Japanese determined by the Ministry of Health, Labor and Welfare, the app provides feedback on the dietary content of the meal, showing the excess and deficiency of nutrients.

The validity of Asken has been confirmed in previous studies. When comparing weighed dietary records with records from Asken, the energy and nutrient intakes were correlated, suggesting the validity of Asken (31). Paper-based dietary records and Asken records have been reported to have a 0.80 median correlation coefficient for nutrient intake (32). Those using this app are health-conscious people, and almost 95% of the users of the app aim to lose weight. This may account for 70% of the users being female. One limitation of using this app is self-efficacy. Dietary self-monitoring induces behavioral changes, and compared to paper-based records, electronic records have been reported to induce stronger changes (33, 34). Therefore, compared to those living under free conditions, our research was conducted on health-conscious people. The average daily nutritional data showed similarity with NNSJ, which reports the average daily Japanese nutritional intake (**Supplementary Figure 1**). We can obtain the NNSJ data only as the average. Lower cholesterol and carbohydrate intake and higher potassium, protein, and dietary fiber intake were observed, which may indicate the "health-conscious" characteristics of the participants compared to the average Japanese people.

In addition to these dietary records, an online survey was conducted in January 2021. This experiment was approved by the Ethics Review Committee on Research with Human Subjects at Waseda University (No. 2020-046) and followed the guidelines laid down in the Declaration of Helsinki. A total of 2491 participants responded at first, and by excluding data missing the reports of all three meals a day or missing basic characteristics such as body mass index (BMI) and sleep, physical activities, and

subjects taking medicine, the final data were 707 for males and 1,695 for females.

Questionnaire

From an online survey, the basic characteristics of the participants (age, gender, BMI) and other lifestyle-related factors (sleep, physical activity, blood pressure) were obtained.

Assessment of Morning Type or Evening Type

Sleep factors such as morningness was assessed by MSFsc (sleep—corrected midpoint of sleep on free days). The computation was as follows: MSFsc = MSF—[(sleep duration on free days)—(sleep duration in workdays)]/2. This assessment is widely used and established (35).

Physical Activity

From an online survey, physical activity was determined by the number of days and hours spent on the three types of activities (vigorous-intensity activity, moderate-intensity activity, and walking). We calculated weekly metabolic equivalents (MET) based on the International Physical Activity Questionnaire (IPAQ) analysis guidelines for each activity intensity for total physical activity (36). The IPAQ is widely used to assess physical activity (37).

Blood Pressure

Participants answered the question; "Please answer the most recent record of maximum blood pressure (systolic blood pressure) by upper arm sphygmomanometer through medical checkup and/or at home." Participants answered their systolic blood pressure by a score of 1–6 (1: <110 mmHg, 2: 111–120 mmHg, 3:121–130 mmHg, 4: 131–140 mmHg, 5: 141–150 mmHg, 6: over 151 mmHg). Blood pressure is usually recorded in the morning. We also asked if they were taking medication for high blood pressure, and the answers of those who answered yes were excluded.

Dietary Data

The dietary data were analyzed using ∼1-month average dietary records of the application. Energy content (kcal), protein, fat, carbohydrate, sodium, potassium, cholesterol, dietary fiber, saturated fat, and alcohol intake were measured for each of the three meals and snack. The intake timings of snacks might be different among participants, because of no check of snack time. From previous research, the validity of the dietary record of this app was high (31). The Na/K ratio was calculated by dividing the amount of sodium intake by potassium intake.

Statistical Analysis

Statistical analysis of the obtained data was performed using a predictive analytics software for Windows (Statistical Package for the Social Sciences; IBM Corp., Chicago, IL, USA), and a p-value of <0.05 was considered statistically significant. To investigate the relationship between dietary patterns and blood pressure, Spearman's rank correlation analysis was conducted. To clarify the relationship excluding other factors, multiple regression analysis among the three meals for each nutrient was conducted. Data are expressed as mean and standard error.

RESULTS

Subject Characteristics

For our analysis, we chose subjects from 2,491 responses to those who answered the questions regarding their blood pressure. The subjects were then excluded for various reasons, such as intake of medicine and outliers or unfilled data to 2,402 people. The mean (SE) age was 45.95 (0.237) years, BMI was 23.12 (0.0778), total PA MET min/week was 32.05 (0.818), and MSFsc was 3.36 (0.0254) (**Table 1A**). All values except MSFsc were significantly higher in men than in women. Large values of MSFsc in females demonstrated more eveningness than males. There were positive relationships between age, BMI, and blood pressure, and a negative relationship between MSFsc and blood pressure (**Table 1B**). Pearson's chi-square test revealed that blood pressure distribution was different between males and females ($p < 0.001$), and males exhibited higher blood pressure than females (**Figure 1A**). There was a positive relationship between age and blood pressure (**Figure 1B**) and between BMI and blood pressure (**Figure 1C**). However, there was a negative relationship between MSFsc and blood pressure (**Figure 1D**). Although there was no relationship between physical activity and blood pressure, individuals with the highest blood pressure showed low physical activity (**Figure 1E**).

Nutrient Intake Volumes in Each Meal

Next, the intake volumes of each nutrient were compared among breakfast, lunch, and dinner in male and female participants (**Supplementary Tables 1, 2**). Intake volumes of snack are shown in **Supplementary Table 3**. All nutrients except for carbohydrates and alcohol in females were significantly taken at dinner and next at lunch in comparison with breakfast in both males and females.

Correlations Analyses of Intake of Nutrients and Blood Pressure

Correlation analysis of the intake of various nutrients and blood pressure was applied for each meal (breakfast, lunch, and dinner) and snack. In addition to the amount of intake for each nutrient, the mean energy ratios of protein, fat, and carbohydrate were compared for each meal and snack. Spearman's correlation analysis showed that blood pressure had a strong positive association with the Na/K ratio for every meal in both males and females (**Table 2**). Nutrients that showed positive associations with blood pressure in both males and females were lunch sodium, lunch and dinner energy, total lipid, lunch carbohydrate, and total and dinner alcohol. Lunch and snack protein ratio and total, dinner and snack dietary fiber intake were negatively associated with blood pressure in both sexes. In females, lipid intake showed a dinner-specific positive correlation (**Table 2**).

TABLE 1 | Basic characteristics of participants and correlation analysis of basic characteristics and blood pressure.

(A) Basic characteristic

	All (N = 2,402)		Male (N = 707)		Female (N = 1,695)		P-value
	Mean	SE	Mean	SE	Mean	SE	(Male vs. female)
Age	45.95	0.237	50.14	0.406	44.17	0.279	<0.001
BMI (kg/m²)	23.12	0.0778	24.21	0.129	22.66	0.094	<0.001
Total PA MET-minutes/week	32.05	0.818	37.55	1.601	29.64	0.939	<0.001
MSFsc	3.363	0.0254	3.076	0.0453	3.489	0.0302	<0.001

(B) Correlation analyses of basic characteristics and blood pressure

	Male (N = 707)		Female (N = 1,695)	
	Correlation	P-value	Correlation	P-value
Age	0.213**	<0.001	0.264**	<0.001
BMI(kg/m²)	0.226**	<0.001	0.315**	<0.001
Total PA MET-minutes/week	−0.100**	0.007	−0.03	0.212
MSFsc	−0.008	0.839	−0.033	0.166

**$p < 0.01$ by Spearman's rank correlation coefficient. BMI, Body Mass Index; PA, Physical Activity; MET, Metabolic Equivalents; MSFsc, sleep-corrected Midpoint of sleep in free-days.*

Association Between the Timing of Each Nutrient and Blood Pressure

Multiple regression analysis showed that even when eliminating the effects of confounding factors, such as sex, age, BMI, MSFsc, and PA MET min/week, various nutrients showed different associations with blood pressure depending on their intake timing (**Table 3**). Focusing on salt intake, lunch and snack Na/K ratio showed a significant positive association, and lunch potassium intake showed a negative association (**Table 3**). Dinner energy, dinner lipid, dinner cholesterol, dinner saturated fat, and dinner alcohol intake were positively associated with blood pressure, whereas breakfast protein and lunch fiber intake showed a negative association with blood pressure (**Table 4**).

DISCUSSION

In this study, we investigated the association between blood pressure and different nutrient intake timing among mobile health app "Asken" male and female users. Our findings confirm previous findings that high Na/K ratio, high lipid intake, high alcohol intake, and low dietary fiber are associated with high blood pressure (15, 17, 18). However, little is known about the influence of intake timing. Our research provides new perspectives on the prevention of hypertension and has shown that various nutrients are associated with blood pressure at different meal timings. In both males and females, lunch and snack Na/K ratio, dinner energy, dinner lipid, and dinner saturated fatty acids showed a meal-specific positive association with blood pressure, and breakfast protein, lunch potassium and lunch dietary fiber showed a meal-specific negative association with blood pressure after eliminating the confounding factors.

From previous studies, dietary records collected by Asken have been reported to be an effective method for estimating the energy and nutrient intakes of Japanese women (31). As stated, the nutrition amount resembles the data calculated from the NNSJ (30) (**Supplementary Figure 1** and Shinto et al.). Focusing on sodium and potassium, dinner sodium intake was lower and potassium intake was higher, resulting in a lower dinner Na/K ratio than that of NNSJ (**Supplementary Figure 2**). This can be explained by the characteristics of the Asken users. With the aim of losing weight, Asken users have higher health consciousness and high self-efficacy (38).

Our results showed that blood pressure had a strong positive association with the Na/K ratio, especially the lunch and snack Na/K ratio. In addition, the negative correlation of lunch potassium shows that promoting excretion of sodium is important for lowering blood pressure at lunch time. Sodium and potassium urine excretion has been previously reported to exhibit circadian rhythm, which is explained by the rhythm of aldosterone (24). Aldosterone, a mineralocorticoid that through ENaC is responsible for the reabsorption of Na and the increase in K secretion through K channels in the distal nephron, has also been reported to exhibit rhythm, low at nighttime and high in the morning (39). Therefore, intake of sodium at dinner time may be excluded in urine, even though sodium intake and the Na/K ratio were high in the dinner. Several previous studies have demonstrated higher excretion of sodium and an increase of Na/K ratio in the evening than in the morning and afternoon (24).

Potassium and dietary fiber are both rich in vegetables. Lunch fiber and potassium intake showed a negative correlation with blood pressure in the current experiment, suggesting the intake of more vegetables at lunch and snack time to protect higher blood pressure. Higher blood pressure has been reported to be

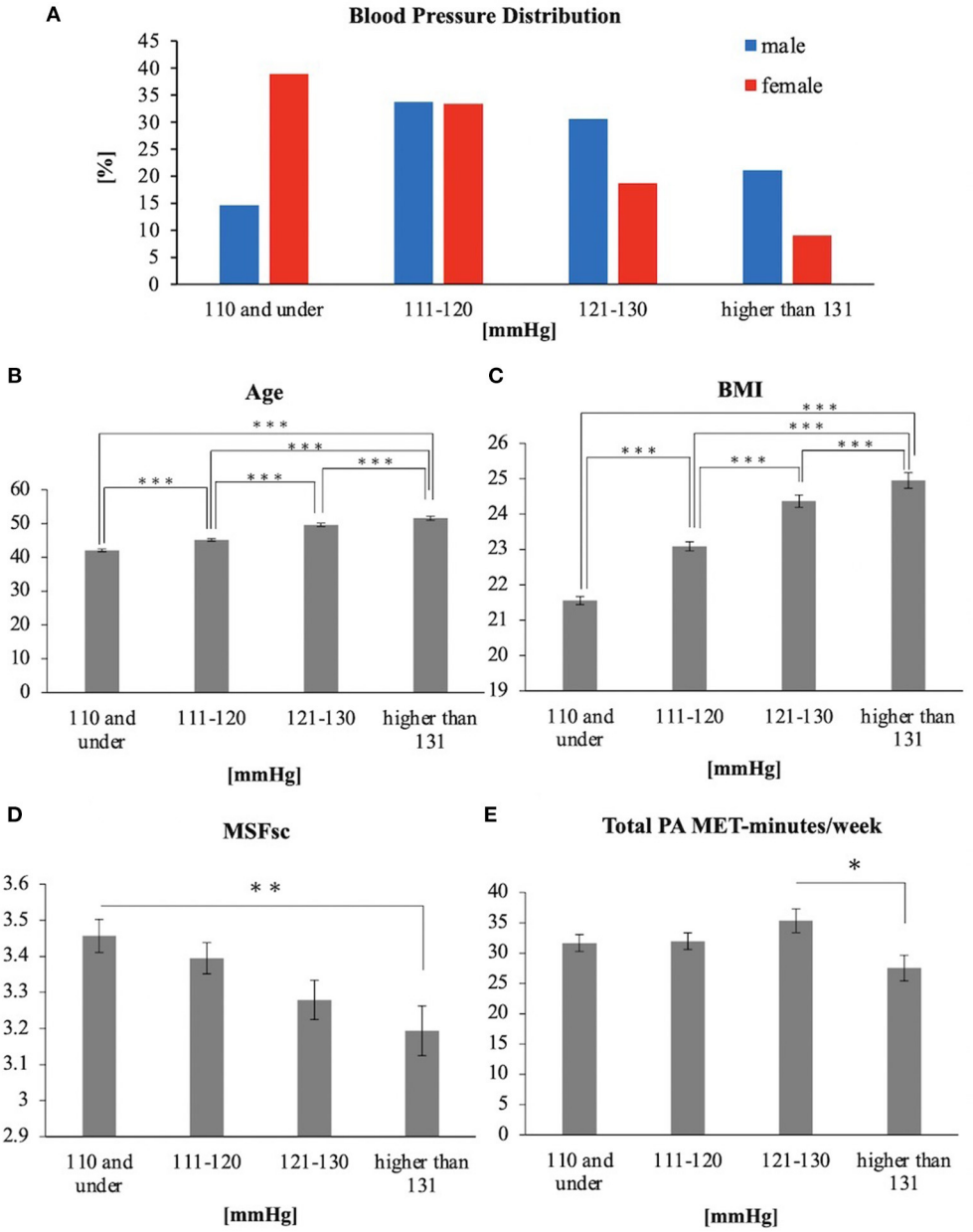

FIGURE 1 | (A) Blood Pressure Distribution **(B)** Age **(C)** BMI **(D)** MSFsc **(E)** Total PA MET-minutes/week. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (Tukey).

associated with lower gut microbiota alpha diversity in many human cross-sectional studies (40). A low intake of dietary fiber leads to reduced microbial diversity (41). Inulin, a water-soluble dietary fiber, has been reported to have greater positive effects on the microbiota in the morning than in the evening (42). Taken together, these results suggest that dietary fibers, especially at lunch and breakfast time, may be more helpful than at dinner time to protect higher blood pressure through an increase in microbial diversity. These results have suggested the importance of vegetables in the prevention of hypertension in a time-specific manner. Vegetables are also known to include many dietary polyphenols such as flavonoids, and their treatment or prevention of hypertension (43). As previously stated, catechin, a type of flavonoid, suppressed the elevation of postprandial glucose more effectively when taken in the evening than in the morning, suggesting that the metabolic response of polyphenols may depend on their intake timing (21). Dietary polyphenols' health promoting effects are reported to be in a hormetic dose-response manner, partly via the upregulation of Nrf2 pathway (44–47). Therefore, in addition to the analysis of nutrients that

TABLE 2 | Correlations analyses of intake of nutrients and blood pressure.

	Male					Female				
	Total	**Breakfast**	**Lunch**	**Dinner**	**Snack**	**Total**	**Breakfast**	**Lunch**	**Dinner**	**Snack**
Na/K	0.128**	0.090*	0.117**	0.128**	0.039	0.051*	0.053*	0.105**	0.051*	0.009
Sodium	0.087*	0.044	0.097**	0.087*	−0.01	0.023	0.055*	0.065**	0.023	−0.022
Potassium	−0.06	−0.044	−0.062	−0.06	−0.080*	−0.02	−0.023	−0.036	−0.02	−0.029
Energy	0.065	−0.011	0.074*	0.106**	−0.019	0.106**	0.052*	0.070**	0.079**	−0.018
Protein	−0.037	−0.062	−0.057	0.012	−0.07	−0.003	−0.04	−0.026	0.021	−0.053*
Lipid	0.085*	0.034	0.055	0.059	−0.029	0.096**	0.043	0.046	0.082**	−0.041
Carbohydrate	0.03	0.005	0.084*	−0.01	−0.007	0.074**	0.068**	0.067**	0.041	0.013
Protein (PFC ratio)	−0.058	−0.049	−0.110**	0.019	−0.081*	−0.079**	−0.094**	−0.077**	−0.04	−0.055*
Fat (PFC ratio)	0.061	0.066	0.019	0.067	0.006	0.045	0.013	0.024	0.054*	−0.041
Carbohydrate (PFC ratio)	0.003	−0.019	0.068	−0.041	0.052	0.036	0.064**	0.048*	0.006	0.063**
Cholesterol	−0.025	−0.01	−0.067	0.05	−0.016	0.024	0.01	−0.002	0.028	0.028
Dietary Fiber	−0.086*	−0.055	−0.063	−0.083*	−0.075	−0.066**	−0.057*	−0.060*	−0.051*	−0.049*
Saturated Fat	0.054	0.044	0.00	0.067	−0.024	0.097**	0.061*	0.037	0.096**	−0.02
Alcohol	0.077*	0.035	0.038	0.113**	0.058	0.063**	0.006	0.052*	0.065**	−0.003

*$p < 0.05$; **$p < 0.01$ by Spearman's rank correlation coefficient.

TABLE 3 | Association between the timing of salt intake and blood pressure including snack.

	β	*P*-value	*R*2	*F*
Breakfast_Na/K	−0.001	0.958	0.187	53.495
Lunch_Na/K	0.072	0.001		
Dinner_Na/K	−0.014	0.514		
Snack_Na/K	0.046	0.022		
Breakfast_Sodium	0.0048	0.812	0.177	52.405
Lunch_Sodium	0.034	0.110		
Dinner_Sodium	0.014	0.518		
Snack_Na/K	−0.018	0.371		
Breakfast_Potassium	−0.017	0.414	0.185	53.764
Lunch_Potassium	−0.043	0.037		
Dinner_Potassium	0.000	0.990		
Snack_Potassium	−0.029	0.142		

Multivariable regression analyses adjusted by age, sex, BMI, Total PA MET-minutes/week and MSFsc.

we have conducted, analysis of the intake timing and volume of dietary polyphenols may provide a clearer view.

Furthermore, breakfast protein was negatively correlated with blood pressure. The beneficial effect of proteins on blood pressure is small, according to previous studies (48). However, a high-protein breakfast has been suggested to accelerate overloading-induced skeletal muscle hypertrophy in mice and have greater skeletal muscle volume in human studies (49). It has also been revealed that breakfast and lunch proteins have a strong positive association with daily physical activity (unpublished observation), and recent studies have supported the role of physical activity in the prevention of hypertension (50). Therefore, the relationship between morning protein intake and exercise may explain its negative correlation with blood pressure. As clinical trials of food-protein-derived peptides in the management of hypertension have been published (51), we should examine the detailed information of protein compositions (cereals, beans, vegetables, fruits, meat, fish milk, egg, etc.) in future experiment.

Dinner cholesterol and saturated fatty acids were positively correlated with blood pressure. A high dinner energy also showed a positive correlation. It is well-known that a high intake of cholesterol and saturated fatty acids causes progression of arteriosclerosis and higher blood pressure (52, 53). However, we do not know the true reason why dinner time intake of these nutrients and energy are related to high blood pressure. As cholesterol synthesis in the body is high in the evening, cholesterol intake and synthesized cholesterol may cooperatively promote arteriosclerosis progression. The intake of saturated fatty acids and energy at dinner time cannot be consumed as an energy source because of sleep. Excess circulation of triglycerides and fatty acids may accelerate the progression of arteriosclerosis (53).

High energy intake and lipid intake at dinner are reported to lead to obesity, which is known to be strongly associated with blood pressure (16). In the present study, we found a positive relationship between BMI and blood pressure. However, the correlation of energy/lipid at dinner time was still observed when eliminating the obesity factor in the present experiment. It has been reported that early time-restricted feeding, with dinner before 3 pm, led to lower blood pressure without inducing weight loss (54). This study, along with our present data, strongly suggests that taking energy/lipid-rich dinner at an earlier clock time may prevent higher blood pressure. As it is well-known that hypertension is related with seriousness of diabetes and

TABLE 4 | Association between the timing of nutrient intake and blood pressure including snack.

	β	*P*-value	*R2*	*F*
Breakfast_Energy	0.007	0.735	0.183	53.700
Lunch_Energy	0.020	0.366		
Dinner_Energy	0.100	<0.0001		
Snack_Energy	0.008	0.703		
Breakfast_Protein	−0.046	0.027	0.183	53.412
Lunch_Protein	−0.032	0.137		
Dinner_Protein	0.019	0.393		
Snack_Protein	−0.017	0.405		
Breakfast_Lipid	0.005	0.792	0.185	54.885
Lunch_Lipid	0.016	0.449		
Dinner_Lipid	0.059	0.007		
Snack_Lipid	−0.013	0.515		
Breakfast_Carbohydrate	0.018	0.382	0.183	53.874
Lunch_Carbohydrate	0.034	0.125		
Dinner_Carbohydrate	0.009	0.699		
Snack_Carbohydrate	0.007	0.725		
Breakfast_Cholesterol	−0.028	0.165	0.179	52.691
Lunch_Cholesterol	−0.025	0.215		
Dinner_Cholesterol	0.041	0.049		
Snack_Cholesterol	−0.001	0.960		
Breakfast_Fiber	−0.034	0.108	0.184	53.605
Lunch_Fiber	−0.048	0.026		
Dinner_Fiber	−0.023	0.285		
Snack_Fiber	−0.026	0.192		
Breakfast_Saturated Fat	0.013	0.523	0.186	55.343
Lunch_Saturated Fat	−0.005	0.814		
Dinner_Saturated Fat	0.072	0.001		
Snack_Saturated Fat	−0.008	0.676		
Breakfast_Alcohol	−0.013	0.509	0.179	51.548
Lunch_Alcohol	0.022	0.304		
Dinner_Alcohol	0.041	0.065		
Snack_Alcohol	0.015	0.475		

Multivariable regression analyses adjusted by age, sex, BMI, Total PA MET-minutes/week and MSFsc.

dyslipidemia, we should investigate the relationships between intake of Na, K lipid, and saturated fatty acids and these diseases in future. In the current experiment, we have not asked if participants had these diseases or not.

There are some limitations to our study. First, the dietary data were collected through self-reports by the subjects, and this self-reporting may have resulted in self-efficacy. Previous studies have discussed that self-monitoring results in behavioral changes (34). The second is blood pressure measurement. Blood pressure was reported by the participants themselves, and this may cause some inaccuracy in the data. We also could not measure the blood pressure at the same time. For more precise data, blood pressure needs to be measured at a standardized time, such as in the morning 1 h after awakening and in the evening before going to sleep according to the Japanese Society of Hypertension have presented in the guidelines for home blood pressure measurement (55). Third, only systolic blood pressure has been reported because many Japanese are unconcerned about their diastolic blood pressure. As both systolic and diastolic blood pressure are used for hypertension diagnosis, in future research, both systolic and diastolic blood pressure should be investigated. Fourth, we could not check the intake timing of snack, however similarity to lunch Na/K ratio suggests many participants may take snack at afternoon. Fifth, the characteristics of the participants being highly health-conscious may have affected the results. Lastly, we could only investigate saturated fatty acids this time, but by looking at the amount of DHA and EPA that work in suppressing arteriosclerosis, we would have been able to have a better understanding.

CONCLUSIONS

We analyzed the relationship between the intake timing of nutrients and blood pressure. In both males and females, a positive association between meals and blood pressure was observed in the Na/K ratio (lunch), energy (dinner), lipid (dinner), and saturated fatty acids (dinner). Moreover, a meal-specific negative association with blood pressure was seen in protein (breakfast), potassium (lunch), and dietary fiber (lunch). Our study provides possibilities to prevent hypertension by changing the timing of various nutrient intakes, especially sodium together with potassium and lipids. However, an intervention study to investigate the effect of the timing of each nutrient on blood pressure needs to be conducted in the future.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

This experiment was approved by the Ethics Review Committee on Research with Human Subjects at Waseda University (No. 2020-046) and followed the guidelines laid down in the Declaration of Helsinki. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MI, HS, YT, TS, SM, MK, AT, NA, MM, and SS designed the research and analyzed the data. MI and SS wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.853118/full#supplementary-material

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REVIEW
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Using Intermittent Fasting as a Non-pharmacological Strategy to Alleviate Obesity-Induced Hypothalamic Molecular Pathway Disruption

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Intermittent fasting (IF) is a popular intervention used to fight overweight/obesity. This condition is accompanied by hypothalamic inflammation, limiting the proper signaling of molecular pathways, with consequent dysregulation of food intake and energy homeostasis. This mini-review explored the therapeutic modulation potential of IF regarding the disruption of these molecular pathways. IF seems to modulate inflammatory pathways in the brain, which may also be correlated with the brain-microbiota axis, improving hypothalamic signaling of leptin and insulin, and inducing the autophagic pathway in hypothalamic neurons, contributing to weight loss in obesity. Evidence also suggests that when an IF protocol is performed without respecting the circadian cycle, it can lead to dysregulation in the expression of circadian cycle regulatory genes, with potential health damage. In conclusion, IF may have the potential to be an adjuvant treatment to improve the reestablishment of hypothalamic responses in obesity.

Keywords: intermittent fasting (IF), hypothalamus, obesity, hypothalamic inflammation, non-pharmaceutical intervention

INTRODUCTION

The neuronal circuits controlling food intake and the endocrine mechanisms involved in this complex modulation network have been widely investigated to clarify the factors associated with the regulation of energy homeostasis. The hypothalamus is considered the central point of this regulatory system. Therefore, impairment of the hypothalamic response generated by signaling disruption in crucial signaling molecules has been associated with the development of morbid obesity, highlighting the importance of controlling the hypothalamic function for health (1–3).

Different central nervous system regions mediate the regulation of food intake, body weight, and energy homeostasis. In this context, the mid-basal portion of the hypothalamus, where the arcuate nucleus is located, is composed of different subpopulations, including the orexigenic neurons,

which are directly involved in the hunger stimulus, and also anorectic neurons, which are mainly involved in response to satiety signals (4). The agouti-related peptide (AgRP) orexigenic neuron and the pro-opium melanocortin anorectic neuron (POMC) are two essential components of energy expenditure, hunger, and satiety control neurocircuits, integrating central and peripheral energy status with metabolic signals (5). It is essential to highlight that the hypothalamus contains other neuronal groups involved in controlling food intake and energy expenditure (6), which are not the focus of this review.

Agouti-related peptide orexigenic neurons co-express the messenger ribonucleic acid (RNA) for the neuropeptide Y (NPY) and the neurotransmitter gamma-aminobutyric acid (GABA). Studies reveal that the intracerebroventricular administration of AgRP (7) or its overexpression is associated with increased food intake (8). In contrast, POMC anorectic neurons are co-located with those expressing the cocaine-and amphetamine-regulated transcript (CART) (2, 9). After its synthesis, POMC is cleaved by different enzymes, generating several peptides responsible for the POMC functions (10). Neurons expressing endogenous melanocortin ligands for POMC and AgRP neuropeptides (antagonists) and neurons containing melanocortin receptors compose the central melanocortin system (8, 11). This system is strictly involved in the control of food intake, glucose metabolism, and energy homeostasis (12, 13), in conjunction with anorectic hormones, primarily leptin and insulin, composing a complex neuroendocrine system to maintain the correct energy and body weight balance, as recently described by Yang et al. (14).

Both POMC and AgRP neurons have the leptin receptor (LepR). When leptin binds to POMC neuronal cell receptors, neuronal depolarization and activation initiate multiple signal translations related to satiety responses. The leptin-mediated signaling is transduced into the nucleus, producing the anorexic POMC and CART neurotransmitters (15, 16). In addition, a cross-inhibitory reaction between AGRP and POMC neurons induces a reduction in orexigenic neurotransmitters in the AGRP neurons.

Insulin is also a crucial hormone for maintaining energy homeostasis by inhibiting pathways associated with NPY/AgRP neurons and their ramifications (16, 17). Therefore, impairments in the central signaling pathways of insulin (18–20) and leptin (21, 22) are associated with energy imbalance and obesity development. In this context, intermittent fasting (IF) is a protocol popularly used as a strategy to promote weight loss (23) and has become a tremendous scientific topic of interest to elucidate the mechanisms that regulate the hypothalamic molecular responses that will reduce body weight and prevent obesity (24, 25).

Previous investigations in human and animal models analyzed the effects of IF on leptin and insulin sensitivity (26, 27), inflammatory pathways (28, 29), the brain-microbiota axis (30, 31), circadian cycle (32, 33), and autophagic pathway (34). All these factors seem to be related to adaptations in POMC and AgRP neuropeptides (35) that can improve energy homeostasis through pathways that are not yet fully understood. The present review explored the molecular and physiological adaptations of leptin, insulin, POMC, and AgRP neuropeptides to IF protocols, mostly performed in animal obesity models.

Leptin and Insulin in Energy Homeostasis: Molecular Pathways Linked to Pro-opium Melanocortin Anorectic Neuron and Agouti-Related Peptide Responses

Insulin and leptin are the main anorectic hormones that act on the arcuate nucleus, activating POMC neurons and inhibiting AgRP neurons (36). Several studies indicate that the loss of hypothalamic insulin signaling (18–20) and leptin (21, 22) can induce changes in energy homeostasis, excessive food intake (hyperphagia), and body weight gain, leading to obesity development. The arcuate nucleus is densely rich in leptin receptors (37). The intracellular signaling cascade begins after leptin binds to its receptors in neuronal cells. An internal conformational alteration in the LepR attracts the next downstream protein, JAK2 (Janus kinase 2) (38). JAK is a cytoplasmic cytokine receptor that can autophosphorylate and promote the phosphorylation of its intracellular tyrosine residue Y-938, associated with the recruitment of the phosphatase SHP2 and its extracellular regulator ERK2, and of the residue Y-1077, which recruits the STAT5 transcriptional and signal transduction activator pathway. The primary effects of leptin on energy homeostasis involve the phosphorylation of the Y-1138 tyrosine residue, which creates a STAT3 binding and recruitment site (39). After its binding and subsequent activation, the STAT3 is transferred to the nucleus of the neuronal cell and promotes the transcription of genes, such as the neuropeptide POMC (40, 41).

Regarding insulin, despite having been discovered in 1921 (42), the complete elucidation of its molecular signaling is still in progress. However, it is known that the insulin receptor (IR) is a tetrameric enzyme that comprises two extracellular alpha subunits and two transmembrane beta subunits. Once the hormone interacts with its receptor, there is activation and consequent phosphorylation of the generated substrates (IR family), leading to activation of its main pathway, the phosphoinositide 3-kinase (Pi3K) pathway, a heterodimeric lipid kinase that binds to tyrosine residues *via* its SH2 domain, generating PI membrane phosphates with PkB/Akt recruitment (43).

Both insulin and leptin can stimulate the Pi3K pathway in the arcuate nucleus with subsequent phosphorylation of their target proteins, leading to hyperpolarization and activation of POMC neurons (44) and inhibition of AgRP (17). Mice with genetic Pi3K deletion in POMC cells did not show activation of POMC neurons in response to insulin or intracerebroventricular leptin administration (45). However, Pi3K deletion in AgRP neurons seems to induce energy expenditure reduction, insulin and leptin resistance, and weight gain (17).

The mechanistic target of rapamycin (mTOR) is one of the Pi3K target molecules through activation of Akt in the hypothalamus (Pi3K/Akt/mTOR pathway) (46). Both leptin and insulin activate hypothalamic mTOR (47, 48), a serine-threonine kinase with an essential role in brain development (49), which

is found in approximately 90% of NPY/AgRP neurons and 45% of POMC/CART in the arcuate nucleus (50). mTOR is known for acting as a metabolic energy sensor and can integrate the variations in the nutrient serum levels with the endocrine responses (51). Thus, in food deprivation (fasting) and with drastic drops in serum glucose and insulin levels, there is a decrease in the phosphorylation of the mTOR active form. On the other hand, increased serum levels of leptin (51) and insulin (52) leads to increased mTOR protein content and expression and reduced food intake in the fed state.

The study of Kocalis et al. (53) observed that the deletion of Rictor-mTOR complex (mTORC2) activation, specifically in POMC neurons, can induce hyperphagia and increase adiposity. Interestingly, the specific deletion in AgRP neurons did not affect energy balance, although it led to mild glucose intolerance. It is known that the p70S6k-mTOR kinase further leads to phosphorylation of AMP-dependent protein kinase α2 (AMPK α2) on serine 491, inhibiting its action and thus limiting the effects of leptin on food intake (52). Thus, in parallel with mTOR activation by food intake, the anorectic hormones leptin and insulin reduce the AMP-dependent protein kinase (AMPK) activity, specifically the AMPKα2 subunit (54, 55). Like mTOR, AMPK is also known as a metabolic energy sensor, being considered an essential protein in the complex system of intracellular energy regulation, which is based on the adenosine triphosphate (ATP)/adenosine diphosphate (ADP) ratio (56).

In conditions of depletion of energy reserves such as hypoglycemia and fasting, AMPK is activated in the hypothalamus (57, 58), leading to increased gene expression of NPY/AgRP in neurons and stimulating food intake (54). AMPK is inhibited in the hypothalamic arcuate nucleus in the fed state in response to increased leptin, insulin, and high levels of serum glucose (54), which consequently inhibits the autophagic pathway in NPY/AgRP neurons, leading to a reduction in food intake by inducing the feeling of satiety and, thus contributing to the eutrophic phenotype (44, 50, 59). Other molecules and pathways are also stimulated by anorectic hormones and contribute to energy homeostasis. Further details about the molecules involved in the signaling pathway of insulin and leptin actions in POMC and AgRP neurons in eutrophic conditions were described in the review article by Varela and Horvath (16).

Regarding the PI3K-mTOR-AMPK pathway, anorexigenic hormones increase the activity of the Pi3K and mTOR pathways, leading to the activation of POMC neurons (44, 45). Additionally, leptin and insulin reduce AMPKα2 activity in the hypothalamic region (15, 54). The p70S6k-mTOR kinase can inhibit the AMPK pathway in the hypothalamus, acting as a counter-regulatory protein (52). The mechanisms by which these molecules modulate the expression of hypothalamic neuropeptides are not fully understood; however, evidence suggests that the autophagic pathway plays a crucial role in this regulation (59–61). Furthermore, Claret et al. (55) showed that mice with genetic deletion of AMPKα2 in AgRP neurons were grown with the eutrophic phenotype. Interestingly, the specific genetic deletion in POMC neurons led to increased body fat and reduced caloric expenditure despite remaining sensitive to leptin. These results suggest that AMPK also plays a regulatory role in POMC neurons by unknown mechanisms. Therefore, evidence suggests that the hypothalamic autophagic pathway is crucial for activating orexigenic and anorectic neurons (59, 61, 62). **Figure 1** summarizes the data described so far.

Autophagic Pathway in Pro-opium Melanocortin Anorectic Neuron and Agouti-Related Peptide Neurons: A Pivotal Point in Energy Homeostasis

It is well known that the neuronal autophagic pathway is crucial for maintaining cellular homeostasis both under basal conditions and in response to stress signals (63, 64). Several studies indicate that the imbalance between activation and inhibition of the autophagic pathway in the central nervous system (CNS) is associated with dysregulation of body energy homeostasis and obesity induction and a greater predisposition to the development of various neurodegenerative diseases (61, 65, 66).

Classically, autophagy can be divided into microautophagy, chaperone-mediated autophagy, and macroautophagy, the latter being the most prevalent and commonly referred to as autophagy (67, 68). The autophagic process begins with capturing cytoplasmic organelles or macromolecules surrounded by a vesicular membrane lining called the autophagosome, which fuses with the lysosome to form autophagolysosome (or autolysosome), and lysosomal enzymes then degrade the sequestered material (69). The autophagosome formation begins with a pre-phagophore structure, which elongates and expands to form the phagophore, which, in turn, will mature in the membrane vesicle, surrounding the substrate that will be degraded (69).

The regulation of this entire autophagic process occurs by activating autophagic molecular complexes, starting with activation of the ULK1 (Unc-51 like autophagy activating kinase 1) complex, followed by the activation of phosphatidylinositol 3-kinase (PI3K), which forms a complex with Beclin 1 after dissociating from lymphoma B cell 2 (BCL-2) (70). Thus, the formed complex activates several proteins of the autophagic family (ATGs) that participate in phagophore elongation and activate the LC3-I protein (light chain 3 of protein 1 associated with microtubules), forming LC3-II. LC3-II is responsible for closing the phagophore and interacting with the p62 protein, which targets the material that the autolysosome will degrade (71, 72). **Figure 2** shows the schematic model of the autophagic pathway.

Evidence suggests that the hypothalamic autophagic pathway is crucial in activating orexigenic and anorectic neurons (59, 61, 62). AMPK is an activating molecule of the autophagic pathway, while mTOR leads to inhibition (73). AMPK can activate the autophagic pathway *in vivo* and *in vitro* by phosphorylating raptor-mTOR (mTORC1) (74) and starting the autophagic pathway initiator complex ULK-1 in AgRP neurons (59). Therefore, AMPK and mTOR directly interact to regulate the autophagic pathway through its complex initiator, ULK-1 (73).

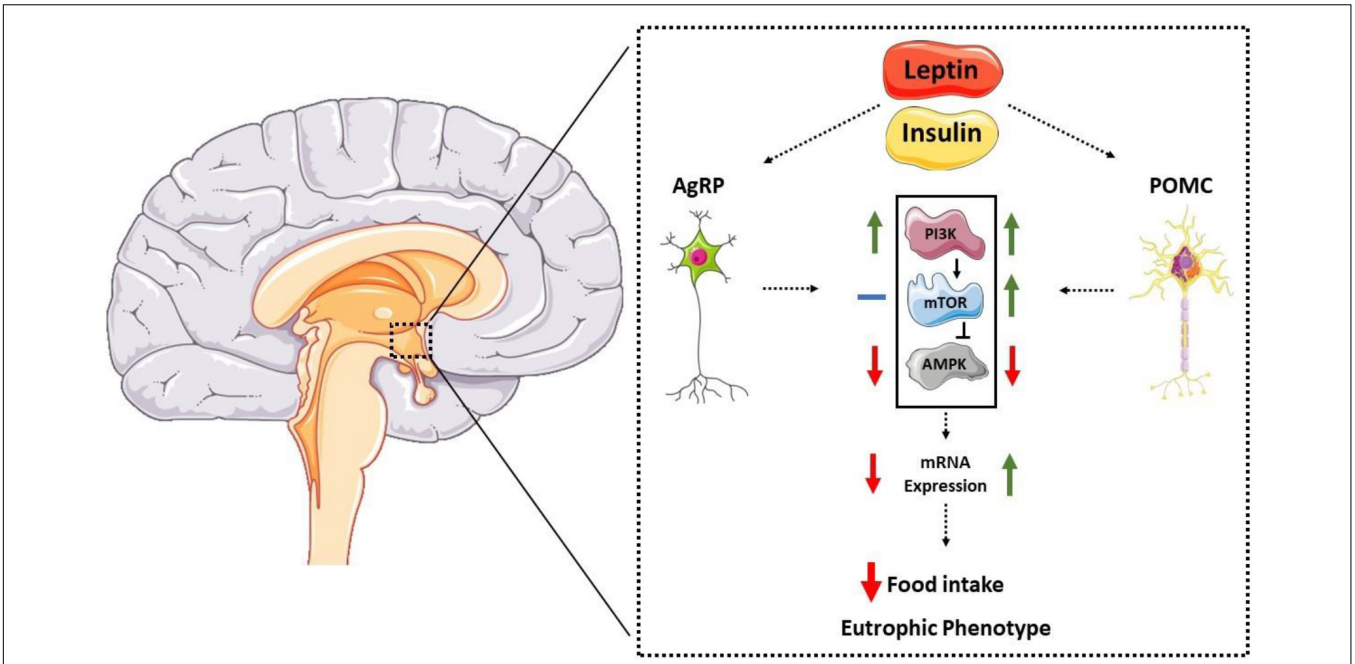

FIGURE 1 | Representative diagram of the interaction between anorectic hormones, molecular pathways, and neuropeptides POMC and AgRP. Anorexigenic hormones (leptin and insulin) act on the hypothalamic arcuate nucleus, reducing AMPK activation in AgRP neurons, thus reducing its expression. In POMC neurons, hormones increase mTOR activity and reduce AMPK, increasing its expression and reducing food intake and eutrophic phenotype.

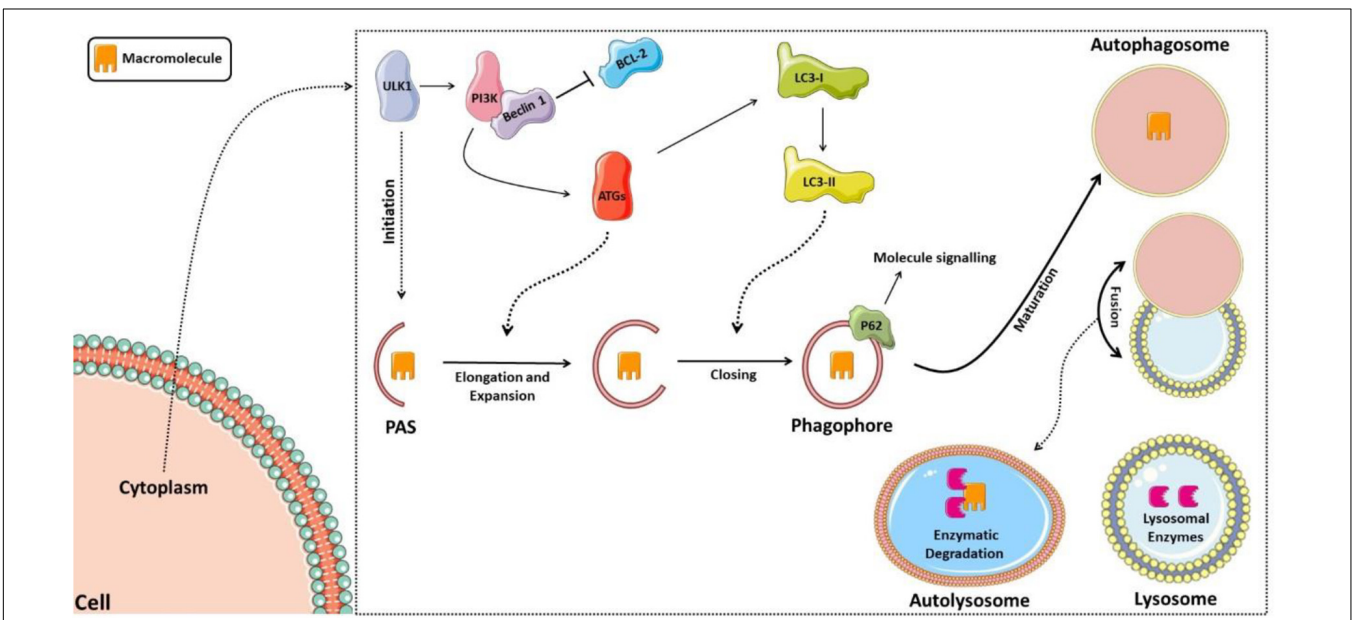

FIGURE 2 | Schematic model of the autophagic pathway. The process starts with activation of the ULK1 complex, then activation of phosphatidylinositol 3-kinase (PI3K), which forms a complex with Beclin 1 after it dissociates from lymphoma B cell 2 (BCL-2). Thus, the complex formed activates several proteins of the autophagic family (ATGs), which participate in the elongation of the phagophore and activation of the LC3-I protein (light chain 3 of protein 1 associated with microtubules), forming LC3-II, responsible for closing the phagophore and interacting with the p62 protein, signaling the material to be degraded. The phagophore matures into the autophagosome, which fuses with the lysosome forming the autolysosome, in which lysosomal enzymes will then degrade the sequestered material.

Specifically, in AgRP neurons, the deletion of Rictor (rapamycin-insensitive companion of TOR), a key molecule in the regulation of the MTORC2 complex, did not change the energy balance (53). However, the deletion of AMPK led to the eutrophic phenotype (55), suggesting that AMPK plays a more expressive role than mTOR in the energy regulation

pathways of this neuronal subgroup. An elegant study published by Kaushik et al. (61) showed that inhibiting the autophagic pathway, specifically in AgRP neurons, in both cells and mice, through the Agt7 gene deletion, significantly reduces food intake and adiposity. In POMC neurons, studies with knockout mice showed that the activity of mTORC1 and mTORC2 complexes are vital factors for the anorectic effects induced by leptin on the neuron and maintenance of the eutrophic phenotype (53, 75).

While the reduction in AMPK activity in AgRP (59) neurons and the increase in mTOR activity in POMC neurons (53, 75) lead to the eutrophic phenotype and considering that AMPK inactivation and mTOR elevation lead to inhibition of the autophagic pathway (73, 76), hypothetically, inhibition of the autophagic pathway in neurons may be associated with the eutrophic phenotype. However, a study showed that the selective deletion of autophagy-related protein 7 (Atg7) in mouse POMC neurons, interestingly, leads to a reduction in melanocyte-stimulating hormone (MSH) and is also associated with increased adiposity and food intake through mechanisms involving resistance to lipolysis (60). In addition, Meng and Cai (77) observed that the suppression of Atg7 in the mediobasal hypothalamus using site-specific lentiviral delivery of shRNA, without distinction of neuronal subgroups, was accompanied by an increase in hypothalamic inflammation, with activation of IKKB and, consequently, increased food intake and reduced energy expenditure (77).

Supporting these data, the AMPK deletion specifically in POMC neurons (55) and the RICTOR/mTORC2 deletion in the arcuate nucleus (53) are also associated with reduced caloric expenditure and obesity. Together, these data reveal that AMPK and mTOR are correlated with the autophagy pathway, which orchestrates a series of coordinated molecular phosphorylations in neuronal subgroups to provide adequate control of hypothalamic inflammation and energy homeostasis, reinforcing that the hypothalamic molecular pathway of obese individuals needs to be further investigated. **Table 1** presents the metabolic phenotypes found according to the deletion or inhibition of molecular pathways in the neuronal subgroups.

What Is Intermittent Fasting?

Intermittent fasting (IF) and caloric restriction are two distinct forms of dietary restriction associated with improving several metabolic parameters, including body weight control (78). Previous studies have shown that the obligation to maintain a daily calorie restriction reduces adherence to the caloric restriction protocols (79). Thus, the presence of *ad libitum* feeding windows in IF protocols emerged as an alternative protocol for dietary restriction interventions. The stress promoted by the low caloric intake is replaced by the metabolic stress induced by intermittent windows of prolonged fasting or alternate days of deficient caloric intake (78, 80). However, it is essential to highlight that both interventions must be carried out with professional supervision. Overfeeding episodes can occur after the fasting window, with the risk of developing eating disorders such as binge eating (81).

Furthermore, caloric restriction programs can also increase the predisposition to the development of psychological disorders (79). The review of Cerqueira et al. (82) pointed out that in animals fed with standardized diets balanced in macro and micronutrients, calorie restriction protocols with daily consumption of 40–60% of energy requirements are associated with micronutrient deficiencies. Deficiency of vitamin B12 and vitamin K, among others, depending on the diet consumed, is observed when the restriction protocols are chronically applied without supplementation with vitamins and minerals (79).

Despite its high popularity, there is no standardization of IF protocols (80). It is established that protocols do not impose water restrictions. All include periods of food restriction, which may refer to total deprivation from food consumption during some hours of the day (fasting window) or a full day containing no-energy food. Recently, some papers (80, 83) have been considering IF in three specific categories: (a) Complete Alternate Day Fasting (ADF) – consists of days with *ad libitum* feeding intercalated by whole days of food restriction, (b) Modified alternate-day fasting (MADF) or alternate-day modified fasting (ADMF) – with two non-consecutive days of total food restriction within the week, or two days of food intake of about 20% of the total caloric necessity with meals distributed throughout the day (c) Time-restricted feeding (TRF) – consisting of a protocol with a fasting window (usually 16 h) followed by a food intake window of approximately 8 h, with the meals distributed within this period, according to individual needs. The main point of the protocols is not to change the average weekly caloric intake but to change the frequency of food consumption (78, 80).

In this sense, the application of IF protocols generally does not change the average calorie intake due to post-fasting compensatory overfeeding. Thus, only a slight reduction in the average percentage of daily intake (84, 85) contributes to the protocol being considered an alternative strategy to improve weight loss and induce positive metabolic adaptations generated by energy stress (84, 85). However, it is essential to mention the warning that IF protocols are not recommended in cases of malnutrition, pregnancy, gastric ulcers, elite athletes, and patients at risk of hypoglycemia, among others (86–88).

Unlike the globally disseminated IF protocols for weight loss and improvement in health-related aspects (23, 24, 89), Ramadan fasting is a protocol with spiritual purpose practiced by Muslim followers of Islam (90). Once a year, according to the Islamic calendar, Muslims abstain from any food or drink, including water, during the period of daylight, having all their meals in the evening or just before sunrise (91). This practice extends for about 30 consecutive days once a year during the Islamic lunar month, which can occur in different seasons depending on the year (92) and the latitude of the geographic region. The fasting window can vary from 11:00 am to 6:00 pm (93). During this practice, most Muslims eat about two bulky meals within 24 h, one just after sunset and the other just before sunrise (93), resulting in a slight but significant reduction in the total calorie intake (94, 95) regarding loss or maintenance of body weight (96). **Table 2** illustrates the main

TABLE 1 | Molecular pathway deletion or inhibition in the neuronal subgroups and the outcomes.

Neuronal target	Deletion or inhibition of neuronal molecular pathways	Species	Outcome	References
AgRP	AMPK	Mice AMPKα2KO	Eutrophic phenotype, light level of glucose intolerance	(55)
	mTOR	Mice lacking Rictor in AgRP	mTORC2 did not change energy homeostasis	(53)
	Autophagic pathway	Atg7F/F-AgRP-Cre mice	Better food intake control and eutrophic phenotype	(61)
POMC	AMPK	Mice AMPK α2KO	Hyperphagia, obesity, hyperglycemia	(55)
	mTOR	Mice lacking Rictor in POMC and C57BL/6JPOMC-rptor-KO	Rictor/mTORC2: decreased energy expenditure and induced obese phenotype, did not induce leptin resistance mTORC1: limited ROS capacity to inhibit food intake	(53, 75)
	Autophagic pathway	$Atg7^{F/F}$-POMC-Cre mice	Limited lipolysis capacity and obese phenotype	(60)
NPY/AgRP and POMC simultaneously	AMPK	*In vitro* and *in vivo* (male C57BL/6)	Dysregulation of autophagic pathway and reduction in body weight	(59)
	mTOR	Mice lacking Rictor in all neurons	Increased adiposity, glucose intolerance, leptin resistance	(53)
	Autophagic pathway	Mediobasal hypothalamus Atg7 K_D mice	Hyperphagia, reduced energy expenditure, and hypothalamic inflammation	(77)

AMP-dependent protein kinase (AMPK), Rapamycin target protein (mTOR), Reactive oxygen species (ROS).

differences between intermittent fasting, caloric restriction, and Ramadan fasting.

Intermittent Fasting as a Possible Adjuvant in the Treatment of Obesity: Modulations in Neuroinflammatory, and Leptin and Insulin Pathways

Obesity is a multifactorial disease usually associated with hyperphagia, hyperinsulinemia, and hyperleptinemia. The high levels of leptin and insulin in the cerebrospinal fluid of obese individuals indicate a chronic state of resistance to the actions of these hormones in the CNS (19, 99). It is essential to highlight the diet quality profile as a significant possible factor in the pathophysiology of obesity. Increased exposure to a high-fat diet (HFD) is associated with a reduction in hypothalamic mTORC1 and leptin resistance (100). There is evidence that an acute lipid infusion for 24 h or exposure to a HFD over 8 – 20 weeks induces markers of inflammation in the hypothalamic NPY/AgRP neurons, which may contribute to a significant alteration in NPY/AgRP expression or content (101) and also, 6 days of exposure to a high-fat diet can induce leptin resistance in mice with a predisposition to obesity (102).

Several studies indicate that the practice of IF for periods longer than 1 month can improve insulin resistance and reduce its serum levels, contributing to the regulation of glucose metabolism (26, 103–105). A recent meta-analysis evaluated 545 participants, most overweight or obese, and observed that IF protocols are associated with reducing the body mass index (BMI) and leptin serum levels, lowering fasting blood glucose, and improving insulin resistance. These results suggest that IF may contribute to prevention/improvement in the resistance of the anorectic hormone observed in obese individuals (106).

Although not fully elucidated, the mechanisms by which IF acts in the insulin signaling pathway are probably different from those observed in caloric restriction protocols since benefits associated with IF can be observed even when there is no reduction in calorie intake and weight loss (84, 103, 107, 108). In addition, there is some evidence that IF protocols may produce more significant beneficial effects on glucose regulation and fasting insulin (103, 108).

It is known that obesity is associated with the chronic low-grade inflammatory process, not only peripheral but also central, highlighted by increased expression of several inflammatory proteins related to impairments in the hypothalamic signaling of leptin and insulin, such as the suppressor of insulin signaling cytokine 3 (SOCS3) (103, 109, 110). Despite SOCS3 being part of a negative feedback system related to this signaling cascade, when it reaches a high concentration induced in an inflammatory scenario, SOCS3 significantly impairs the anorexic leptin cascade. This cytokine can bind to an intracellular region of LepR, attenuating the ability of JAK2 to autophosphorylate and recruit the STAT3 pathway (111). In addition, the C-terminal portion of SOCS can recruit the ubiquitin transferase system, promoting the degradation of JAK receptor complexes (112). Thus, SOCS3 impairs the reduction in the activity of the AMPK protein threonine 172 by leptin (113), stimulating autophagic activity in AgRP neurons and appetite (59). It is also known that SOCS3 can impair the insulin signaling pathway by binding directly to the insulin receptor (114) and/or degrading both substrates of insulin receptors 1 and 2 (IRS1/2) (115). The study of Mori et al. (109) observed that hypothalamic suppression of SOCS3 could prevent central insulin resistance generated by the chronic high-fat diet.

In this sense, although several studies show that IF protocols can reduce plasma levels of pro-inflammatory proteins in obese or overweight individuals (27, 116), few studies have assessed the adaptation of inflammatory proteins in the hypothalamic region. Spezani et al. (27) evaluated the effects of a 24-h fasting protocol interspersed with days of *ad libitum* high-fructose diet in mice with induced obesity (eight-week protocol with a high-fructose

TABLE 2 | Main differences between intermittent fasting, caloric restriction, and Ramadan fasting.

	Intermittent Fasting protocols	Caloric restriction	Ramadan fasting
Caloric intake	Considering the food consumption throughout the week, there is a slight caloric intake restriction (97)	TRF: intake of 40% to 60% of the total energy expenditure, or daily restriction of 500 kcal to 1000 kcal (82) ADF: includes days containing absolute fasting of food (83). MADF: includes 2 days a week with no-energy intake or days with severe restriction of food intake (less than 25% of daily necessity) (80)	Slight caloric intake restriction (300 kcal) (95)
Meals daily distribution	2 to 7 food restriction windows weekly. Usually composed by 16 h-fasting or 2 days of the week with a caloric intake lower than 20% of the TCI (78)	Daily caloric restriction with a variable number of meals (TRF, MADF) (82) or days without any meals (ADF, ADF)	Fasting during daylight period (from 11 AM to 6 PM). Generally two meals a day, one after sunset and one before sunrise (93)
Related risks	Binge eating and hypoglycemia (81)	Vitamin and mineral deficiency (82)	Risk of dehydration and accidents at work (98)
Liquid intake	No restriction (84)	No restriction (82)	Restricted, including water restriction. Liquid intake is allowed only at night (90)

diet). After 4 weeks of intervention, a reduction in the expression of hypothalamic SOCS3 was observed. However, animals fed a standard diet and submitted to an IF protocol showed an increase in SOCS3 compared to control animals with a standard diet without applying the IF protocol (27). Controversially, the study of Zangh et al. (117) did not observe changes in the expression of hypothalamic SOCS3 or alteration in plasma insulin in female mice fed with a standard diet and submitted to chronic IF protocols for 24 h performed only one to two times a week, during a period of 13 or 42 days.

It is also known that the increase in tumor necrosis factor-alpha (TNFα) attenuates the anorectic effect of leptin and increases the expression of SOCS3 in the hypothalamus (118). Despite studies showing that caloric restriction (116) and Ramadan fasting (119) can reduce plasma TNFα levels, particularly in obese or overweight individuals, a recently published meta-analysis (28) evaluated serum levels of inflammatory markers in response to different IF or caloric restriction protocols. After applying the exclusion criteria, the meta-analysis included only one study with obese individuals and IF (alternating every 24-h between consuming 25% or 125% of energy needs), which did not reduce TNFα levels (120). However, it is crucial to consider the lack of papers published in this area.

Regarding animal studies, Spezani et al. (27) evaluated the effects of IF for 24 h. The authors observed that obese mice submitted to fasting curiously showed a greater expression of hypothalamic TNFα when compared to the control group. Therefore, the data are still contradictory, and further studies are needed to assess the content and expression of TNFα, specifically in the hypothalamus of obese animals submitted to different IF protocols.

Another relevant inflammatory pathway involved in the etiology of obesity is the IKKb/NF-kb pathway (110). Zhang et al. (110) showed that mice submitted to a high-fat diet developed obesity accompanied by increased concentrations of IKKB, which can activate the nuclear factor kb (Nf-kb), leading to endoplasmic reticulum stress in the hypothalamus and consequent resistance to leptin and insulin (110). IKKB phosphorylation in the mid-basal portion of the hypothalamus can impair the action of insulin by inducing tyrosine phosphorylation and the consequent inactivation of the insulin receptor (IR). In addition, it can limit the activity of its target proteins: phosphatidylinositol-3-kinase (Pi3K) and protein kinase B (Akt) (110, 121), which are involved in the control of the hypothalamic autophagic pathway (70). In a complementary way, the increase in IKKB contributes to the elevation of the expression of hypothalamic SOCS3 (110), impairing the central signaling of leptin and insulin (41, 122). However, although IKKB is a protein widely studied in obesity models, no investigations have evaluated IKKB in the hypothalamic region in response to IF protocols.

The IKKb/NF-kb inflammatory cascade can also be activated by lipopolysaccharides (LPS) when bound to their Toll 4 membrane receptor (TLR-4) (123, 124). Previous studies have shown that obese individuals present increased levels of LPS in the bloodstream, causing a condition called metabolic endotoxemia, which is associated with systemic inflammation and an increased risk of developing chronic diseases (125), favoring the development/worsening of obesity (125, 126). Additionally, prolonged treatment with LPS seems to increase JNK and limit the hypophagic effects in response to central insulin administration, regardless of the increase in body weight (127). Although not fully understood, the increase in LPS plasmatic levels is probably due to intestinal dysbiosis and changes in the permeability of the intestinal wall (128, 129).

Dietary factors seem to modulate endotoxemia, and the use of prebiotics could contribute to attenuating its progression (31), while chronic exposure to a high-fat diet intake could worsen progression (125). In this sense, a recent review article proposed that IF protocols can also be used as a nutritional strategy, affecting the brain-microbiota axis of obese individuals (31). An elegant study demonstrated that the removal of the intestinal

microbiota with the use of antibiotics reduced the protective effects of IF on the cognitive function of the evaluated mice, with the subsequent administration of microbiota metabolites, such as short-chain fatty acids and 3-acid propionic indole, which were able to improve cognitive function and insulin sensitivity (30). Together, we hypothesized that IF might play a supporting role in attenuating inflammation in the CNS through actions on the microbiota-brain axis. However, this hypothesis needs to be evaluated.

Additionally, it is known that during acute fasting periods, there is an increase in β-hydroxybutyrate (βHB) production (130), leading to increased phosphorylation of IRS1 and Akt in their active forms, a reduction in serum insulin levels, and a better response to the intraperitoneal insulin tolerance test (131), as well as being able to modify hypothalamic leptin and insulin signaling pathways in type 2 diabetic rats (132). It is essential to highlight that βHB is also involved in inflammatory control (133). The oral administration in Crohn's disease patients exerts an anti-inflammatory response through downregulation of NF-kb (134). Cerniuc et al. (135), evaluating an IF protocol (2 non-consecutive days of total fasting per week) in healthy women, also identified a significant increase in blood βHB levels. To date, despite not directly evaluating insulin response associated with βHB production and hypothalamic responses in IF protocols, data suggest that the increase in butyrate levels may also contribute to improving insulin sensitivity in response to IF.

Although the relationship of neuroinflammation with insulin and leptin signaling in response to IF protocols need to be further explored, data suggest that: (1) the mechanisms of action of IF seem to be different from those observed in calorie restriction protocols (103, 108); (2) IF appears to be able to improve insulin and leptin sensitivity (105, 106); (3) IF seems to be able to modulate inflammatory pathways in the brain (27, 29) and attenuate the levels of LPSs in the plasma (29), which we hypothesize could be associated with an improvement in hormonal and neuronal sensitivity; and (4) the increase in βHB production in IF (135) may also contribute to better insulin sensitivity considering the relationship of βHB and the insulin pathway (131, 132).

The Influence of the Circadian Cycle on the Modulation of Leptin and Insulin Pathways in Different Intermittent Fasting Protocols

Although several studies show that IF protocols are capable of improving insulin and leptin sensitivity, it is essential to emphasize that the time when the fasting window and the eating window are performed significantly interferes with metabolic responses and autophagic stimulation due to their influence on the hormonal rhythm guided by the circadian cycle (33, 136). However, the habit of skipping breakfast is associated with greater consumption of food at night (breakfast skipping and late-night eating pattern), increasing the risk of developing insulin resistance and cardiometabolic risk (33, 137, 138).

Jamshed and coworkers (33) submitted overweight or obese individuals to IF protocols. The authors evaluated the differences between the protocol carried out with the food window from 8 AM to 2 PM (Early Time-Restricted Food – eTRE) with a second protocol containing the last meal at 8 PM, both on a controlled diet. After four consecutive days of intervention, the authors observed increased BMAL1 expression in the morning, activation of Akt2, reduced fasting plasma insulin, and glucose concentrations in the eTRE group compared to the group that had the last meal at 8 PM (33). These results corroborate another study carried out with humans by the same research group that observed that the eTRE group improved insulin sensitivity, assessed by the glucose tolerance test, compared to the group fed at night (107).

The IF protocol performed without respecting the circadian cycle can induce a dysregulation in the expression of the circadian cycle leading to a significant increase in the natural peak of mRNA expression of genes involved in glucose regulation (i.e., *Gck, Slc2a2*, and *Pdk4*) and also lead to a higher plasma leptin levels when compared to an IF protocol applied to respect the circadian cycle (with a distributed feeding window in the active period of mice) (32). On the order hand, an IF protocol respecting the circadian cycle seems to reverse the obese and hyperphagic phenotype of heterozygous knockout mice of brain-derived neurotrophic factor (BDNF) and re-established insulin sensitivity and brain BDNF levels after 3 weeks of intervention (139). Thus, due to its influence on the circadian cycle, the effects of IF on endocrine responses and body weight may vary according to the time of day in which each food and fasting window is held. It seems better for healthy improvements not to skip breakfast and start the fasting window close to sunset to improve sensitivity to anorectic hormones and help prevent obesity (137, 140, 141).

Therefore, it is essential to point out that these data warn us regarding popular IF models disseminated in social media that encourage avoiding breakfast and starting the eating window at lunch. It is essential to reinforce the importance of a scientific basis to achieve better dietary prescriptions at the individual and population levels. This topic was deeply explored in the recent review published by Moon et al. (142).

Intermittent Fasting and the Autophagic Pathway

Autophagy is an essential mediator of physiological responses associated with the generation of ROS and cellular protein damage (143), being directly involved in maintaining energy homeostasis through the increased expression of neuropeptides (144) and in the control of the neuronal inflammatory response (77). Energy stress and IF-induced oxidative stress can activate the autophagic pathway (145) through the increase in sirtuins (SIRTs) (146, 147), associated with increased phosphorylation of AMPK in threonine (148, 149), correlated with phosphorylation of the ULK1 protein and autophagic pathway (150). Therefore, the application of IF protocols is a non-pharmacological alternative capable of activating the autophagic pathway (33, 34).

During prolonged fasting, lower glycemic values and changes in the adenosine monophosphate/adenosine triphosphate (AMP/ATP) ratio induce SIRTs activation in tissues such as the

kidney, skeletal muscle, and blood samples from overweight individuals (145, 147, 151). Sirtuins are known to induce the autophagic pathway through phosphorylation of AMPK, FOXO1, or deacetylation of autophagic family proteins (145, 149). A study evaluating the IF and autophagic pathway in adults observed an increase in serum levels of SIRT1 and the autophagosomal membrane component LC3A, thus suggesting autophagic stimulation, accompanied by improved insulin sensitivity (33).

AMP-dependent protein kinase is an essential protein associated with the neuronal autophagic pathway and energy homeostasis (59). An interesting study by Kaushik et al. (61) observed that acute fasting could increase the content of free fatty acids in the hypothalamus, with consequent phosphorylation and activation of AMPK and ULK1, increasing autophagic flow in AgRP neurons with hunger induction. Furthermore, the authors observed that impairment of the autophagic pathway in cultures of hypothalamic cells through the deletion of the protein related to autophagy 7 led to a reduction in AgRP levels, food intake, and adiposity (61). The increased availability of fatty acids can induce the hypothalamic autophagic pathway and increase NPY expression (144).

Additionally, the acute fasting protocol can also lead to phosphorylation of mTOR and its target protein, ribosomal protein kinase S6 (S6K) at serines 240 and 244 in the hypothalamus, thus inactivating the mTOR/S6k pathway (50). During fasting, the increase in AMPK associated with the reduction in mTOR contributes to the regulation of food intake (50, 52) and activation of ULK1 and the autophagic pathway (150). These data corroborate the work of Chaix et al. (152), who evaluated IF protocols in obese mice, and observed an increase in the levels of the homolog of ATG8, Gabarap1, a key regulator of autophagic flow during fasting. The animals showed significant weight loss compared to the control group, although food intake did not show any significant difference.

Although several studies have shown the effects of caloric restriction programs or fasting periods on the autophagic pathway, it is essential to emphasize that, to our knowledge, few studies have assessed the impact of IF protocols on the autophagic pathway. To date, we understand that fasting periods in general lead to increased availability of free fatty acids in the hypothalamic region, reduced levels of glucose and serum amino acids, leading to activation of sirtuins (33) and the AMPK pathway (148), and inactivation of the mTOR pathway, thus stimulating the autophagic complex (50, 73). There are no studies evaluating the autophagic pathway in the hypothalamic nucleus in response to chronic IF protocols.

Intermittent Fasting, Pro-opium Melanocortin Anorectic Neuron, and Agouti-Related Peptide Neuropeptides

The mechanisms by which IF alters the expression of hypothalamic neuropeptides are not fully understood; however, it is known that IF can improve sensitivity to leptin and insulin (106) and stimulate the autophagic pathway (33, 145), which is related to the activation of hypothalamic neuropeptides and energy homeostasis (27, 55, 59, 60). Another relevant factor is the increase in reactive oxygen species induced by the IF protocol (146). ROS in the hypothalamus is also a factor that leads to the electrical activation of neuropeptides POMC and inactivation of Npy/AgRP (153).

The suppression of reactive oxygen species decreases the activation of POMC cells and increases the activity of NPY/AgRP neuropeptides (153). During fasting, the mechanisms of oxidative protection performed in the mitochondria protect the exacerbated increase in ROS in AgRP neurons. Uncoupling protein 2 (UCP2) is a protein abundantly expressed in arcuate nucleus neurons associated with energy homeostasis and involved in controlling oxidative stress in mitochondria (154, 155). A study shows that the function of UCP2 *via* AMPK seems to be a key point for the electrical activation of NPY/AgRP neurons during a fasting period (156). In contrast, ROS levels are low during fasting in POMC neurons, and the transient increase in ROS favors satiety and the action of leptin *via* mTOR (75, 157). However, these data were evaluated using acute fasting protocols. There is still no evidence about the content of ROS in neuronal groups and its impact on energy homeostasis in response to IF protocols.

Low serum leptin values during fasting contribute to an increase in the expression of AgRP and NPY (156, 158, 159), stimulating the autophagic pathway and consequently inhibiting activation of the hypothalamic-pituitary-thyroid axis to reduce caloric expenditure and save energy (160). However, contrary to the results observed after a single fasting period window, the study of Chausse et al. (161) submitted eutrophic rats to a 24-h IF protocol and observed that rats that underwent IF for 3 weeks, even with an excellent response to leptin, curiously showed increased expression of the orexigenic neuropeptide AgRP both during fasting periods and on feeding days. Increased energy expenditure, reduced energy efficiency factor, and lower weight gain were also observed when compared to the control group. These findings suggest that AgRP neuron responses to the IF protocol may differ from those observed after a single fasting window, and further studies are needed.

It seems that during the IF protocol, the response of neuropeptides can change. A study with eutrophic mice evaluating the response to IF protocols after 13 days and again after 42 days found that the first intervention increased food intake in the feeding windows accompanied by an increase in NPY mRNA expression. However, interestingly, after 42 days, there was a reduction in NPY mRNA expression that returned to baseline values. There was no difference compared to the control group (without IF intervention), thus suggesting a new late hypothalamic adaptation of NPY in response to the chronic application of the IF protocol (117), which may be related to the popularly described hunger adaptation. Regarding POMC neuropeptides, there were no significant changes.

Additionally, the time that the fasting window is performed can also influence the expression of neuropeptides. Animals exposed to a high-fat diet for 2 weeks and then submitted to the IF protocol of 16 h for 1 week, with the feeding period performed during the rest period, disregarding the circadian cycle, showed increased hypothalamic expression of the orexigenic genes NPY

and AgRP. Higher food intake and higher serum levels of leptin were also observed, thus suggesting possible resistance to leptin compared to animals that followed the IF protocol with the feeding window performed in the active period (referring to the night period for mice). Therefore, it can be concluded that when the IF is carried out with the food period during the rest period, disregarding the physiological circadian cycle, there is a possibility that the protocol can trigger dysregulation of the neuroendocrine mechanisms of hunger control, which may harm health (32).

Few studies have assessed the effects of IF and neuropeptide expression in obese individuals. Gotthardt et al. (35) studied

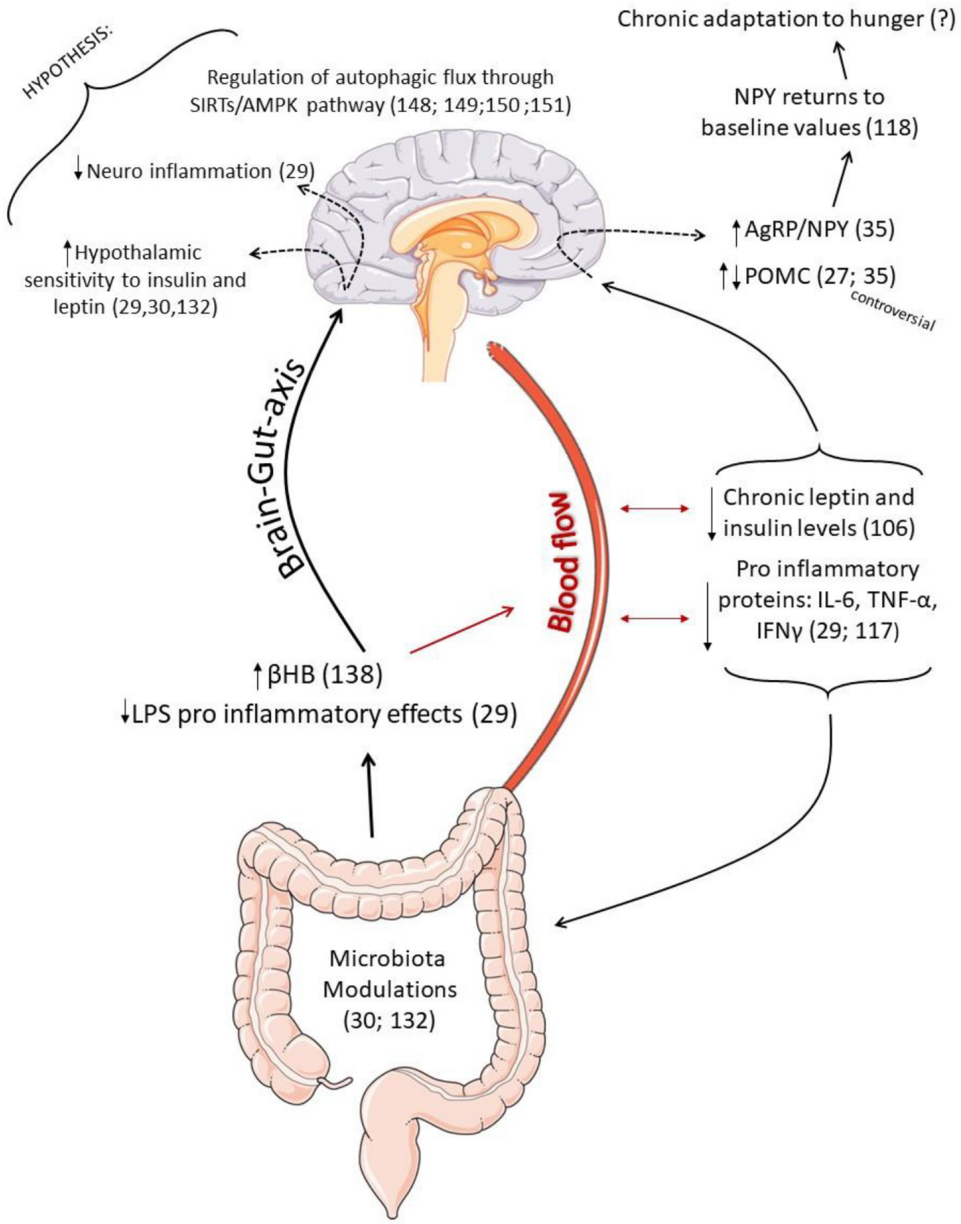

FIGURE 3 | Schematic representation of the possible effects of intermittent fasting (IF) as an adjuvant treatment to partially rescue hypothalamic responses in obesity. Once IF prevents the pro-inflammatory effects in the hippocampus caused by LPS (29), and it is also able to increase the production of β-Hydroxybutyrate (βHB) (136), correlated to inflammatory control (134) and insulin sensitivity, it is possible to hypothesize that modulations in the microbiota may be helpful to reduce hypothalamic inflammation and increase hormonal sensitivity. Low serum leptin and insulin values during fasting contribute to an increase in the expression of AgRP and NPY. However, interestingly, the NPY mRNA expression returned to baseline values as a chronic response to IF, which may be is an adaptation to hunger. Furthermore, some studies found an increase in the expression of POMC neuropeptide, which is controversial. Such neuronal sensitivity to the IF protocol is perhaps also associated with better regulation of hypothalamic autophagic response since IF can activate the autophagic pathway in other tissues (146), and autophagic flux is an essential mediator of neuropeptide responses.

obese mice submitted to an IF protocol where the mice were food deprived every other 24-h period beginning at 9:00 AM (fasting day), 2 h into the light cycle, for 4 weeks. The results showed increased expression of mRNA of hypothalamic NPY and increased energy expenditure compared to the control group that consumed a high-fat diet *ad libitum*. Regarding the expression of POMC neurons, the group that performed the IF showed a significant reduction in the expression of the POMC neuropeptide when compared to the control group with an *ad libitum* high-fat diet, which was also accompanied by a decrease in serum levels of leptin, improvement in insulin sensitivity, and weight loss.

Interestingly, a study evaluating the effects of IF in animals fed with a standard diet observed that after 4 weeks of application of the IF protocol (24 h fed, 24 h fasting), the group with standard diet and fasting presented reduced expression of POMC when compared to its respective control (standard diet without fasting) (27). However, the authors also looked at the effects of IF on two other types of diet: obese animals fed a high-fat diet and obese animals fed a high fructose diet. After 4 weeks of applying the IF protocol (24 h fed, 24 h fasting), the fasted obese animals showed increased expression of POMC in both protocols (27).

Therefore, we suggest that the content of neuropeptides in IF seems to occur differently from that observed in caloric restriction protocols, and the adaptations of the CNS seem to differ according to (1) the duration of time that the IF protocol is being applied, with the orexigenic neuropeptide NPY being able to return to baseline values as a late adaptation (117); (2) it is essential that the distribution of feeding and fasting periods respects the circadian cycle to avoid possible health risks (32); (3) concerning POMC neuropeptides, the IF protocol interestingly seems to reduce the expression of POMC neurons in some models (27, 35), but the results are still contradictory. Therefore, further studies are necessary to elucidate the effects of IF on hypothalamic responses and energy homeostasis. In addition, it is necessary to investigate long-term changes. **Figure 3** summarizes the possible effects of IF as an adjuvant treatment to partially rescue hypothalamic responses in obesity.

CONCLUSION AND FUTURE PERSPECTIVES

Evidence indicates that IF protocols can be used as a strategy to promote weight loss, as they induce an increase in energy expenditure (35, 161) and improve the peripheral response to anorectic hormones (33, 162), which can significantly interfere with the hypothalamic autophagic pathway (33) and also in the expression of neuropeptides (27, 35). Thus, the literature reviewed allows us to hypothesize that IF could help reestablish, at least in part, the control of hypothalamic molecular responses in obese individuals, alleviating neuroinflammation and improving hypothalamic sensitivity anorectic hormones, thus helping to enhance reestablishment of energy homeostasis. However, when the IF protocol is performed without considering the circadian cycle, it can impair energy metabolism regulation (32). These associations require more research, mainly when obese individuals submitted to long periods of IF are evaluated regarding the responses of the autophagic pathway and hypothalamic neuropeptides. In conclusion, considering the favorable results of IF in obesity, the protocol may be an adjuvant treatment to partially rescue hypothalamic responses in obesity.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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Breast Milk and the Importance of Chrononutrition

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During pregnancy the human fetus receives timed cues from the circadian rhythms of temperature, metabolites, and hormones from the mother. This influence is interrupted after parturition, the infant does not secrete melatonin and their circadian rhythms are still immature. However, evolution provided the solution to this problem. The newborn can continue receiving the mother's timed cues through breastmilk. Colostrum, transitional, and mature human milk are extraordinary complex biofluids that besides nutrients, contain an array of other non-nutritive components. Upon birth the first milk, colostrum, is rich in bioactive, immunological factors, and in complex oligosaccharides which help the proper establishment of the microbiome in the gut, which is crucial for the infants' health. Hormones, such as glucocorticoids and melatonin, transfer from the mother's plasma to milk, and then the infant is exposed to circadian cues from their mother. Also, milk components of fat, proteins, amino acids, and endogenous cannabinoids, among others, have a markedly different concentration between day and night. In the present review, we give an overview of nutritive and non-nutritive components and their daily rhythms in human milk and explore their physiological importance for the infant. Finally, we highlight some interventions with a circadian approach that emphasize the importance of circadian rhythms in the newborn for their survival, proper growth, and development. It is estimated that ~600,000 deaths/year are due to suboptimal breastfeeding. It is advisable to increase the rate of exclusive breastfeeding, during the day and night, as was established by the evolution of our species.

Keywords: chrononutrition, melatonin, glucocorticoids, circadian feeding, tryptophan, cannabinoids, oligosaccharides, secretory IgA

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INTRODUCTION

Being active and eating during typical times of rest, as with night-shift work, disrupts circadian clocks and is related to a higher risk of several metabolic disorders (1). Recent studies have also found that restricting eating to certain times of day can be beneficial to health (2), and from these observations the field of chrononutrition has developed (3). The goal of chrononutrition is to adjust nutrition quality and intake to coordinate with an individual's biological clock, so that one consumes the optimal type and quantity of food at the correspondingly optimal time of day (3). Most of the current understanding of chrononutrition arose from work in adults and thus does not encompass the lifespan of developing humans. Rest and activity patterns and nutritional needs

change as humans develop. Unlike adults, newborns ingest milk during the day and night, and this has an important biological significance: the concentration of milk components changes according to the circadian rhythms of the mother. Moreover, the infant is sensitive to milk and environmental circadian cues. In the present contribution, we review the current evidence about these two topics related to rhythms in breastmilk and environmental conditions and discuss their relevance for the proper establishment of the infant's circadian rhythms. Some studies label the temporal changes of milk components as circadian but, in most cases, they describe only different concentrations between specific hours of the day and night; in the present review, we will refer to them as daily rhythms. Our main emphasis will be on human studies.

In the womb, the fetus is exposed for approximately 9 months to the circadian, physiological, metabolic, and behavioral rhythms of the mother. This circadian milieu is abruptly interrupted upon birth, but nature developed the perfect substitution: maternal milk whose composition changes according to the circadian rhythms of the mother (4–7). Not surprisingly, in humans the newborn ingests milk during the day and the night, and the nutritive and non-nutritive components change accordingly. The world health organization recommends exclusive breastfeeding for at least the first 6 months of age to improve child survival, healthy growth, and development but unfortunately, breastfeeding rates in the world are low (8).

NUTRIENTS IN HUMAN MILK THROUGH LACTATION

Human milk is an extremely complex biofluid with dynamic composition, beginning with the first milk, colostrum, through transitional and mature milk; it changes significantly depending on the maternal diet, environmental factors, and whether milk is produced for preterm or term infants (9–11). Mature human milk composition contains 88% water, 7% carbohydrates, 4% fat, and 1% protein (9). Protein and total amino acid concentration is highest in colostrum 14–16 g/L, then steadily decreases to 7–8 g/L in mature milk (12). Caseins and whey proteins are the main proteins in milk, but proteomic analysis has identified ∼1,700 different proteins (13). Fat concentration has an increasing trend from 26 g/L in colostrum to 37 g/L in mature milk (11). Carbohydrates are the most abundant macronutrient, in which lactose concentration is very stable during lactation with a mean concentration of 61.4 g/L (4), with a lower concentration in colostrum (56 g/L) than in mature milk in which it reaches its highest concentration (68 g/L)to support the increasing growth of the infant [**Figure 1**; (16)]. Moreover, milk also has vitamins, enzymes and coenzymatic factors, hormones, and immunological factors [(14); **Figure 1**].

NON-NUTRITIVE COMPONENTS AND IMMUNOLOGICAL FACTORS

The immune system of the newborn is immature and human milk is rich in immunological factors. It contains immunoglobulins, immune cells, complement proteins, and immunomodulatory and antimicrobial factors (17–19). Immunoglobulin A (IgA), Immunoglobulin G (IgG) and Immunoglobulin M (IgM) are present in colostrum, but IgA is different from that found in plasma. It is a dimer with a joining J chain protein and a secretory component named secretory IgA (SIgA), which increases its resistance to proteolytic degradation in the intestine (20). Consistent with this finding, significant quantities of SigA are found in the infant stool, which decreases in parallel with that in milk (21). Levels of sIgA are much larger in colostrum, in comparison with IgG and IgM. Their levels decline from 5.4 g/L in colostrum to 1.3 g/L in mature milk [(17); **Figure 1**]. Lactoferrin, an iron-binding glycoprotein with bacteriostatic and bactericidal activity against *Escherichia coli*, Candida *albicans,* and other pathogens, such as viruses (22), is higher in colostrum (5 g/L) than in mature milk [1.4g/L; (17); **Figure 1**]. In addition, human breast milk contains pluripotent stem cells and leucocytes (23–26), plus inflammatory and anti-inflammatory cytokines (27). Polyunsaturated fatty acids, such as docosahexaenoic (DHA) and arachidonic (ARA) acids, have immunomodulatory properties and participate in several developmental and cognitive processes (28). Contrary to the common belief that human milk is sterile, it has been established that in mature milk, the infant consumes ∼8 X 10^4-10^6 commensal bacteria, fungi, and viruses that colonize the infant gut to form a microbiome that allows proper development of the immune system (9, 29). Also, breastmilk contains ∼1,400 species of mature microRNAs that are absorbed by the intestinal epithelial cells and are involved in immunomodulatory and epigenetic regulation (30, 31). Milk also contains a complex array of approximately 200 oligosaccharides, which have important immunological properties and are essential to the development of a "healthy" microbiome. Their concentration is highest in colostrum (20–25 g/L) and decrease through lactation to 5–20 g/L (14, 32). Cannabinoids are among the several neurochemicals found in milk; these include anandamide (AEA) and 2-arachidonoyl glycerol (2-AG), the latter in much larger concentration [(33); **Figure 1**]. Thus, milk is a complex biofluid that contains myriad nutritive and non-nutritive components. Human milk has an addition allevel of complexity: it reflects circadian rhythms of the mother.

DIURNAL CHANGES IN MILK COMPONENTS

In human milk, there are circadian hormonal variations, including glucocorticoids (GLUC), melatonin, (MEL), Leptin, Ghrelin, and others. Plasma GLUC increases during the last phase of the night, reaches a peak during the morning, and then decreases (34). In contrast, MEL increases during the night and it is almost negligible in plasma during the day (35). GLUC and MEL may communicate "time of day and night" to the body; they are associated with alertness and sleep phases. GLUC and MEL are transferred to breast milk, and their concentration mirrors that in plasma. GLUC concentration in human milk is higher in the morning. MEL levels are low in the evening

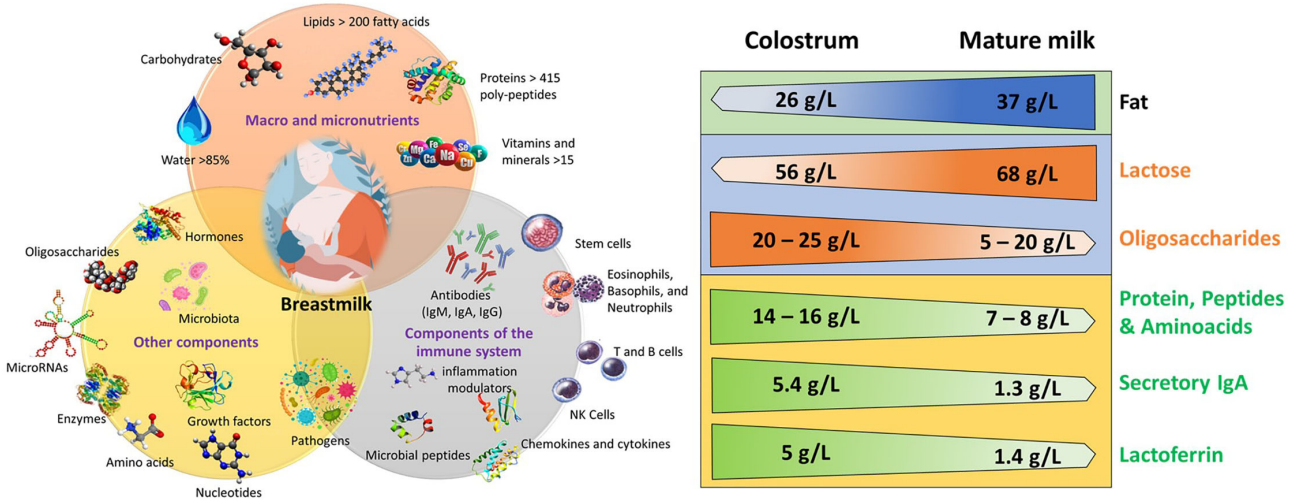

FIGURE 1 | Components of human milk and differences between colostrum and mature milk. **(A)** Macro and micronutrients, components of the immune system, and other components. Modified from (7, 9, 10, 14, 15). **(B)** Concentration of some components in colostrum and in mature milk. Modified from (4, 11–14, 16).

and the first part of the night, then increase again (5, 36). At 3–4 days after parturition MEL is below the limit of detection between 1,400 and 1,700 h, but during the night between 0200 and 0400 it reaches a concentration of 99 ± 26 pmol/L (6, 35). Concentration in human milk of another hormone, Leptin, is significantly higher during 10 pm and 4 am compared to 10 am−10 pm (37). Lipids increase in the morning, reach a peak from midday to evening, and reach lower values at night (38–40). Ghrelinisalso present in human milk as well as insulin, adiponectin, obestatin, and other metabolic hormones (15, 41). No diurnal differences have been found among these latter hormones or in total proteins, carbohydrates, and lactose, or there are controversies about their diurnal differences (42, 43). However, of 17 amino acids explored, only a clear rhythm of tryptophan was observed in colostrum, transitional, and mature human milk with an acrophase at approximately 0400 h, and then its levels decrease and reach a nadir during the afternoon (7). In mature milk, clear rhythms were detected also in methionine, aspartic acid, histidine, phenylalanine, and tyrosine, with an acrophase at different times of the day (7). In mature milk, there are also rhythms of nucleotides, adenosine 5'monophosphate (5'AMP), guanosine 5'monophosphate (5'GMP), uridine 5'monophosphate (5'UMP),cytidine5'monophosphate (5'CMP) and inosine 5'mono'hosphate (5'IMP), the first three with higher levels during the night and the latter two during the day (44). In mature milk, 2-AG showed significantly higher concentration during the day than during the night, which mirrored plasma levels in the mother [(33); **Figure 2A**].

DIURNAL CHANGES IN IMMUNOLOGICAL FACTORS

Earlier studies reported no differences in concentration of IgA, IgM, and IgG between day and night (46, 47). Further, in a study of 36 mothers, colostrum, transitional and mature human milk, sIgA was higher during the day at 12:00 h than during the night at 24:00 h. IgG and IgM were also higher during the day than during the night in transitional and mature milk [(48); but see (19)]. C3 and C4 complement proteins, which opsonize pathogens and participate in the innate and adaptive response, increased significantly in colostrum during the diurnal period in humans (48). There are also reports about significant differences between day and night concentrations of cytokines IFNγ, IL4, IL5, and IL10 (49, 50). No diurnal differences have been found in Lactoferrin concentrations (46).

PHYSIOLOGICAL IMPORTANCE OF DIURNAL CHANGES IN MILK COMPONENTS

During late pregnancy the fetus expresses rhythms of total activity, heart rate, and general and breathing movements (51–53). This is not only a response to maternal rhythms, as the fetal brain is necessary for the integration of the mother's cues (54). Disruption of circadian rhythms during pregnancy leads to an array of negative pregnancy outcomes, such as increased frequency of miscarriage, preterm infant delivery, and low birth weight (55). Upon birth the newborn is exposed to a variety of manipulations and environmental changes and time of the establishment of circadian rhythms of CORT, MEL and day/night rest and sleep rhythm vary widely at 3–6 months of age (56). In a study in which the infant was breastfed on demand during the day and night and exposed to light only during day time, the circadian rhythms of temperature, sleep/wake, and MEL were detected during the first week and at 30 and 45 days of life, respectively (57), significantly earlier than most reports. Accumulating evidence reveals the benefits of changes in milk components and environmental conditions for the infant.

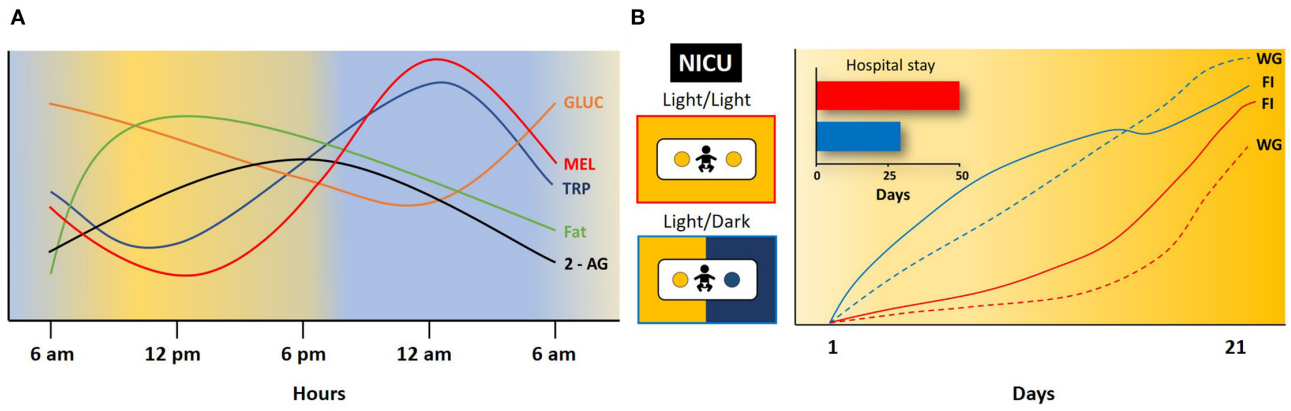

FIGURE 2 | Daily rhythm in some milk components and effect of light/dark conditions on infant development. **(A)** Changes in concentration of glucocorticoids (GLUC), melatonin (MEL), tryptophan (TRP), fat and 2-arachidonoyl glycerol (2-AG) in 24 h. Modified from (5, 6, 33, 35, 36). **(B)** Effect of constant light (Light/Light) or 12 h light and 12 h dark (Light/Dark) on food intake (FI), weight gain (WG), and length of hospital stay in infants at the neonatal intensive care unit (NICU). Blue line and dashed lines infants in Light/Dark; Red line and dashed lines infants in Light/Dark. Note the shorter hospital stay and the increase in food intake and weight gain in infants in Light/Dark cycle in comparison to infants in Light/Light conditions. Modified from (45).

The higher concentration of tryptophan and nucleotides 5'AMP, 5'GMP, and 5'UMP may play an important role as sleep promoters. It has been established that adenosine decreases cellular activity and is a somnogen (58). In agreement, there is an increase of 5'GMP in adult human plasma during sleep in the night in comparison to the period of wakefulness (59). In human milk, the concentration of 5'AMP, 5'GMP, and 5'UMP is higher during the night than during the day (44). The effect of the differential concentration of tryptophan and the nucleotides was tested in children. Sleep-wake cycle in newborns is not established and they nurse during the day and the night (60). They ingest tryptophan and nucleotides during the night, when the concentrations are highest. Tryptophan is a precursor of the hormone melatonin, and its ingestion corresponds with the variable levels of 6-sulfatoxymelatonin in urine, a metabolite of melatonin (61). To test the effect of this amino acid on sleep, tryptophan was added to formula milk, along with 5'AMP and 5'UMP to children 4–20 weeks of age. Children that received formula with this amino acid and nucleotides from 18:00 to 06:00 h showed a reduction in their latency to sleep as well as an increase in their hours of sleep (62). In another study of the same group, 8–16 month old children were fed a cereal enriched with tryptophan, 5'AMP, and 5'UMP during the night. Actigraphic recording revealed an improvement in sleep parameters (63). These experiments support the assumption that tryptophan, 5'AMP, 5'UMP, and MEL in human milk during the night improve infant sleep and help to consolidate their sleep/wake cycle. Vitamin B12 also may improve sleep in children. In humans, it has been associated with the modulation of sleep and circadian rhythms (64–66), and its deficiency during pregnancy has been associated with a subsequent increase in the infants' crying (67). Together the evidence indicates that there are chronobiotic substances in milk that contribute to the establishment of the sleep-wake cycle of the infant. Indeed, a recent study demonstrates that breastfed infants achieved a circadian rest-activity rhythm at 6th week age in contrast to 12 weeks in mixed, formula and breastmilk-fed babies (68). Exclusively breastfed infants had better sleep parameters in comparison to formula-fed infants (69). Infants at 2 months of age who were breastfed, in contrast to formula-fed infants, had a significantly lower frequency of colic attacks and severity of irritability attacks, which was associated with the night-time consumption of MEL through milk (70).

MEL has a strong interaction with the immune system. Human lymphocytes contain membrane MEL receptors which suggest that they can detect physiological changes in this hormone (71). *In vitro* studies have demonstrated an effect of MEL on the phagocytic activity of mononuclear and polymorphonuclear lymphocytes from colostrum. When exposed to *Escherichia coli*, in the presence of MEL, these cells increase their phagocytic, and bactericidal activity by stimulating cellular oxidative metabolism (6). In another example, TNF-α, a regulator of inflammatory processes, which is present in human milk, inhibits the *in vitro* synthesis of MEL in the ratpineal gland (72). This information is relevant, as cesarean section delivery in humans increased the production of TNF-α in colostrum, in parallel with a suppression of the nocturnal MEL increase (50). This can lead to an inflammatory process and to a disruption of the beneficial actions of MEL in the newborn. There are reports that the addition of nucleotides to formula milk significantly increased weight gain (73) and the rate of the occipitofrontal head circumference gain in infants 2 months of age (74). Infants fed with milk supplements enriched with DHA had a lower incidence of bronchiolitis and bronchitis during the first year of age (75).

The high level of 2-AG cannabinoids in human milk may modulate the infant's food intake, and this also can be influenced by the weight of the mother. Milk of overweight and obese mothers has a larger concentration of 2-AG and this may have an impact on the body mass index of the infant because of the effect

of endocannabinoids on food intake and the hedonic properties of food (33, 76, 77). Overweight mothers give birth to overweight babies (78). Cannabinoids are involved in development, as the administration of an antagonist of the CB1 receptor within the first day after birth in mice; inhibited milk ingestion due to an impairment of the pup's suckling response (79). This evidence and that cannabinoids are one of the few compounds in human milk with diurnal variation; suggest that they play an important role for the infant. It is noteworthy that breastfeeding also has important benefits for the mother, as it reduces the risk of ovarian cancer, mammary cancer, and postpartum depression (80).

EFFECTS OF ENVIRONMENTAL LIGHT/DARK CYCLE ON NEONATES

The importance of circadian rhythms for the wellbeing of infants has been reported in preterm infants in a neonatal intensive care unit. After parturition, premature infants were separated from their mothers and usually did not ingest their mother's milk. The infant's pineal in humans does not secrete MEL, and those infants were maintained in constant light conditions (81). This strategy severely affected their survival. Infants in this facility maintained in constant light had a lower weight gain, spent more days on the ventilator and on phototherapy, displayed lower motor coordination, and showed a delayed response to be fed orally, in contrast to babies maintained in a cycled light-dark condition (81). In another study, a personalized helmet over the head of the infant that covered their eyes but permitted airflow was used to maintain 12:12 h, light/dark condition, and infants were fed with mother's milk. Infants under this procedure showed faster weight gain, improved oxygen saturation, more rapidly developed a melatonin rhythm, and attained a shorter discharge stay in the hospital [(45); **Figure 2B**]. These studies are examples of the importance of circadian rhythms after birth in infant. Fortunately, the compelling evidence of the benefits of exposing premature infants to a light/dark cycle is leading neonatal care societies to recommend this practice for clinical applications (82).

CONCLUSION

The complexity of milk stands in sharp contrast to the concept of simple food for the infant. The analysis of milk components and their diurnal changes has led to the enrichment of milk substitutes. Unfortunately, there is only limited research on the role of breastfeeding and control of light-dark conditions in the establishment of infant circadian rhythms and wellbeing. Based on the evidence reviewed herein, we consider this issue to deserve attention, as regarding infant nutrition, there is increasing use of milk supplements, which are devoid of myriad nutritive and non-nutritive components, and lack the diurnal rhythms inherent in breastmilk. Long-term studies demonstrate that breastfeeding, in contrast to infant formula, has multiple benefits: decreased risk of developing obesity and type 2 diabetes, gastrointestinal, ear, and respiratory tract infections, and improved cognitive neurodevelopment and mental and behavioral health (83–85). Many of these benefits are epigenetic, *via* the components of breast milk, including the infant's microbiome (86). In a recent survey of 130 countries, it was concluded that suboptimal breastfeeding causes ∼600,000 child deaths/year primarily from pneumonia and diarrhea (87). Moreover, breastfeeding since birth is effective in preventing death in premature babies, in contrast to conventional care practices (88). A milk substitute cannot replace the complex composition and diurnal dynamic changes of breastmilk, so major approaches must be developed to promote the benefits of breastfeeding over commercial formula to optimize infant nutrition and subsequent health. Thus, the control of the light/dark conditions (89) in addition to the circadian variations in milk components through exclusive breastfeeding every 2–5 h (57), is strongly recommended as an important strategy to improve health and proper infant development.

AUTHOR CONTRIBUTIONS

MC-F, AR-L, AC-M, CM-V, RV-C, and MC contributed to the conception, writing, and figures design. All authors approved the submitted version of this article.

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Is the binge-eating disorder a circadian disorder?

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Introduction

There is a wide range of eating disorders (EDs) which span from restrictive eating patterns such as anorexia nervosa, to the compulsive eating spectrum such as bulimia nervosa (BN), night-eating syndrome (NES), and binge-eating disorder (BED), all with complex multifactorial pathogenesis including biological, environmental, and psychological factors. In general, EDs enclosed within the compulsive spectrum include disordered bouts of feeding at different times of the day. Many aspects of these disorders are modulated by the circadian system, such as meal timing, mood, compulsive behavior, and sleep quality.

We conducted a systematic search combining binge eating studies with circadian/sleep analyses to assess the main aspects of the BED and its relationship with the circadian system. This was conducted on the electronic database MEDLINE/PubMed using the search string (binge eating) AND [(circadian) or (rhythm)]. We searched the literature references to find additional articles. We kept only the papers that treat binge eating as a disorder and not as a component of other disorders, such as the binge eating episodes in bulimia nervosa. In view of the scarce literature on the subject, here we propose a call to action for examining circadian patterns in more detail within BED.

Binge eating disorder (BED)

Binge eating disorder (BED) is the most frequent eating disorder, affecting 1–3% of the population, with onset between 15.5–27.2 years of age and an average duration of 4–8 years (1, 2). Like most eating disorders, BED is more common in women (3.5%) vs. men (2.0%) (2). Despite being the most common eating disorder, many patients fail to receive a proper diagnosis or do. Indeed, in a representative sample of American adults, only 3.2% of respondents who met DSM-5 criteria for BED had received an appropriate formal diagnosis in the previous 12 months (3). Originally described by Spitzer (4), BED is characterized by the presence of recurrent binge-eating episodes with a minimum duration of 3 months and a frequency of > once a week. Another important fact to consider is the unpleasant nature of binge eating and the absence of purging behaviors or subsequent compensation.

An episode of binge eating is characterized by the ingestion of more food than what most people would eat in a similar period and circumstances, as well as a feeling of lack of control over what is ingested. Its clinical description represents a specific eating disorder, different from other mental disorders after its inclusion in 2013 in DSM-5 (5). The differential diagnoses of this disorder include Bulimia Nervosa (BN), the binge-eating and purging subtype of Anorexia Nervosa (BP-AN), and night eating syndrome (NES).

In BN, episodes of binge eating are usually followed by self-induced purging behaviors (generally vomiting). In BP-AN, the same thing happens, but body weight is noticeably decreased due to the intense restriction of meals. In both disorders, an intense fear of gaining weight is clearly present. Finally, the NES is characterized by recurring episodes of food intake at night, manifested by eating when waking up during the night or by excessive consumption of food after dinner (6). Clearly, in the NES the most important factor is the time of day in which the ingestion occurs. Described in the 1950s, the chronobiological aspects of NES have received more consideration: NES patients tend to express evening chronotype with a higher frequency of insomnia and overweight conditions (41, 42).

Patients with BED present a high prevalence of psychiatric and physical comorbidities. Between 30.0 and 80.0% of individuals with BED present lifetime comorbid mood and anxiety disorders (7, 8). Other common comorbidities reported in individuals with BED comprise numerous addiction disorders such as substance use/abuse (22.0%) (9), gambling problems (5.7–18.7%) (10) as well as compulsive buying (7.4–18.5%) (11).

The prevalence of binge eating disorder in individuals with obesity attending weight loss programs was found to be between 16–52% (12).

Individuals with obesity and comorbid eating disorders are at high risk of several medical and psychosocial complications such as diabetes, hypertension, and chronic pain (1, 13). A study with 152 treatment-seeking individuals with obesity found that those with binge eating disorder had higher BMIs, more severe levels of depression and obsessive-compulsive symptoms, and stronger feelings of inadequacy and inferiority than those without binge eating disorder (14).

Chronotype and chrono-nutrition

Humans differ in their preferences for activity and sleep patterns during the day, reflecting interindividual differences in their daily physiological organization, defining morning and evening chronotypes (15, 16).

There is a clear association between mood and chronotypes (17, 18). In particular, the eveningness dimension could be considered a vulnerability factor to depressive symptoms and Major Depressive Disorder (19).

Different studies investigated the relationship between personality traits and chronotypes (20); evening types were associated with anxious, hostile, impulsive and depressive personality traits, while a morning chronotype was associated with traits such as conscientiousness and with the tendency to be compliant (21, 22).

The term chrono-nutrition refers to energy distribution processes including feeding-fasting rhythm, meal frequency, the duration of the eating period, and the relationship with metabolic health (23, 24). Meal regularity establishes the pattern of energy intake and distribution, which is crucial for health outcomes. On the other hand, meal irregularity, defined as food consumption at different times and in varying amounts throughout the day (25), is associated with obesity and metabolic-related disease (26). Several recent studies indicate that food intake at later times is highly associated with increased adiposity, obesity, and metabolic risk (26, 27). Moreover, eating a large amount of food during the evening increased the odds of obesity and metabolic syndrome (28). An increased ratio of evening-to-morning meals has been associated with an increase in the body mass index (BMI), while a higher morning-to-evening ratio has the opposite effect (29–31). The conclusion is that eating when the body is not ready to manage a large amount of energy (i.e., when we are preparing to sleep), has a great impact on our health (28). The proximity of food consumption to the nocturnal rise of melatonin was associated with impaired glucose homeostasis and increased adiposity (32, 33). Eating behavior has been linked to circadian rhythms (34), since clock genes may synchronize not only the feeding-fasting rhythm but also metabolism itself (35, 36). In this sense, the individual differences in chronotype could influence eating behavior as a zeitgeber (37). For instance, late chronotype or late mealtime were associated with an increased desire for high-fat foods and more appetite (38).

The relationship between the circadian system and specific eating disorders remains unclear in part, this may be due to nosographic aspects related to the diagnostic systems of EDs, particularly regarding the differential diagnoses between BED and NES.

Although NES is described as an eating disorder in the DSM, many authors have questioned its validity as a nosographic construct (39, 40), even when it represents the most relevant results associated with eating at night and its consequences. Individuals with BED or NES share the feeling of a loss of control over food consumption, but both syndromes differ in their timing. Several studies demonstrate that energy intake in NES patients tended to occur during the night, affecting the sleep-wake cycle (41, 42). In contrast, in BED patients, the available literature fails to systematically collect and inform the time of the binge behavior that may occur at any time of the day (43, 44). Only a few studies report circadian preferences in patients with eating disorders (37, 40, 45). A circadian approach to the BED indicated that subjects with either BED or NES were more likely to have an evening chronotype (40), as well

as higher rates of anxious and depressive symptoms. Subjects with NES have similar sleep onset and offset times to those of controls (46, 47), suggesting that their delay in chronotype may be due to their eating behavior and not to the circadian system. Indeed, more work is needed to examine ***when*** binge episodes occur and what is their relationship with the feeding-fasting rhythm, the subject's chronotype. Social aspects should also be considered, e.g., if the binge occurs when people are alone, or if there is some preference for binge during week-days or during weekends which can contribute to social jetlag, another circadian disruption with metabolic consequences. In this sense, both NES and BED can act as a promotor of- as well as a consequence of- circadian disruption, inducing a vicious cycle between both alterations.

Binge behavior and sleep

Sleep disturbances has been studied in patients with NES, which tend to present insomnia and periodic limb movements. Moreover, night eaters reported higher ratings of sleep perturbations and use of sleep medications (41). However, NES was not associated with day-time sleepiness. A rest-activity pattern study found no differences on sleep-onset time or total sleep duration in NES, although more awakenings and later sleep offset time have been reported (41).

A study of the rest-activity pattern in patients with BED demonstrated some alterations in their circadian behavior. BED patients exhibited a lower MESOR (mean estimate of circadian rhythm, derived from fitting the data to a cosine wave) and amplitude than control subjects (48). Patients also presented low sleep-efficiency values, but this was also present in an obese control group, indicating that it could be ascribed to overweight conditions (45). Different studies analyzing obese patients with and without BED confirmed this finding: sleep architecture abnormalities in patients with BED were also found in the obese control group (45). Despite this, another study demonstrated that BED patients show more minutes of wakefulness during the sleep period than normal-weight controls (49), presumably due to their binge episodes. In addition, significantly higher sleep-disruption parameters were found in children with BED than in obese non-BED and normal-weight controls (49). More work needs to be done to clarify the relationship between BED and the circadian pattern of sleep, sleep quality, and its dependence on obesity.

Addictive-like eating behaviors

Binge eating disorder is recognized as an eating disorder involving compulsive food intake. Some authors point out that this type of behavior could be included within a theoretical construct called Food Addiction (FA) (50, 51), employed to describe addictive-like compulsive overeating which involves cravings and difficulties in abstaining from high-calorie foods (52). Even though FA could be present in patients with BED, it is important to note that while BED is a classified and diagnosable mental disorder, FA remains a controversial concept that has not yet been recognized as a diagnostic entity. FA shows some similarities with substance abuse disorders; indeed, both drug consumption and excessive food intake share neural changes in the brain reward system (53–56). Dopaminergic activity disruption appears to be a common root for drug intake and compulsive eating, supporting the food addiction concept (57).

As we mentioned, feeding is rhythmically distributed over a 24h period (58). Interestingly, a circadian rhythm for caloric intake has also been described (59–61); and, in accordance, a binge eating behavior rhythm was found in rats with an increase in dopamine receptor density in the nucleus accumbens (62). This "caloric preference" rhythm is regulated by the circadian system (59, 63–65), while its hedonic component depends on the rhythmic activity of DA, which in rodents peaks at night, corresponding with their nocturnal phase of activity and food consumption (66, 67). This DA release in the striatum depends on the circadian-controlled VTA rhythmic activity (68, 69). Indeed, more information is needed on the relationship between feeding and reward rhythms in humans.

There is also a correlation between chronotype and food preference: an evening chronotype is correlated with greater consumption of high calorie-beverages, caffeine, alcohol, nicotine, and fast food (70–72), and lower consumption of fruit, vegetables, and fish (73). Moreover, subjects with an evening type show a higher energy intake at night, and this effect was even larger during weekends (71, 74). The metabolic and hedonic regulation of feeding is rhythmic and depends on the circadian system; the timing of food consumption is crucial for the regulation of food intake, and the loss of this circadian pattern might lead to disorders that display compulsive eating, obesity, and metabolic syndrome as a part of its symptomatic complex (75).

Conclusion

In this work, we focused on the literature exploring the main physiological aspects controlled by the circadian system that affect or are affected by the binge eating disorder, and how the circadian system may play a significant role in the development of more severe outcomes. We summarize the current state of the literature studying the circadian aspects of BED, to find out that is a topic yet to be explored and offer our suggestions for future research in BED to include designs capable of collecting sufficient and necessary information to study the role of the circadian system on the BED progression. The circadian system modulates different aspects of mood and mood-related behavior, including emotion, compulsive behavior, and regulatory control (76). All these features are in close relationship with BED and should be studied from a circadian perspective. Given the higher prevalence of BED in women, it would also be important to delve

into the influence of neurohormonal systems in the genesis of the disorder and its relationship with biological rhythms.

One of the main difficulties to study circadian aspects of BED is the differential diagnostic overlapping between BED and NES. Food is a strong signal for the circadian system; both BED and NES alter the feeding-fasting rhythm causing a circadian disruption which, in turn, will affect feeding, creating a vicious cycle of important consequences for the patient's well-being. In this sense NES and BED can be consequence of- or promotor of- a circadian disruption and an evening chronotype. Future studies need to address the crosstalk between BED, NES and the circadian system, including some objective measures of sleep-wake cycle, circadian status and energy consumption, as well as some subjective measures to cover aspects related to sleep and chronotype. In this sense, the mechanisms behind binge timing, mood, compulsive behavior, and sleep alteration need to be acknowledged to create a more accurate BED vs. NES diagnosis.

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Obesity in male volcano mice *Neotomodon alstoni* affects the daily rhythm of metabolism and thermoregulation

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The mouse *N. alstoni* spontaneously develops the condition of obesity in captivity when fed regular chow. We aim to study the differences in metabolic performance and thermoregulation between adult lean and obese male mice. The experimental approach included indirect calorimetry using metabolic cages for VO_2 intake and VCO_2 production. In contrast, the body temperature was measured and analyzed using intraperitoneal data loggers. It was correlated with the relative presence of UCP1 protein and its gene expression from interscapular adipose tissue (iBAT). We also explored in this tissue the relative presence of Tyrosine Hydroxylase (TH) protein, the rate-limiting enzyme for catecholamine biosynthesis present in iBAT. Results indicate that obese mice show a daily rhythm persists in estimated parameters but with differences in amplitude and profile. Obese mice presented lower body temperature, and a low caloric expenditure, together with lower VO_2 intake and VCO_2 than lean mice. Also, obese mice present a reduced thermoregulatory response after a cold pulse. Results are correlated with a low relative presence of TH and UCP1 protein. However, qPCR analysis of Ucp1 presents an increase in gene expression in iBAT. Histology showed a reduced amount of brown adipocytes in BAT. The aforementioned indicates that the daily rhythm in aerobic metabolism, thermoregulation, and body temperature control have reduced amplitude in obese mice *Neotomodon alstoni*.

KEYWORDS

***Neotomodon alstoni*, obesity, metabolism, UCP1, thyroxine hydroxylase, body temperature, daily rhythm**

Introduction

Circadian physiological responses anticipate daily environmental changes, such as light-dark or temperature cycles, so that predictive homeostasis in energetic balance favors survival. Circadian thermoregulation in mammals is achieved by changes in behavior and metabolism that compensate for heat fluctuations (1). In acute exposure

to cold, mammal's heat production is regulated by changes in motor activity intensity and metabolic heat production by shivering as well as non-shivering thermogenesis, respectively (2). Mammal thermoregulation is under hypothalamic control. It is maintained through metabolic, energy, and endocrine adjustments (3) that may vary depending on the time of day, the season, sex, or age. Exposure to cold triggers a metabolic activation to preserve body temperature (BT) by an integrated response in diverse hypothalamic nuclei, such as the preoptic area and the dorsomedial hypothalamus (3, 4), complemented by the activity of the brown adipose tissue (BAT). This response is modulated along the day-night cycle by the arcuate and suprachiasmatic nuclei (5).

Mitochondrial uncoupling protein 1 (UCP1) is a functional protein that contributes to thermogenesis in Brown Adipose Tissue (BAT). UCP1 is produced by multiple pathways (6–8). Among the most important is the release of noradrenaline from the innervating nervous system in BAT; its synthesis is dependent on the enzyme tyrosine hydroxylase (TH), the limiting enzyme of catecholamines (9).

Obesity affects thermoregulation in different ways, i.e., by thermal insulation of enhanced subcutaneous fat, low heat production due to reduced motor activity (10, 11), endocrine dysregulation, i.e., thyroid (12) and leptin resistance (13). At the same time, obesity in mice negatively affects circadian rhythms; for example, the molecular circadian clock modulates metabolic mechanisms in active bioenergetic tissues, including BAT, whereas the regulation of BT changes according to the time of day (14, 15). Mechanisms affected by obesity in the circadian thermoregulatory responses still need research. A better understanding of the physiological and behavioral changes in different animal models adapted to face seasonal changes may enrich our knowledge of how different species of rodents handle thermoregulation.

The volcano mouse *Neotomodon alstoni* (Merriam, 1898), a nocturnal rodent of the Neotominae subfamily, genus *Peromyscus*, subgenus *Neotomodon* (16), it is restricted to the Transverse Neovolcanic Ridge of the central zone of Mexico, where inhabits pine forests, in grass lands. It can be found between 2,600 and 4,600 Meters above sea level (m.a.s.l.) (17). *N. alstoni* lives in burrows and feeds on grains and insects (18). Adult mice have a nasoanal length is 100–130 mm, ears are almost bare; hair of the dorsal region is dense and gray while the ventral fur is whitish. Adults usually weigh from 40 to 50 g (19). A cytogenetic analysis shows that its chromosome number is 2n = 48 with a fundamental number NF = 66, like *Peromyscus* (20). *Neotomodon alstoni* is listed as Least Concern species, according to the red list of the International Union for Conservation of Nature (IUCN) (21). It may adapt favorably to captive conditions (22, 23) and it can live up to 5 years in laboratory conditions (24). When captive or raised in vivarium conditions, part of the animals raised or captured spontaneously develop obesity when fed regular chow food, this characteristic seems to be congenital because potential conditions, other than *ad libitum* food access and isolation are equal for all mice, and intermediate phenotypes of pre-obese mice that suggest a heterozygous phenotype (25). Obese adult mice usually weigh more than 60 g and, compared with lean mice (BW∼45 g), have alterations in the amplitude of the circadian rhythm of motor activity, as well as in the daily signaling of various hormones involved in metabolism such as insulin, leptin (26, 27) and ghrelin (28). The behavioral and endocrine changes previously observed suggest that obesity status in this species may also be associated with a deficit in the nictemeral behavioral and metabolic thermoregulation and its thermogenic response to a cold environment. We aimed to determine whether obese *Neotomodon alstoni* mice exhibit a deficit in metabolic rate and thermoregulation compared to lean mice over the 24 h profile in light/dark cycles (LD). In addition, we explored its possible relation with a change in the relative presence and gene expression of the mitochondrial Uncoupling Protein UCP1, and the relative presence of Tyrosine Hydroxylase (TH) in interscapular Brown Adipose Tissue (iBAT). Histological analysis of iBAT was also performed. Finally, to understand potential differences in thermoregulation defense in a cold challenge, we explored changes in body temperature when mice were exposed to a 2 h cold pulse, both at noon and midnight.

Materials and methods

Animals

Adult male mice *Neotomodon alstoni* were born and raised in the vivarium of Science Faculty at the National Autonomous University of Mexico (UNAM), as indicated elsewhere (29). Maintenance conditions included regulated photoperiod (Light: Dark cycles 12:12, 350–500 lx, lights on at 06:00 h), and controlled room temperature (23 ± 1°C). After weaning, mice grew individually in acrylic boxes with a metal grill lid and free access to regular rodent food (Purina, 5001) and tap water. Then, after 7–12 months, mice were separated into two groups: Lean (46 ± 3 g), and obese mice (65 ± 3 g) and used in experimental protocols indicated below. Each set of results indicates the number of animals used for every protocol. LD schedule is indicated in Zeitgeber time (ZT), considering ZT0 when the lights were on and ZT 12 when the lights were off.

Mice *Neotomodon alstoni* that show obesity can be distinguished from normal-weight mice when at ca. 7 months old (26, 27). Therefore, young adult mice in lean or obese groups were males between 7 and 12 months old. At the end of the protocols, mice were euthanized by decapitation, and interscapular adipose tissue was collected, kept in deep freezing, or prepared for histological analysis. All experimental protocols and procedures were performed following the Declaration of Helsinki and the Guide of the National Institutes of Health

for the Care and Use of Laboratory Animals, NIH Publication No. 8023; guidelines and the General Health Law for Research Studies in Mexico (NOM-062-Z00-1999), based on ethical management for chronobiology studies (30) and the Committee on Academic Ethics and Scientific Responsibility of the Sciences Faculty, and the Neurobiology Institute UNAM.

Indirect calorimetry

Indirect calorimetry was determined in intact lean ($n = 10$) and obese mice ($n = 5$) in a room with environmental conditions controlled (12:12 photoperiod, 06:00–18:00 h, photophase, 21 ± 1°C) at the Neurobiology Institute, UNAM. Respirometry parameters such as oxygen consumption (VO_2), production of carbon dioxide (VCO_2), respiratory quotient ($RQ = VCO_2/VO_2$), and energy expenditure (EE) were measured as indicated elsewhere (31) utilizing an OxyletPro system and the Metabolisms software v.3.0.01 (Panlab Harvard Apparatus, Barcelona, Spain). Mice were first acclimatized for 2 days in individual acrylic cages (Oxylet LE 1305 Physiocage, Panlab) with food and water. Then, respirometer parameters were obtained every 3 min for 3 days at a controlled flow rate of 700 ml/min (Oxylet LE 400—supplier air, Panlab). During all procedures, mice had access to food and water *ad libitum*, and both parameters were also monitored.

Body temperature loggers

According to previous studies, intraperitoneal body temperature was recorded using the data logger iButton (Thermochron DS1921H-F50#; Dallas Semiconductor, Dallas, TX, USA) (32). It has been proven elsewhere that iButtons have been useful in studies using smaller mice CF1, BALB/c, and C57BL/6N (∼35 g) (33) than in lean adut *Neortomodon* (∼45 g). Loggers were programmed to record and store temperature data every 10 min, and recording was programmed to begin 10 days after implantation. Surgery was performed on anesthetized mice with intraperitoneal ketamine/xylazine (Anesket/Procin, Pisa, Mexico) 100/10 mg/kg weight. The abdomen's skin was shaved, and the area was cleaned with 70% ethanol and iodine solution. A longitudinal incision of ∼2 cm was made along the midline, and the previously disinfected iButton covered with sterile wax (surgical specialties Co. Mx) was inserted into the abdominal cavity. The muscle and skin incision were separately sutured with nylon thread. After surgery, mice were kept on a warm bed until recovery from anesthesia; analgesia was provided by topic xylazine. Animals were allowed to recover from surgery for at least 10 days. Daily curation of the wound and cleaning cages were provided. Only mice that were full recovery from surgery with a locomotor activity pattern typical of intact animals were used for the temperature recordings. BT was monitored for at least 3 days (LD 12:12 at 23°C, LD 12:12 at 23°C with a 2 h cold pulse at noon and midnight). During the recordings the cages and substrate (clean wood chips) were replaced weekly. At the end of the experiment, mice were sacrificed by decapitation. The interscapular and gonadal adipose tissue was immediately frozen or preserved in paraformaldehyde, and their data loggers were removed to collect data using the iButton-Thermochron® software.

Control of environment and exposure to cold

Mice were kept in a controlled temperature environment using a Plant-Growth Chamber (Adaptis 1000, CONVIRON), which provided a stable and programmable temperature maintained at 23 ± 1.5°C, humidity 40%, with a photoperiod LD 12:12 using white LED light (300 lx). Then, cold exposure pulses were programmed as a drop in temperature from 23 to 10°C for 2 h; the pulses were programmed to take place at midnight (24:00–02:00 h) and noon (12:00–14:00 h) in LD 12:12 (06:00–18:00 h, photophase).

Protein immunodetection by western blot

The relative presence of UCP1 protein was analyzed using Western Blot, as previously indicated (34). The iBAT samples from mice euthanized at noon were frozen in dry ice and kept at −80°C until analyzed. The adipose tissue was removed and homogenized in 4 volumes of RIPA buffer (Cell Signaling, Danvers, MA) supplemented with complete protease inhibitors (Roche Diagnostics, Indianapolis, IN) using a Potter-Elvehjem Teflonglass homogenizer (70 rpm for 15–20 s). The homogenate was centrifuged at 2900 rpm for 5 min at 4°C, and the infranatant (intermediate layer) was carefully recovered, aliquoted, and stored at −80°C until used. Total protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA). Samples of 50 μg of protein mixed with 2X Laemmli buffer (Bio-Rad) and incubated at 80°C for 5 min were used. Proteins were separated on a 7.5% polyacrylamide gel, transferred to a nitrocellulose membrane (Bio-Rad), and blocked with Blotting-Grade Blocker (Bio-Rad) for 3 h. Then, the membranes were incubated separately overnight at 4°C with different rabbit antibodies (anti UCP1 EPR20381 ab 209483 Abcam dilution 1:1,000, and anti-Tyrosine Hydroxylase AB152 Sigma-Aldrich; dilution 1:5,000). The membranes were washed and incubated for 2 h with alkaline phosphatase (AP) conjugated antibody: donkey anti-rabbit IgG-AP (1:5,000 dilution; sc-2315; Santa Cruz Biotechnology, Dallas, TX, USA). According to the manufacturer's instructions, the bands were visualized using

the AP conjugate substrate kit (Bio-Rad Laboratories, Hercules, CA, USA). Transfers were digitized, analyzed with Image J® software (version 1.38, NIH, USA), and normalized to the α-tubulin signal (anti-α-tubulin ab 15246, Abcam, 1:1,000 or anti GAPDH 1:5,000 Abcam 9485) as a loading control.

Hematoxylin-eosin staining

Interscapular and gonadal adipose tissue (epididymal) was fixed in paraformaldehyde 4% for 24 h, then transferred to a 10% formalin fixative solution for 1 week, and then to zinc-formalin (1% zinc sulfate) (35). Subsequently, the tissues were dehydrated for paraffin waxing, following the previous description indicated elsewhere (36), using a 12 h automated dehydration process in a Histokinette equipment (Leica TP 1020) at the Veterinarian Histopathology facilities of the Autonomous University of Querétaro, México. Tissue in 20 μm sections was counterstained with hematoxylin-eosin staining. Three slides from each sample of interscapular brown adipose tissue (iBAT) and gonadal WAT were taken from lean and obese mice ($n = 3$ from each group), and analyzed with Image J® software (NIH, USA) to measure the size of gonadal adipocytes and the content in brown adipocytes (25 mm^2), the average weight of interscapular adipose tissue was compared (lean $n = 6$, obese $n = 7$).

Ucp1 gene expression by RT-qPCR

Total RNA was isolated from *Neotomodon alstoni* from iBAT, quickly dissected and frozen in dry ice and kept at −80°C until further processing (37). We use the TRIzol™ Reagent (Invitrogen Life Technologies, Carlsbad, CA) following the manufacturer's instructions, with minor modifications (38, 39). Genomic DNA contamination was removed by treating 1 μg of total RNA per sample with 10 units of DNase I (#04716728001; Roche, Basel Switzerland), and cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (#4374966; Applied Biosystems™ Inc., Foster City, CA) following the manufacturer's instructions. We included negative controls without a template or reverse transcriptase, cDNA was diluted 1:5 before use.

Currently, the genome sequence for non-traditional animal model *N. alstoni* mice is not available. However, from a previous study (16) upon comparative taxonomy of muroid species *Peromyscus maniculatus* and *N. alstoni*; primers were designed using GenBank® genetic sequence database for *Peromyscus maniculatus bairdii*, using NCBI Primer-BLAST, for ucp1 gene [Uncoupling protein 1 (mitochondrial; proton carrier) mRNA; Accession Number: XM_006995737.2] and for tbp gene (TATA-box binding protein mRNA; Accession Number: XM_042261953.1), for normalization of mRNA expression (40). All primers were designed to span exon-exon boundaries and were analyzed *in silico* with NetPrimer (PREMIER Biosoft International, Palo Alto, CA) software). Oligonucleotide primer sequences are given in Table 1.

TABLE 1 Oligonucleotide primer sequences for RT-qPCR.

Primer name	Synthesis direction	Sequence (5′-3′)
naUcp1	Forward	ACACTCTGGAAAGGGACAAGC
	Reverse	CACACTTGGGTACTGTCCCG
naTbp	Forward	GGCATCACTGTTTTATGGTGTGT
	Reverse	GGAGTCATGGCACCCTGTG

All real-time PCR reactions were carried out in the StepOne™ Real Time System (ThermoFisher Scientific, Waltham, MA), using KAPA SYBR® Fast qPCR Kit (KAPA BIOSYSTEMS, Woburn, MA, USA), according to Martínez-Moreno et al. (41). The reactions were performed in duplicate under the following conditions: initial denaturation at 95°C for 3 min followed by 45 cycles (*Ucp1*) or 40 cycles (tbp) of 95°C for 3 s, 61°C (ucp1) or 62°C (*tbp*) for 20 s and 72°C for 10 s. A dissociation curve was run to ensure that there was only one specific amplification product for NTC or negative controls; there was no detection signal. cDNAs standard curves were prepared using serial dilutions to obtain the primer efficiencies, which were 90% for ucp1 and 90.5% for tbp. We used a relative standard curve quantification method (42, 43) to normalize *Ucp1* mRNA expression levels between experimental lean and obese mice groups, to the mRNA level of the reference gene *tbp*, by generating standard curves for each gene using pooled cDNA.

Data analysis

The results are expressed as mean ± SEM of each parameter. For statistical analysis and plotting, we used the GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA). Intergroup comparisons were tested with a two-way ANOVA for independent measures with a factor for each group, a factor for time, and a Fisher *post-hoc* test. To assess the mean total area under the curve (AUC) in the 24 h daily profile, a comparison of the number and size of adipocytes and relative expression of proteins from iBAT between lean and obese mice was performed with a Mann-Whitney U-Test. Normalized U*cp1* mRNA expression in BAT of relative quantification method from RT-qPCR data was averaged and compared between groups by non-parametric Mann Whitney *U*-Test. For all analyses, statistically significant differences were considered at $P < 0.05$.

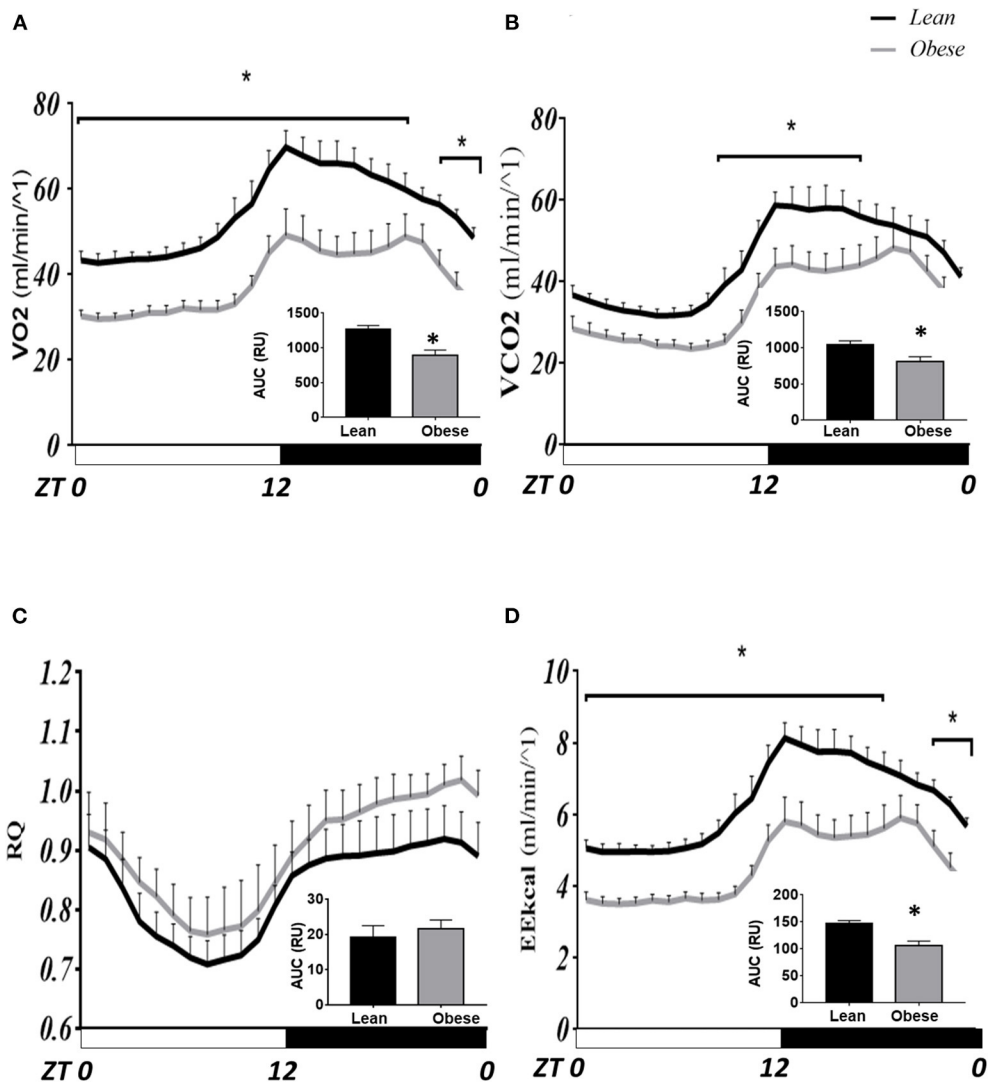

FIGURE 1
Comparative daily profile of calorimetry parameters in mice *N. alstoni*. Data are presented as mean ± SEM, $n = 10$ lean, $n = 5$ obese. Lean (black line), Obese (gray line). Bars depict the LD cycle. *Significant differences intergroup (two-way ANOVA, $P < 0.05$). **(A)** VO_2 intake; **(B)** VCO_2 production; **(C)** Respiratory ratio; **(D)** Energy Expenditure (kCal).

Results

Metabolic rate

Figure 1 shows the hour-by-hour mean ± SEM along the LD cycle on the parameters evaluated in intact mice inside metabolic cages on LD 12:12. The VO_2 and VCO_2 profiles (ml/min^{-1}, Figures 1A,B, respectively) show a daily rhythm, with a rise before lights off, fades throughout the night, and falls before the onset of lights. Obese mice display a lower volume of oxygen intake and CO_2 production in most of the schedules tested. The bimodal pattern shown by the obese mice during the night is more consistent with previously observed locomotor activity patterns (34). Significant differences found between groups simultaneously are indicated with a star ($P < 0.05$). The hourly average VO_2 intake was not different only at ZT 20 and 21. In contrast, VCO_2 production was different over several hours, between ZT 10–18, corresponding to the end of the photophase and the first half of the scotophase. The AUC was lower (31%) in obese VO_2 intake (893.4 RU) than in lean mice (1,295 RU, $P < 0.05$); while in CO_2 production, AUC in obese was lower (26%) (797.6 RU) than in lean mice (1,077 RU, $P < 0.05$), indicating low aerobic metabolic demand in obese mice (inside graphs).

In Figure 1C, the Respiration Coefficient (RQ) tends to be higher in obese than in lean mice; although, no significant differences were detected in these parameters between groups

nor in AUC. However, energy expenditure (EE kcal/min-1) was different at most times of the day except for ZT 21 and 20, similar to VO_2 intake (Figure 1D). The AUC for EE showed a similar pattern to that for VO_2 intake, where obese mice had lower energy expenditure (21%) (3,177 RU) than lean mice (4,041 RU; $P < 0.05$).

Figure 2A shows the daily profile of food intake. The average intake in obese mice (5.8 ± 0.7 g/day) was not statistically different from lean mice (5.0 ± 0.4 g/day). However, obese mice eat more than lean mice during the day at ZT 1, 2, and 9; and at ZT 19 and 22 during the night ($P < 0.05$). Water intake (Figure 2B) was not different during the day, but it was mainly during the night, from ZT 15- to ZT 23, where obese mice drank nearly twice more water than lean mice ($P < 0.05$).

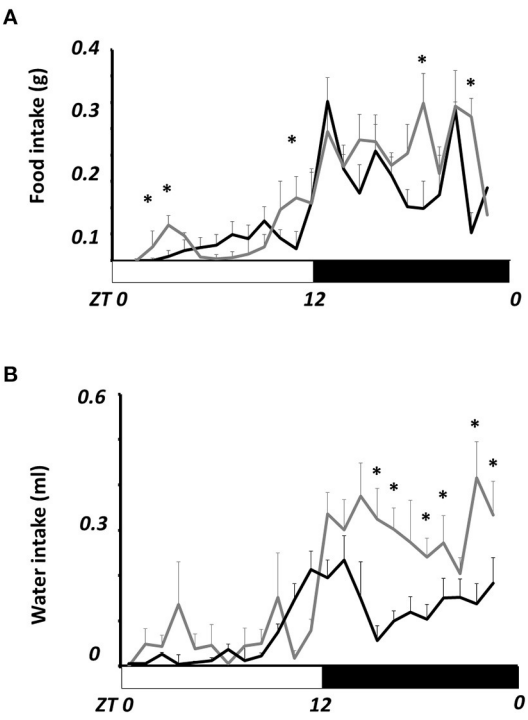

FIGURE 2
Comparative daily profile of daily food **(A)** and water **(B)** intake in lean and obese N. alstoni. Data are presented as mean ± SEM, $n = 10$ lean, $n = 5$ obese. Lean (black line), Obese (gray lines). Bars depict the LD cycle. *Significant differences intergroup (two-way ANOVA, $P < 0.05$).

Body temperature and response to cold exposure

Figure 3A shows the 3-day average (±SEM) of body temperature (BT) in LD from lean (black lines, $n = 7$) and obese mice (gray lines, $n = 5$) in a 24h LD profile. Significant differences between groups are noted in brackets ($P < 0.05$). Obese mice show lower BT than lean mice at onset and offset of the scotophase. Average BT in obese mice during the day was lower (34.9 ± 0.06°C) than in lean mice (35.7 ± 0.06°C), a reduction of 2.4%; while during the night, obese mice presented 36.4 ± 0.20°C, and in lean mice 37.6 ± 0.1°C, a reduction of 3.5%, nearly 1°C below the average in day and night ($P \leq 0.05$). The AUC was smaller in obese mice (820.9 ± 13.7 RU) than in lean mice (844.9 ± 2.4 RU), with no significant differences.

When exposed to cold in a 2-h drop from 25 to 10°C in ambient temperature, the thermoregulatory response was evidently different between lean and obese mice. Figure 3B shows the 3-day average of BT's daily profile (mean ± SEM), and gray bars indicate the time when the ambient temperature falls (cold pulses). Differences in BT were found right after the pulse at either noon or midnight. The change in BT during the first day of the protocol is shown in Figure 3C. Data from each mouse are plotted with a line in black (lean $n = 7$) and gray (obese mice $n = 5$) before the pulse (time 0), then during the first and the second hour during the cold pulse (hours 1 and 2), and after the pulse (hours 3 and 4), respectively. The results showed a variable response mainly among obese mice; some showed a clear drop in BT during the pulse that remains low after the pulse, while few obese mice could defend BT from cold. At midnight, lean mice (black dots) compensated BT from the first hour of cold exposure, and at noon, only one out of six mice showed a drop in BT. The average (±SEM) of the percent change regarding the time 0 is shown in Figure 3D. Even though there is a fall during the pulse regarding the time 0, there are no significant differences between lean (black bars) and obese mice (gray bars) until after the cold pulse finished. Obese mice reduced their BT by nearly 10%, and the change seems to be persistent 2 h after cold exposure.

Relative expression of the protein UCP1, TH, and histology of interscapular adipose tissue

Figure 4A shows the mean ± SEM of the relative presence of UCP1 detected by WB with an α-tubulin signal as the protein of reference from samples of iBAT ($n = 14$ lean, $n = 8$ obese mice) collected at noon. Representative blots are shown in the right panel (Figure 4B). Obese mice showed nearly 50% less relative presence of UCP1 than lean mice, which may be related to a thermoregulatory deficiency seen before, shown in Figure 3C. Unexpectedly, the relative expression of normalized *Ucp1* shows a nearly 11-fold increase in obese ($n = 4$) than in lean ($n = 6$) mice ($P < 0.01$; Figure 4C). On the other hand, the protein thyroxine hydroxylase relative to GADPH in iBAT evaluated by

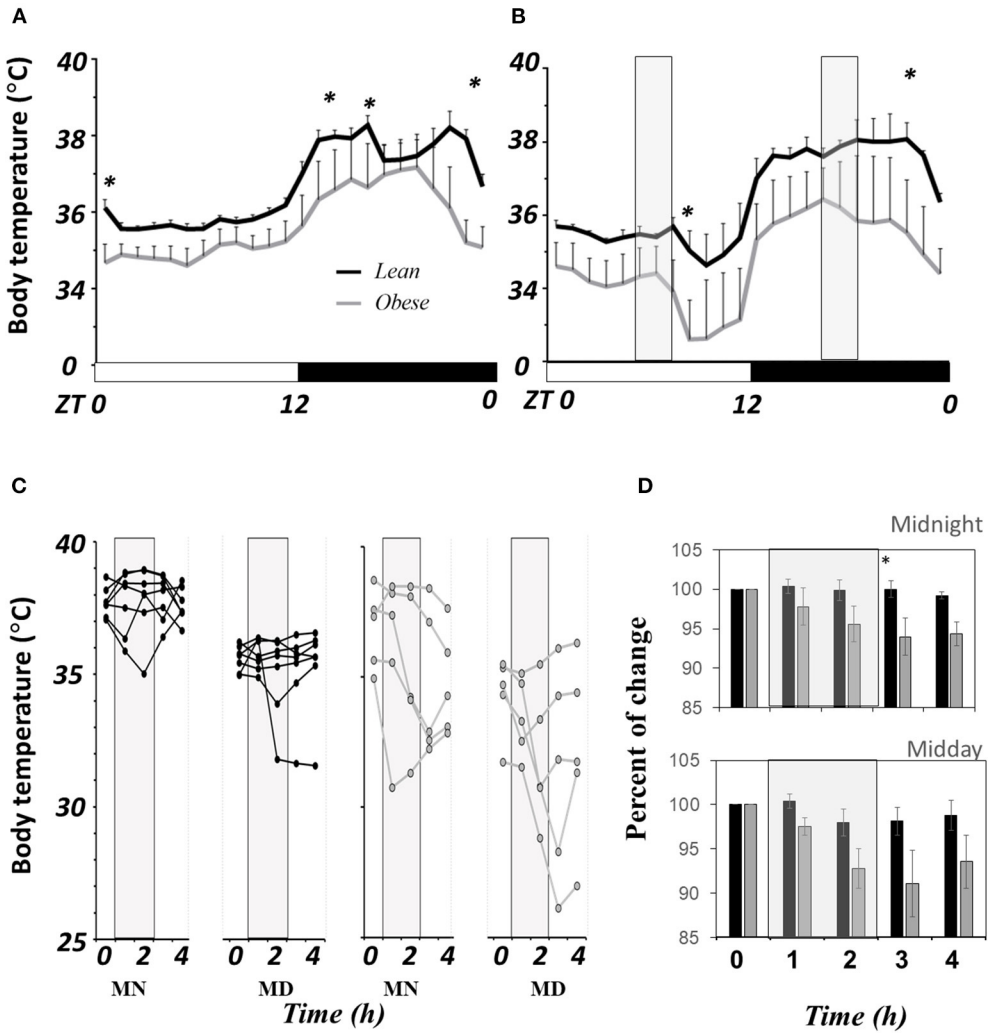

FIGURE 3
Comparative of core body temperature in *N. alstoni*. Lean (black lines and bars, $n = 7$), Obese (gray lines or bars, $n = 5$). **(A)** Hour by hour average ± SEM of 3 days in LD; **(B)** Daily profile temperature and exposure to a drop in environment temperature (12°C, light gray bars), **(C)** first day individual changes in BT before during and after the cold exposure; **(D)** Percent change of body temperature before, during and after cold pulses vs. Time 0. *Significant differences intergroup ($P < 0.05$).

Western Blot (Figure 4D) show a reduction of nearly 20% in obese mice ($n = 5$, each group).

Figure 5 shows the quantitative histological analysis of slides contrasted with hematoxylin and eosin of staining in samples from iBAT and compared with gonadal white adipose tissue (WAT). Obese mice (gray bars) had nearly 4 times more adipose tissue than lean mice (black bars) at the interscapular zone (Figure 5A). Lean mice had a brown compact mass of adipocytes (Figure 5D). In contrast, obese mice showed adipose tissue with a lighter color (white-yellowish) than that of lean mice, consistent with the low proportion of brown adipocytes observed on slides (Figure 5E). The iBAT in obese does not show abundant blood vessels as it is in lean mice. The histological composition of iBAT in lean mice is mainly composed of brown adipocytes (small cells in dark gray), contrary to obese mice, in which brown adipocytes are scarce (nearly 75% less than in lean mice, Figure 5B). White adipocytes tend to be larger in gonadal adipose tissue of obese than lean mice (Figures 5C,F,G).

Discussion

In vertebrates, thermoregulatory needs are closely related to energy balance, and endothermic animals always prefer normothermal conditions to reduce energy costs. Obesity in mammals has an influence on thermoregulation in different ways, increasing the thermal insulation conferred by subcutaneous fat (10), reducing motor activity (11), as well as modifying

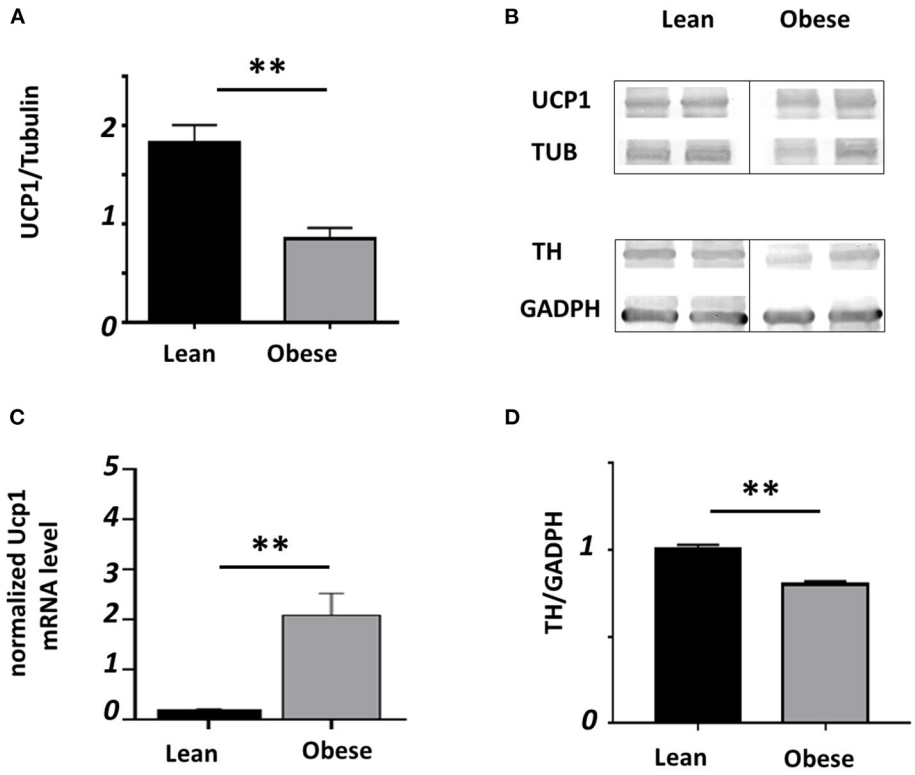

FIGURE 4
Relative presence of UCP1 and TH in iBAT. Black bars represent lean and gray bars obese mice. **(A)** Relative presence to tubulin lean ($n = 14$) and obese ($n = 8$). **(B)** Representative blots. **(C)** Relative mRNA for Ucp1 ($n = 6$ lean, $n = 4$ obese) analyzed by RT-qPCR and normalized to the reference gene tbp. **(D)** Relative abudance of TH ($n = 5$ each group); (**$P < 0.01$).

endocrine thyroidal metabolism (12) and leptin signaling (13). Thermoregulation in obese rats is lower than in lean rats (44), and studies about the metabolic rate and obesity in rodents rely on models where the genetic structure or diet is modified to highlight the overweight condition (45, 46).

The present work describes the difference in daily thermoregulation and energy metabolism between lean and obese *N. alstoni* mice. This species is interesting because spontaneous obesity occurs in some mice raised in captivity, isolated in cages, and with *ad libitum* access to bioterium food. The biological causes for developing this condition are still uncertain. Along with previous studies [reviewed in (25)], this work helps to understand the circadian affectations in behavior, metabolism, and thermoregulation in obese mice. It is also relevant in line with circadian dysregulations related to the obesity condition, such as reduced locomotor activity, alterations in glucose regulation, oxidative stress, leptin, and ghrelin signaling, as well as hepatic lipid dysregulations that may be linked to metabolic disorders.

In the present work, we show that in *N. alstoni*, metabolism and thermoregulation are down-regulated in obese mice. Indirect calorimetry assays showed that the main differences between lean and obese mice occur in the ratio VO_2/VCO_2 throughout the day. However, the RQ showed no differences, and we cannot conclude that different energetic substrates may be differentially used in obese mice. Nonetheless, a tendency to be higher in obese mice suggests that more carbohydrates are used instead of lipids during the night. The RQ involves Vol CO_2 released/Vol O_2 absorbed; carbohydrates are oxidized through aerobic respiration resulting in an equal ratio of VCO_2/VO_2 (RQ = 1). Subsequently, the RQ for fat and protein metabolism is 0.7 and 0.8, respectively. If a mixture of the substrates is consumed, the RQ ratio collectively is ∼0.8 (47). Results in Figure 1C suggest that lipids are more likely to be used during the rest phase of the day in lean mice, and obese mice may have to use a mixture of substrates. This interpretation could be related to the excess of adipose tissue detected in obese mice.

Energy expenditure (EE) in kCal/min^{-1} (Figure 1D) was clearly reduced in obese mice. In previous studies, we observed that obese *N. alstoni* mice show half the amount of locomotor activity during the night and sometimes more activity during noon than lean mice (27, 37), results that are consistent with a reduced EE observed during the night in the present work.

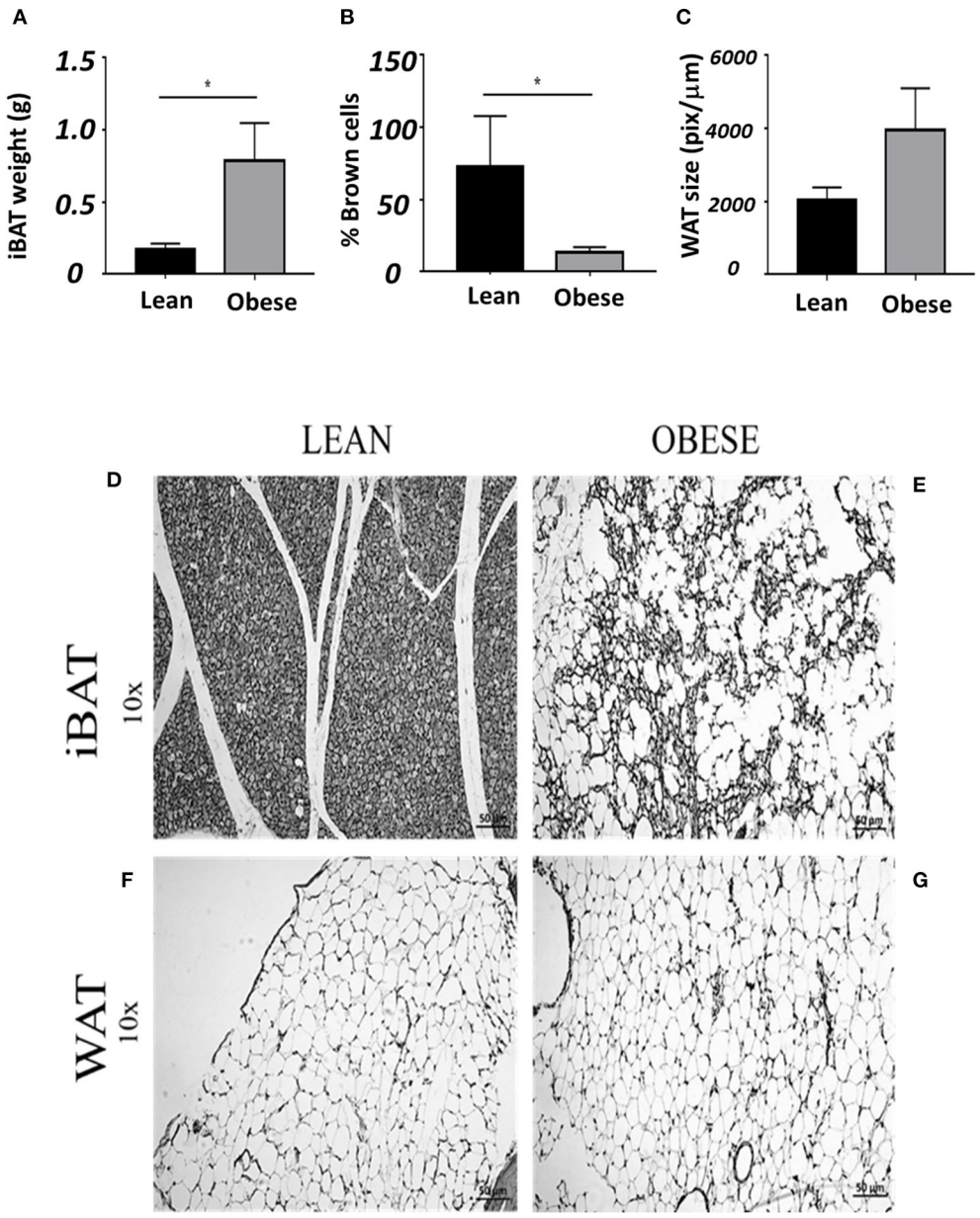

FIGURE 5
Average ± SEM of: **(A)** weigh of iBAT, **(B)** % of brown adipocytes **(C)** size of white gonadal adipocytes. **(D)** Slide of iBAT from a lean, **(E)** obese iBAT. **(F)** Gonadal white adipose tissue from lean and **(G)** obese mice. *Significant differences intergroup lean and obese male mice ($P < 0.05$).

Nevertheless, during the rest phase, EE in obese mice is still reduced compared to lean mice, indicating that metabolic use of energy during rest is also different, as observed in body temperature results (Figure 3A) as well as it may be related with the enhanced food intake observed during the day in obese mice (Figure 2A). Also, polydipsia observed in obese mice (Figure 2B) could be related to a diabetogenic tendency detected previous reports (26), since it is recognized that diabetic organisms present polydispsia (48). The diabetic db/db mice present a spontaneous mutation of the leptin receptor (49) also show polydipsia as well as increased food intake. Eating and drinking is an activity mediated by lateral hypothalamic area (LHA) and a subpopulation of LHA neurons expressing Neurotensin also present the leptin receptor (50). LHA neurons may promote water intake in mice (51), in *Neotomodon* reduced hypothalamic leptin receptors and leptin transduction previously observed (27) may affect the leptin regulation of water intake.

Reduced BT in obese mice was on average, almost 1°C colder than that observed in lean mice, while during the active phase, there were times when the difference reached even 3°C below, such as the end of the night. These data suggest that obese mice might present reduced oxidation of fat deposits and thus lower heat production, implying a different homeostatic set point. A similar response has been previously observed in ob/ob mice. However, it has been discussed that such differences in thermoregulation may not include a shift in the homeostatic set point (52).

Metabolic phenotypes of obese mice may be useful to better understand the relationship between genetic disorders or physiological processes and energy metabolism *in vivo*. Understanding thermoregulatory alterations across different thermic challenges may reveal specific deficiencies in responses to cold and heat challenges (53). In obese *N. alstoni* mice, the exposure to a cold pulse did not change body temperature in lean mice, but a diversity of responses was observed in obese mice, especially during the resting phase. A decrease in body temperature in obese mice could be related to a possible facilitation of torpor in obese animals; this hypothesis may be tested by analyzing the circulating pyruvate in obese mice after a cold pulse that seems to facilitate the torpor (54). Daily torpor facilitates survival when animals are faced with limited energy resources, usually in adverse environments. In other *Peromyscus* species, torpor occurs when starvation is sufficient to significantly reduce body weight, and this is most notable in animals that fatten under laboratory conditions (55), as well as ob/ob obese mice (56). In obese *Neotomodon alstoni*, restricted access to feed for 4 h during the day produces a significant reduction in feed intake and body weight, but does not significantly reduce motor activity (57) or induce torpor. Obese *Neotomodon* seems to be more sensitive to cold rather than lack of food. The possible resistance to leptin in obese *Neotomodon* (27) could be involved in the torpor response to cold. In ob/ob mice, leptin has been shown to reduce the torpor response to a period of fasting (58).

The reduced thermoregulatory response appears to be linked to a deficiency of the UCP1 protein in iBAT (Figure 4A), which could be related to the different proportions of brown and white adipocytes in lean and obese mice. It has been demonstrated that transcription of the UCP1 gene is up-regulated by adrenergic stimulus (59), in our experiments, we found low presence of TH in obese mice. The UCP1 is a member of the mitochondrial carrier protein superfamily, all of which have similar molecular weights and structural similarities (60). We used a monoclonal antibody specific for rat and mouse UCP1 protein; however, we do not know if it recognizes *Neotomodon*'s protein; therefore, we cannot exclude the possibility that the antibody recognizes any of the UCP1 members of the mitochondrial superfamily. To understand whether *Ucp1* gene expression was proportional to the relative protein, we performed the mRNA analysis using a sequence for a phylogenetically related species *Peromyscus maniculatus*. BLAST analysis of the *Ucp1* sequence shows only one gene for *Peromyscus*, as for *Mus musculus*. As long as there is a lack of information in *Neotomodon*'s genome, approximation of these mechanisms at the genetic level depends on their homology with the closest species. The qPCR results indicate an elevation in the relative presence of *Ucp1* mRNA in obese mice, which does not correspond to the discrete relative presence of the protein. Despite the few samples analyzed ($n = 5$ for each group), the observed differences were striking. This result indicates that the canonical pathway of adrenergic induction of UCP1 might not be involved in such expression and it will be necessary to explore various transcription factors such as Pgc1α, Prdm16, and Pparα (7) to better understand the regulation of *Ucp1* expression in obese mice. UCP1 is also regulated either at the transcription of gene and protein activity in mitochondria as well as its possible regulation by cAMP, retinoids, thyroid hormone, and other factors (6). In this scenario, our data could be explained by events that need further studies: (a) a strong inhibitory regulation of the traslational activity (at ribosomal level, nuclear transport, etc.), (b) a fragility or rapid exchange of the synthetized protein (covalent modifications, intracellular transport, etc.). In order to understand the difference observed in mRNA and protein, future research is required.

Brown adipose fat is innervated by the sympathetic nervous system. Adrenergic-stimulated breakdown of triglycerides in the lipid vesicles of the brown adipocyte provides rapid activation of thermogenesis, followed by the efficient distribution of heat released from brown fat for delivery to vital organs (61). A first approximation considering TH relative presence in iBAT indicates a slight drop in TH presence in iBAT, which is in line with the decrease in UCP1 protein. It is possible that in obese *N. alstoni*, reduced sympathetic tone may be a consequence of leptin resistance in the hypothalamus. Obese *N. alstoni* show high circulating leptin, while there is low expression of leptin receptors in the hypothalamus and their correspondent activity (27). Leptin can modulate thermogenesis and regulate energy expenditure. The action of leptin on LepRb neurons in DMH/DHA and mPOA mediates sympathetic tone (11), which in turn regulates the metabolic activity of brown adipose tissue, mainly due to UCP1 activity.

In addition, this process allows heat production and its dissipation (derived from non-shivering thermogenesis) with the release and use of fatty acids from the brown adipose tissue (62, 63). Sensitivity to the thermoregulatory effects of leptin appears to be integrated in part at the level of DMH (64).

The physiological basis of the substantial differences in metabolic efficiency between lean and the obese mice requires further study and may play a key role in understanding the pathophysiology of obesity (65). The plasticity of the adipose tissue is well known; recent research provides insight that BAT undergoes "bleaching," a brown-to-white conversion induced by lipase and leptin receptor deficiency,

as well as impaired β-adrenergic signaling, leading to brown adipocyte expansion, death, and obesity-related inflammation (66). Furthermore, BAT malfunction and tissue inflammation, including macrophage activity, appear to decrease thermogenic expression by low sympathetic innervation and norepinephrine activity (67). In the present work, we observed that obese iBAT exhibit large numbers of adipocytes resembling white adipose cells, suggesting similar bleaching. Also, low relative TH expression may be related to low sympathetic innervation and norepinephrine activity.

Obese *Neotomodon* resembles the ob/ob mouse deficient in leptin production (68). The ob/ob mouse is about three times the body mass of the lean mouse while its daily food intake is similar. At ambient temperature BT in obese is ∼1–2°C lower than in the lean mice and presents a reduced capacity for thermogenesis due to exposure to cold without prior acclimatization (69, 70). Hypothermia in the ob/ob mouse is the result of a deficiency in heat production in brown adipose tissue (70, 71) consistent with a deficit in noradrenergic stimulation (72). On the other hand, mice with a leptin receptor deficiency (db/db) show also reduced energy expenditure in thermoregulation and thermogenesis such as in ob/ob mice (73). The deficit in noradrenergic stimulation of subcutaneous white and brown adipose tissue appears to be a consequence of a lack of leptin signaling in neurons expressing brain-derived neurotropic factor in the paraventricular nucleus of the hypothalamus (BDNFPVH), a descending neural pathway that it is crucial for energy homeostasis (74). The obese *Neotomodon alstoni* present hyperleptinemia, more clearly in females (26) and low leptin signaling in the hypothalamus (27). Given that in *Neotomodon* the obesity condition occurs spontaneously only in a subset of the captive mice, may be cause of some genetic deficit related to leptin signaling or leptin resistance that in this species still needs to be identified.

Although the causes may be multiple, it is essential to further investigate the relationship between thermoregulation deficit and obesity. For example, a deficit in TRPM8 cold receptors has been associated with low BT in obese mice (75). Furthermore, when given a cold pulse, lean Neotomodon showed a greater burst of locomotor activity than obese mice (data not shown), which involves the reduction of producing extra heat through exercise.

Obesity is the consequence of larger energy intake than energy expenditure. We noted in *N. alstoni* that mice eat more food. It is possible that the obesity in these mice may be a combination of a reduction in energy expenditure with a higher energy intake, considering that obese mice show reduced BT, locomotor activity, and low thermogenesis to compensate for a cold pulse than in lean mice, and less lipid oxidation during fasting; however, at this stage, we cannot distinguish which is cause and which is consequence.

Also, calorimetric assessment suggests that metabolic pathways regulating energy substrates and circadian organization of metabolism are impaired, and lipid catabolism is impaired in obese *N. alstoni* mice (34). The above is consistent with increased free fatty acids (FFA) and out of phase or reduced relative expression of hepatic PPARs, suggesting a potential enhanced lipolysis, but also possible deficient FFA uptake and/or reduced fatty acid oxidation in peripheral organs. A decreased metabolism and reduced heat production appear to be part of a circadian disruption in obese mice (76, 77). Obese animals are regulating their body temperature at a lower level than lean animals and are unable to maintain a stable body temperature in the face of a cold challenge. However, they are still able to maintain a daily rhythm; therefore, it does not appear to be a severe circadian dysfunction, but a rather a putative different set point in regulation. The causes of such change suggest hypothalamic differences that may target nuclei controlling thermoregulation, i.e., a function of heat-sensitive neurons in the preoptic anterior and dorsomedial hypothalamus that directly control the dissipation of heat (78).

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Bioethics Committee, Neurobiology Institute, and the Committee on Academic Ethics and Scientific Responsibility of the Sciences Faculty UNAM.

Author contributions

Conceptualization: MM-A, MD-M, and AH-G. Methodology: MM-A, AH-G, MP-M, EA-L, DG-M, and AC-C. Formal analysis: AH-G, MM-A, and DG-M. Resources: MM-A and MD-M. Review and editing: All authors. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

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Snack timing affects tissue clock and metabolic responses in male mice

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Snacking of small quantities of palatable food items throughout the day is common in modern societies and is promoted by 24/7 lifestyles. Long-term mistimed high-caloric food intake disrupts endogenous circadian rhythms and supports the development of obesity and other metabolic disorders. However, less is known about the time-of-day dependent effects of snacking. We hypothesized that already a single snacking episode may affect the circadian regulation of metabolic parameters, in particular when the snack is consumed during the daily rest phase. We performed an acute snack experiment in mice by providing access to chow or chocolate either at day- or nighttime and assessed snack effects on core body temperature, locomotor activity, and gene expression in metabolic tissues. Our results show that daytime chocolate snacking leads to a higher body temperature and locomotor activity increase compared to chow and nighttime intake. This goes along with altered clock and metabolic gene expression in peripheral tissues. Changes in nutrient uptake transporter gene expression in the small intestine suggest increased glucose resorption after daytime snacking. Our results indicate an early mechanism for the adipogenic effect of mistimed high-calorie snacking.

KEYWORDS

caloric intake, circadian clock, body temperature, locomotor activity, snack

Introduction

Eating palatable calorie-dense snacks during various phases of the day is a common behavior in Western societies with unlimited access to all kind of foods. A high-calorie diet is known to disrupt endogenous circadian rhythms and to shift food intake into the normal rest phase (1). While it is known that 1 week on a high-caloric diet is sufficient to shift endogenous rhythms in the liver (2), the acute effects of snacking on physiological parameters and circadian rhythms is much less understood.

Circadian clocks are internal timekeepers that synchronize physiology and behavior to external environmental rhythms such as the light-dark cycle (3). A master circadian pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus resets subordinate cellular clocks throughout the body to coordinate rhythmic functions within and between tissues and organs (4). At the molecular level, these clocks consist of interlocked transcriptional-translational feedback loops of clock proteins such as brain and muscle ARNT-like protein 1 (BMAL1 or ARNTL), circadian locomotor output cycles kaput (CLOCK), Period (PER1-3) and Nuclear receptor subfamily 1 group D member 1/2 (or NR1D1/2 or REVERBα/β) (3, 4).

While light is the main synchronizer of the SCN clock, altered food intake patterns can uncouple peripheral clocks from the SCN, thus leading to internal circadian desynchronization (5). In mice, daily food intake rhythms peak in the beginning of the active phase in line with the time of highest energy expenditure (6, 7). If food intake is restricted to the normal rest (i.e., light) phase energy expenditure is reduced and mice gain weight compared to animals with *ad libitum* food access (6). In contrast, appetite for highly palatable foods peak in the early inactive phase in mice, and also in humans, the drive to snack is higher toward the end of the active phase (i.e., in the evening) (7, 8). Sweet craving increases throughout the day in humans (9) and snacking rather than consuming one hot meal is common in night-shift workers (10). Studies on total energy intake in shift workers are controversial (11, 12), but disturbed eating patterns and increased snacking are consistently observed in night shift workers (13). Food intake-dependent signals such as insulin may provide feedback to the circadian system adjusting clock rhythms in metabolic tissues (14–16). However, the interaction of meal timing and type on the circadian clock system is still poorly understood.

We hypothesized that—in line with the increased vulnerability to snacking at this time—high-energy food intake would have stronger effects on circadian rhythms in the inactive phase. For this purpose, we offered mice chow or chocolate snacks at different times of day and investigated the impact on physiological parameters such as core body temperature and locomotor activity as well as on peripheral clock and clock target gene expression. Our data show that chocolate snacking has a stronger impact compared to chow on physiological parameters and tissue clock effects are largely restricted to the normal rest phase in mice.

Materials and methods

Animals and housing conditions

All experiments were performed with adult male wildtype mice on C57BL/6JRj background. Mice were purchased from Janvier labs (Le Genest-Saint-Isle, France). They were housed individually under standard laboratory conditions in a 12h: 12h light-dark cycle with *ad libitum* access to normal chow and tap water unless otherwise indicated. Experimental groups were age- and weight-matched. Experiments were carried out in accordance with the German Law for Animal Welfare and approved by the Ministry of Energy, Agriculture, Environment, Nature, and Digitalization (MELUND) of the State of Schleswig-Holstein, Germany.

Core body temperature/locomotor activity recordings and acute snack experiment

All animals were implanted with G2 E-mitters (Starr Life Sciences, Oakmont, USA) into the intraperitoneal space to measure body temperature and activity. Mice were anesthetized with isoflurane (4% in air), injected with 4 mg/kg Carprofen (Rimadyl, Zoetis, Parsippany, USA) and Bepanthen (Bayer, Leverkusen, Germany) was applied on the eyes. The abdomen was shaved, disinfected and the abdominal cavity opened by first cutting the skin and then the muscular layer. The sterilized E-mitter was implanted and the two layers of the abdominal wall were closed separately. After 1 week of recovery, temperature and activity were recorded on the experimental day in 1-min intervals using ER4000 receivers (Starr Life Sciences) and the Vital View software, version 5 (Starr Life Sciences). On day four, mice were fasted for 12 h and then received a snack for 20 min (or nothing for the control cohort). Nighttime snack mice were fasted from *zeitgeber* time (ZT; ZT0 = light onset) 2/2.5 on day four onwards, receiving a snack at ZT14/14.5 whereas daytime snack mice fasted from ZT14/14.5 onwards and received a snack at ZT2/2.5 on the next day. Mice had access to the snack for 20 min. Control mice were fasted but received no snack. All animals were sacrificed after another 40 min (ZT3/3.5 or ZT15/15.5).

Tissue and serum collection

Mice were sacrificed by cervical dislocation and trunk blood and tissues [subcutaneous white adipose tissue (scWAT), epididymal white adipose tissue (eWAT), intrascapular brown adipose tissue (iBAT), duodenum, jejunum, ileum, liver, pancreas] were collected. Tissue samples were stored in RNAlater (Invitrogen, ThermoFisher Scientific, Waltham, USA) and kept at 4°C for 11–12 h before transfer to storage at −20°C. Blood clotting was allowed at room temperature for 20 min followed by 30 min centrifugation at 664 rcf and 4°C. Serum samples were stored at −80°C until further processing.

Food intake measurements

Food intake was determined for the 20-min re-feeding period on the experimental day. Snacks—chow [breeding diet #1314, Altromin, Lage, Germany (14% fat, 27% protein, 59% carbohydrates, metabolized energy: ∼3,339 kcal/kg)] or chocolate (RUF milk chocolate drops, RUF, Quakenbrück, Germany; per 100 g: 2,099 kJ = 503 kcal, fat 27.1 g from which 16.7 g saturated fatty acids, carbohydrates 54 g from

which 47.6 g sugar, protein 6.5 g)—were weighed before and after mice had access. It was confirmed for each mouse that food was not crumbled. Nutrient composition in chow and chocolate was calculated using the manufacturer's indication of food composition (Supplementary Figure 1).

Serum levels of glucose, triacylglycerides, free fatty acids, insulin, and leptin

Serum glucose concentrations (mg/dL) were measured with a glucometer (ACCU-CHECK, Aviva, Roche, Mannheim, Germany). Triacylglyceride (TAG) concentrations were determined in duplicates using a triglyceride colorimetric assay kit (Cayman Chemical, Ann Arbor, USA) following the manufacturer's instructions. Free fatty acid (FFA) levels were measured in duplicates according to the kit's manual (serum/plasma fatty acid kit, non-esterified fatty acids detection, Zenbio, Durham, USA) with samples diluted 1:10 in dilution buffer. Serum insulin concentrations were measured in duplicates using a mouse insulin ELISA (Mercodia, Uppsala, Sweden) following the manufacturer's 5 µL protocol. A four-parameter logistic curve was fitted to calculate the concentrations. Due to limited material three samples were measured in singlets and the number of samples (n = 2–6) is not matching the n = 6 of glucose, FFA, and TAG. The blank absorbance value was subtracted from the mean of each duplicate and the concentration calculated according to the TAG, FFA or insulin standard curve, respectively. The insulin concentrations were converted as follows: 1 µg ≙ 174 pmol and 6 pmol/L ≙ 1 µIU/mL. Leptin levels were determined in duplicates using a mouse leptin ELISA kit (Crystal Chem, Elk Grove Village, USA) according to the kit's manual. Two samples were measured in singlets due to limited material. Data were analyzed in GraphPad Prism 8 (GraphPad, San Diego, USA).

Quantitative real-time PCR

Total RNA was isolated from tissue homogenates (Omni Bead Ruptor 24, Omni International, Kennesaw, USA) by Trizol (Ambion, Life Technologies, Austin, USA) chloroform (≥99.5%, Honeywell, Charlotte, USA) extraction. RNA was reverse transcribed using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Waltham, USA) according to the manufacturer's protocol. Go Taq qPCR master mix kit (Promega, Madison, USA) was used for qPCR. Plates were run on a CFX-96 or CFX-Connect thermocycler (Bio-Rad, Hercules, USA) and analyzed with the $\Delta\Delta C_t$ method using *Eef1α* as reference gene. Data were normalized to the mean ratio of the "no snack" group. Primer sequences were: *Bhlhe40* forward 5'-CTCCTACCCGAACATCTCAAAC-3', *Bhlhe40* reverse 5'-CCAGAACCACTGCTTTTTCC-3', *Bmal1* forward 5'-CCTAATTCTCAGGGCAGCAGAT-3', *Bmal1* reverse 5'-TCCAGTCTTGGCATCAATGAGT-3', *Clock* forward 5'-ATGGTGTTTACCGTAAGCTGTAG-3', *Clock* reverse 5'-CTCGCGTTACCAGGAAGCAT-3', *Cry2* forward 5'-AGATGGCCTCAGGTTTTCTCAG-3', *Cry2* reverse 5'-TTACGGCCCACTCTACCTTCT-3', *Eef1α* forward 5'- TGCCCCAGGACACAGAGACTTCA-3', *Eef1α* reverse 5'- AATTCACCAACACCAGCAGCAA-3', *Lipe* forward 5'-GGCTCACAGTTACCATCTCACC-3', *Lipe* reverse 5'- GAGTACCTTGCTGTCCTGTCC-3', *Per1* forward 5'-AGTTCCTGACCAAGCCTCGTTAG-3', *Per1* reverse 5'-CCTGCCCTCTGCTTGTCATC-3', *Per2* forward 5'-GCCAAGTTTGTGGAGTTCCTG-3', *Per2* reverse 5'-CTTGCACCTTGACCAGGTAGG-3', *Pnpla2* forward 5'-CAACGCCACTCACATCTACGG-3', *Pnpla2* reverse 5'-TCACCAGGTTGAAGGAGGGAT-3', *Nr1d1* forward 5'-AGCTCAACTCCCTGGCACTTAC-3', *Nr1d1* reverse 5'-CTTCTCGGAATGCATGTTGTTC-3', *Slc2a2* forward 5'-TCAGAAGACAAGATCACCGGA-3', *Slc2a2* reverse 5'-GCTGGTGTGACTGTAAGTGGG-3', *Slc5a1* forward 5'-TCTGTAGTGGCAAGGGGAAG-3', *Slc5a1* reverse 5'-ACAGGGCTTCTGTGTCTTGG-3'.

Data and statistical analysis

Data are represented as group mean ± SEM. Statistical analyses were performed in GraphPad Prism 8 (GraphPad, San Diego, USA) whereby p-values < 0.05 were considered significant. To compare data between groups and different time points 2-way analysis of variance (ANOVA) was used with Bonferroni *post-hoc* tests to compare data at one ZT between groups or within one group at different ZT's, respectively.

Temperature and activity data were recorded in 1-min intervals. The 15 min before the snack was given to the mice were taken as baseline measures for temperature and activity. Mean temperature or activity were compared to the maximum change in body temperature or activity after "snack in." Data were statistically analyzed by repeated measurement 2-way ANOVA with Bonferroni *post-hoc* tests. To further analyze changes in temperature and activity as well as the kinetics of these changes, each data point was normalized by subtracting the mean of the 15-min baseline. Such normalization allows a correction for individual differences in baseline body temperature and locomotor activity. For noise reduction in activity data a 10-min moving average was applied. Analyses were performed for each mouse individually. A non-linear fit (one site) was laid through the data for each group to determine the logIC50 value representing the half-time (in minutes) to the maximum change in temperature or activity. Analyses were performed with n = 6 mice for all cohorts; however, one no snack/daytime and one chow/nighttime animal were excluded due to technical reasons during the recordings. To calculate the mean change

in temperature and activity, the mean of the 15 min before the snack was subtracted from the mean of the 1 h after "snack in" until sacrifice. To compare changes in temperature and activity (logIC50), non-linear fits were performed for each mouse individually. During fitting of temperature data, a constraint was set to $0 < \text{logIC50} < 60$, but one no snack/nighttime and one chow/daytime mouse had to be excluded in addition to the two above mentioned mice because it was not possible to fit a sigmoidal curve with a plausible logIC50 through the data. For activity data the most accurate fits were obtained with a 10-min moving average over the non-normalized total counts. To not distort the fit due to decreasing activity in the second half of the experiment, only data including a moving average of 30.5 min were used to determine the half-time of activity change as a direct response to the snack. Constraints were set for some curves to ensure that the correct part of the curve was fitted compared to other mice in the group. In addition to the animals excluded for technical reasons, one no snack/nighttime mouse was excluded from activity logIC50 analysis as a reasonable fit could not be obtained. logIC50 values were statistically compared between cohorts and across time by 2-way ANOVA.

qPCRs were performed with $n = 6$ per cohort, but individual values were excluded based on Grubbs outlier tests. To analyze peripheral tissue gene expression profiles, the mean relative mRNA expression of the respective gene in every group was determined for each tissue, e.g., relative *Bmal1* mRNA expression in liver for the no snack/daytime group. Therefore, each data point in Figure 4 represents one tissue. Heat maps how the mean gene relative mRNA expression for each tissue. To investigate the relationship between clock gene mRNA levels against glucose transporter gene expression, simple linear regressions were performed using GraphPad Prism 8 (GraphPad, San Diego, USA).

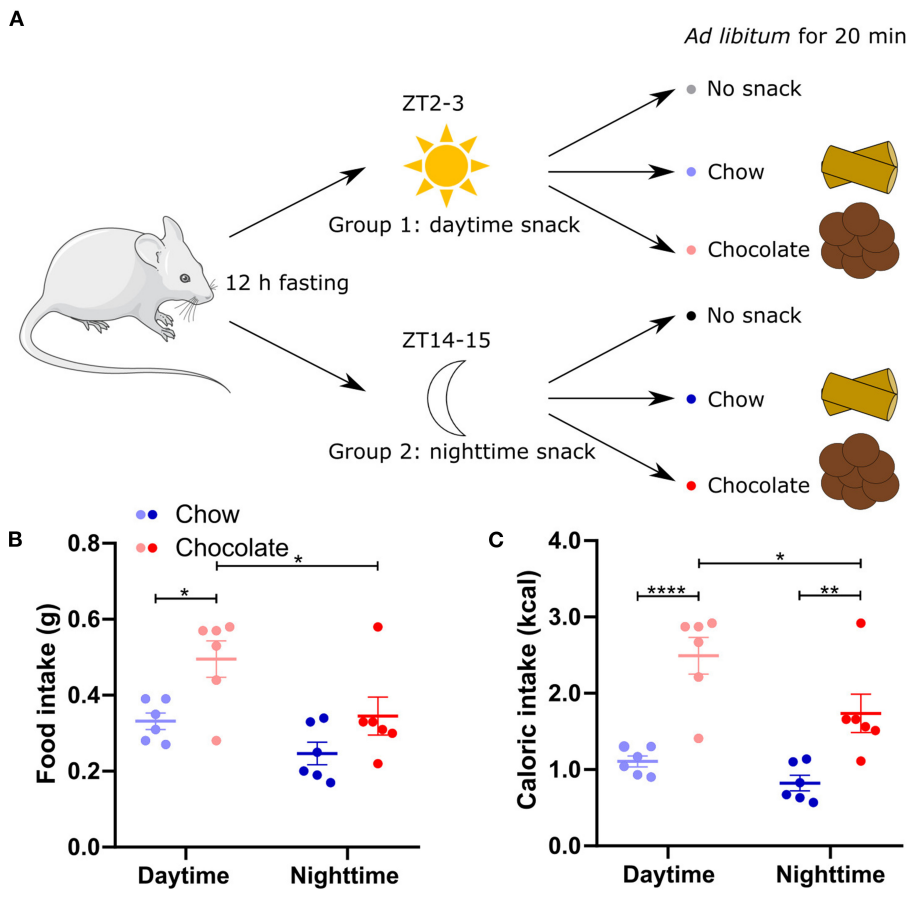

FIGURE 1
Food and caloric intake is higher after chocolate compared to chow snacking. **(A)** Experimental outline. Mice were separated in two groups to receive either a daytime (group 1) or nighttime (group 2) snack. After 12-h-fasting no snack (control cohort), normal chow, or chocolate was provided *ad libitum* as a snack for 20 min. **(B)** Food intake during 20 min chow or chocolate snacking. **(C)** Caloric intake during chow and chocolate snacking [calculated from **(B)**]. Data are shown as mean ± SEM; $n = 6$ per group; Bonferroni *post-hoc* test: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.0001$; 2-way ANOVA: **(B)** time, group $p < 0.01$, interaction $p > 0.05$, **(C)** time $p<0.05$, group $p < 0.0001$, interaction $p > 0.05$. Mouse image: smart.servier.com.

Results

Chocolate snack effects on caloric intake are higher during daytime

To assess the time-dependent effect of different snacks, mice were separated into two groups of three cohorts each. After 12 h of fasting, they received either no snack (control cohorts), a chow snack (chow cohorts) or a chocolate snack (chocolate cohorts) with access for 20 min (Figure 1A). The first group received the snack (or no snack) in the beginning of the light phase (ZT2-3, daytime) whereas the second group received the snack (or no snack) in the beginning of the dark phase (ZT14-15, nighttime). Mice consumed more energy during daytime compared to nighttime snacking for chocolate but not for chow. Caloric intake was always higher in mice receiving the chocolate compared to the chow snack independent of snack timing (Figures 1B,C). These data suggest that both, snack type and timing, influence appetite regulation, but temporal regulation is more pronounced for more palatable snack types.

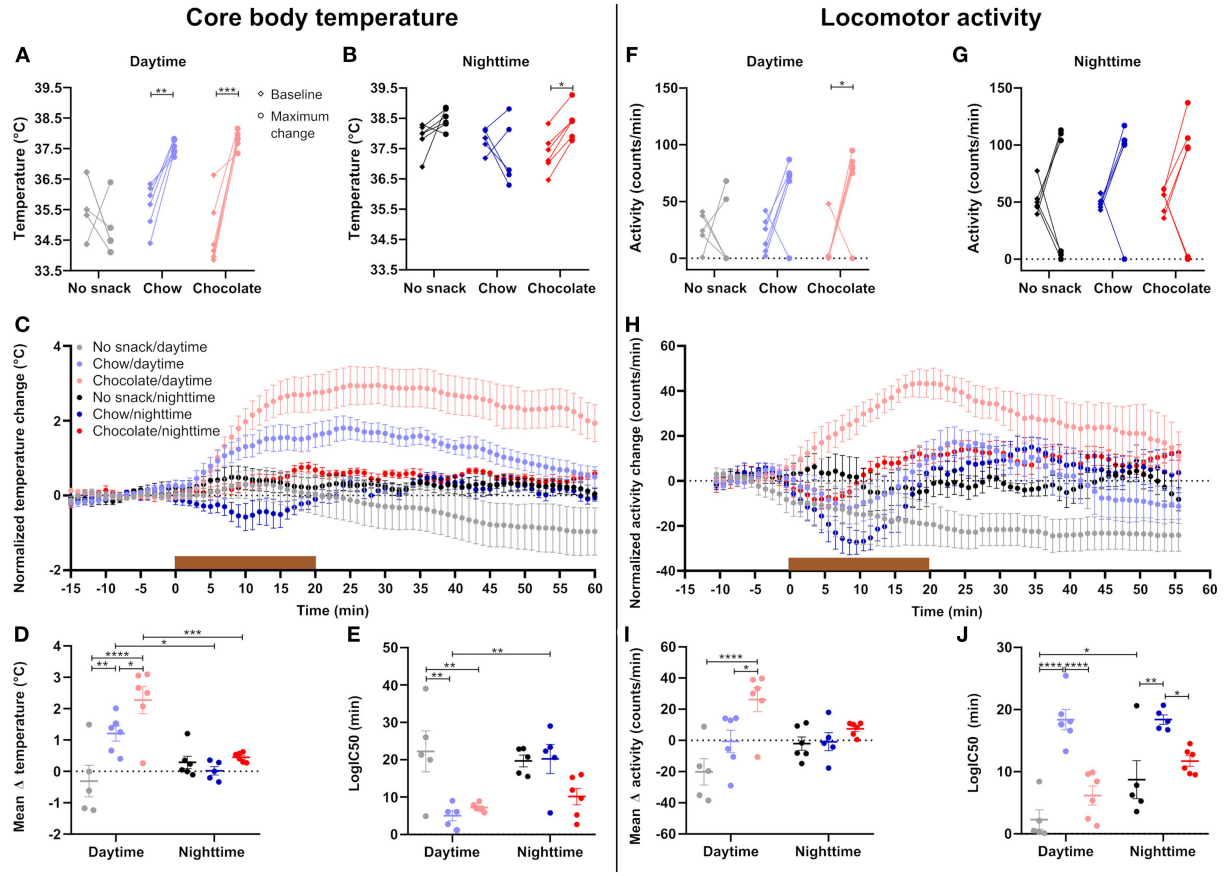

FIGURE 2
Daytime snacking leads to temperature and locomotor activity upregulation with a step-wise increase in chow and chocolate. Mean body temperature during the 15 min baseline (◊, left) and maximum change (○, right) in body temperature upon **(A)** daytime and **(B)** nighttime snacking for each mouse. **(C)** Change in body temperature upon snacking. Data were normalized by subtracting the mean temperature during the 15 min baseline. **(D)** Mean change in body temperature. **(E)** logIC50 from individual non-linear fits of body temperature curves. Mean activity during the 15 min baseline (◊, left) and maximum change (○, right) in activity upon **(F)** daytime and **(G)** nighttime snacking for each mouse. **(H)** Change in activity shown as 10-min moving average. Data were normalized by subtracting the mean activity during the 15 min baseline. **(I)** Mean change in activity. **(J)** logIC50 from individual non-linear fits of activity curves. Data are shown as mean ± SEM; $n = 5–6$ per group; **(A,B)** Bonferroni *post-hoc* test: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; **(A)** Maximum change no snack vs. chow $p < 0.0001$, maximum change no snack vs. chocolate $p < 0.0001$; **(B)** Maximum change no snack vs. chow $p < 0.05$; Repeated measurement 2-way ANOVA: **(A)** time, group, interaction $p < 0.001$; **(B)** time, group $p > 0.05$, interaction $p < 0.05$; **(C,H)** Non-linear fit. Brown bar indicates snack access for 20 min. **(D,E,I,J)** Bonferroni *post-hoc* test: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$; 2-way ANOVA: **(D)** time $p < 0.01$, group $p < 0.001$, interaction, $p < 0.01$, **(E)** time $p < 0.05$, group $p < 0.001$, interaction $p < 0.05$; **(I)** time $p > 0.05$, group $p < 0.001$, interaction $p < 0.05$, **(J)** time $p < 0.01$, group $p < 0.0001$, interaction $p > 0.05$. **(F,G)** Bonferroni *post-hoc* test: $*p < 0.05$; Repeated measurement 2-way ANOVA: **(F)** group $p < 0.05$, time, interaction $p > 0.05$; **(G)** time, group, interaction $p > 0.05$.

Snack type dependent upregulation of core body temperature and locomotor activity is restricted to daytime

Food intake leads to a transient increase in body temperature and locomotor activity, and both have been implicated in the postprandial regulation of satiety (17, 18). We thus examined the interaction of time and snack type on these two physiological parameters. After daytime snacking body temperature significantly increased compared to baseline in the chow and chocolate cohorts (Figure 2A) whereas a temperature increase after nighttime snacking was only observed in the chocolate cohort (Figure 2B). We next investigated the kinetics of the temperature increase upon snacking. While body temperature increased comparably early upon, both, chow and chocolate snacking during daytime, this increase was higher, and temperature stayed elevated for longer in the chocolate cohort (Figure 2C). In comparison, body temperature in the chow/daytime snacking cohort dropped back to baseline levels 1 h after the snack was provided (Figure 2C). In contrast to daytime, body temperature changes in the nighttime cohorts were smaller and largely comparable between snack types (Figures 2C,D). We did not observe differences in temperature increases between the control cohorts at different ZTs, but we found much larger temperature effects in the chow/daytime and chocolate/daytime compared to the respective nighttime cohorts (Figure 2D). Daytime snacking led to a stronger increase in body temperature in the chocolate cohort compared to chow (and no snack) with mean temperature changes of 2.6°C, 1.5°C and −0.3°C, respectively (Figure 2D). To compare the kinetics of body temperature changes upon snacking we performed a non-linear fit for each individual curve and determined logIC50 values. Daytime snacking with chow and chocolate induced a fast increase in body temperature (logIC50 < 10 min; Figure 2E). Additionally, chow/daytime snacking induced a faster body temperature increase than in the chow/nighttime cohort (logIC50 of 5 and 20 min, respectively; Figure 2E). These temporal effects were much less pronounced for the chocolate snack cohorts [logIC50 values of 7 (daytime) and 10 min (nighttime); Figure 2E]. Together, these data suggest that daytime snacking induces larger changes in body temperature and that these changes differ for snack type.

Similar to what we observed for body temperature, locomotor activity was affected mostly by snacking during daytime, while little effects were observed at nighttime (Figures 2F–H). Significant increases in locomotor activity compared to baseline activity were restricted to the chocolate/daytime cohort (Figures 2F,G). While the no snack/daytime cohort became less active due to extended fasting, chow-fed animals showed on average stable activity and chocolate/daytime mice a marked increase in activity peaking at 20 min after snack access (Figures 2H,I). Individual non-linear curve fits revealed delayed changes in activity in the chow cohorts at both time points compared to control and chocolate fed animals (Figure 2J). Overall, we observed a time-of-day effect for activity kinetics with a significant *post-hoc* comparison for the control cohorts (Figure 2J). In summary, the extents of locomotor activity changes upon snacking are in line with body temperature effects with larger changes induced by daytime snacking and chocolate snacks. However, marked differential effects were observed with regard to kinetics suggestive of distinct regulatory mechanisms.

Daytime snacking alters serum glucose and free fatty acid levels

We next analyzed the impact of timed chow or chocolate snacking on postprandial nutrient levels in the circulation (Figure 3). 1 h after the snack serum FFA levels were significantly decreased in the chow/daytime cohort and reduced with borderline significance ($p = 0.0540$) after chocolate snacking during the day compared to no snack controls (Figure 3A). Serum FFA concentrations were not altered in the nighttime cohorts (Figure 3A). We did not observe any snack induced changes in serum TAG levels apart from a general time effect with slightly higher levels at daytime compared to nighttime (Figure 3B). Serum glucose concentrations were increased after chow and chocolate daytime snacking compared to controls with higher levels in the chow cohort (Figure 3C). Nighttime serum glucose concentrations did not differ between the cohorts. Serum insulin levels were higher at daytime compared to nighttime, but we did not observe snack induced differences (Figure 3D). However, the insulin concentrations roughly followed serum glucose concentrations (Figures 3C,D). We did neither observe a significant effect for time nor snack group in serum leptin levels (Supplementary Figure 2). Together, snacking decreased FFA and increased glucose serum levels specifically during the day hinting at temporal differences in lipid mobilization and glucose uptake.

Time and snack dependent regulation of clock and metabolic gene expression in peripheral tissues

Considering the observed time and snack type specific responses in body temperature as well as in locomotor activity against a background of circadian regulation of both processes, we hypothesized that tissue clock gene expression might likewise be affected by snacking with higher changes after chocolate snacking and during daytime. We focused on peripheral tissues important for the processing of nutrients such as liver, pancreas, adipose tissues (eWAT, scWAT,

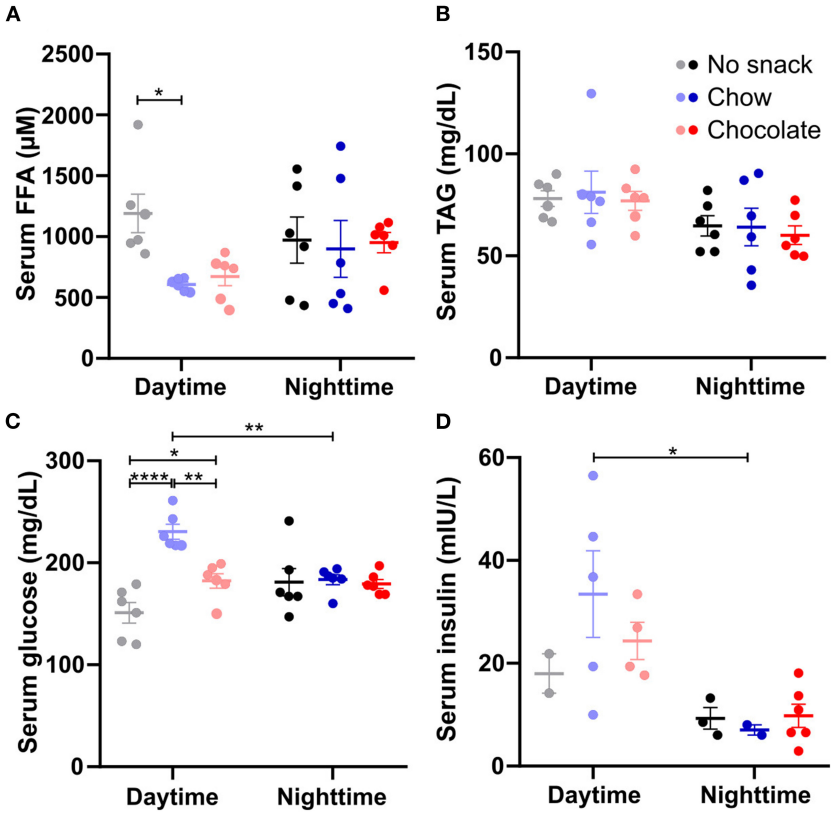

FIGURE 3
Daytime snacking alters serum free fatty acid (FFA) and glucose levels. Concentrations of **(A)** FFAs, **(B)** triacylglycerides (TAGs), **(C)** glucose, and **(D)** insulin in serum. Mice were fasted for 12 h and received no snack (control cohort), normal chow, or chocolate *ad libitum* for 20 min. Data are shown as mean ± SEM; **(A–C)** $n = 6$, **(D)** $n = 2$–6 per group; Bonferroni *post-hoc* test: *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$; 2-way ANOVA: **(A)** time, group, interaction $p > 0.05$, **(B)** time $p < 0.01$, group, interaction $p > 0.05$, **(C)** time $p > 0.05$, group, interaction $p < 0.001$, **(D)** time $p < 0.01$, group, interaction $p > 0.05$.

and iBAT), and small intestine (duodenum, jejunum, and ileum). Overall, results were largely comparable across all these tissues with marked temporal differences for *Bmal1*, *Per1*, *Per2*, *Cry2* and *Nr1d1* in the control cohorts and snack effects confined mostly to *Bmal1* and *Nr1d1* expression during daytime and *Per1*, *Per2* and Cry2 expression during nighttime (Supplementary Figure 3). To provide a more general overview, we plotted a heat map showing the mean relative mRNA expression for the respective gene and determined specific clock gene responses across metabolic tissues by averaging the mean relative clock gene mRNA expression for each group (Figure 4). A detailed analysis of each single gene in each tissue is provided in Supplementary Figure 3. Across all tissues, *Bmal1* expression was higher at daytime compared to nighttime with antiphasic regulation of *Per1*, *Per2*, and *Cry2* in the periphery in all snack cohorts (Figures 4A,C–E). *Bmal1* expression was decreased at daytime after chocolate but not after chow snacking while levels were stable during nighttime (Figure 4A). In contrast, *Per1* expression was decreased in the chow and chocolate cohorts during nighttime snacking with no effect during daytime (Figure 4C). For *Nr1d1*—and similar to *Bmal1*—we observed a snack induced decrease in expression during daytime, but no snack effect in the night (Figure 4B). Effects on *Per2* and *Cry2* expression were restricted to nighttime (Figures 4D,E). *Per2* was increased in the chow/nighttime cohort compared to the control and chocolate cohorts (Figure 4D). *Cry2* was decreased after chocolate/nighttime snacking compared to the chow and nighttime cohort (Figure 4E). *Bhlhe40* expression was comparable between different tissues at day-/nighttime after different snacks, however, it was increased in scWAT in the chow cohorts as well as in iBAT after chow/daytime snacking (Figure 4F). *Clock* mRNA expression did not respond to snack intake, but slightly higher daytime mRNA levels compared to nighttime were observed in the chow cohorts (Figure 4G). In summary, time and snack type dependent effects on clock gene mRNA expression were observed in metabolic tissues. Regulation of *Bmal1* and *Nr1d1* was restricted to daytime, while *Per1*, *Per2*, and *Cry2* responses were seen only after nighttime snacking.

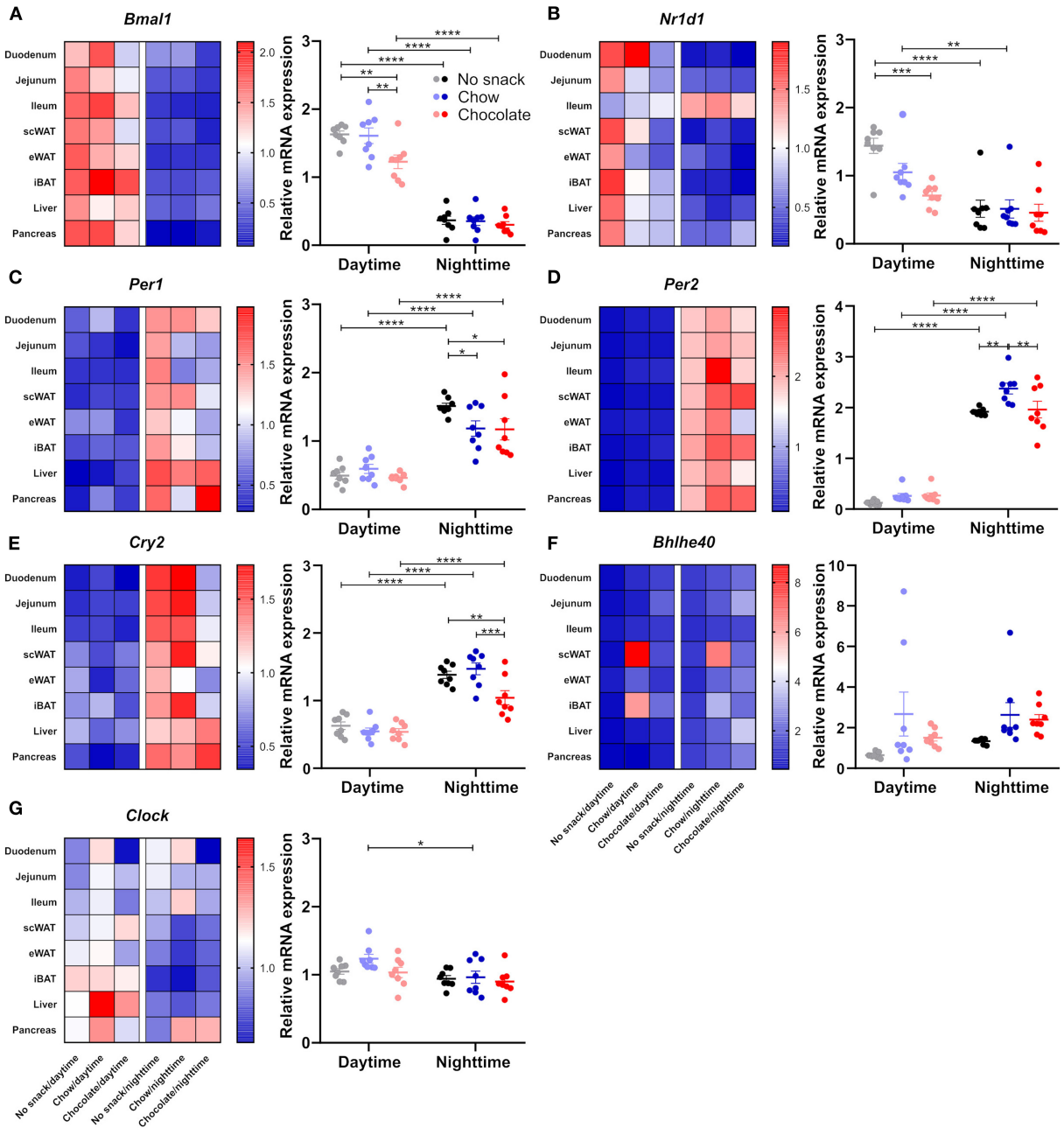

FIGURE 4
Snacking affects clock gene expression in peripheral tissues. Mice were fasted for 12 h and received no snack (control cohort), normal chow, or chocolate *ad libitum* for 20 min either at daytime or nighttime. Clock gene expression was determined 1 h after the snack was provided. Heat map of mean gene relative mRNA expression for each tissue and mean gene relative mRNA expression of **(A)** *Bmal1*, **(B)** *Nr1d1*, **(C)** *Per1*, **(D)** *Per2*, **(E)** *Cry2*, **(F)** *Bhlhe40*, **(G)** *Clock* in peripheral tissues (duodenum, jejunum, ileum, subcutaneous and epididymal white adipose tissue, intrascalpular brown adipose tissue, liver, pancreas). Each data point represents one tissue. Data are shown as mean ± SEM; $n = 3$–6 within $n = 8$ tissues per group; Bonferroni *post-hoc* test: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; 2-way ANOVA: **(A)** time $p < 0.0001$, group $p < 0.01$, interaction $p > 0.05$; **(B)** time $p < 0.0001$, group $p < 0.01$, interaction $p < 0.05$; **(C)** time $p < 0.0001$, group, interaction $p > 0.05$; **(D)** time $p < 0.05$, group $p < 0.01$, interaction $p < 0.05$; **(E)** time $p < 0.0001$, group $p < 0.01$, interaction $p < 0.05$; **(F)** group $p < 0.05$, time, interaction $p > 0.05$; **(G)** time $p < 0.01$, group, interaction $p > 0.05$. Separate analysis of each gene in each tissue as well as statistics are provided in Supplementary Figure 3.

Reduced FFA levels after snacking during daytime suggested an effect on lipolysis in adipose stores (Figure 3A). We therefore compared responses of clock and lipolysis associated gene expression in scWAT (Supplementary Figure 3, Figure 5). *Bmal1* expression was higher at daytime compared to nighttime for all groups and was reduced after chocolate/daytime snacking

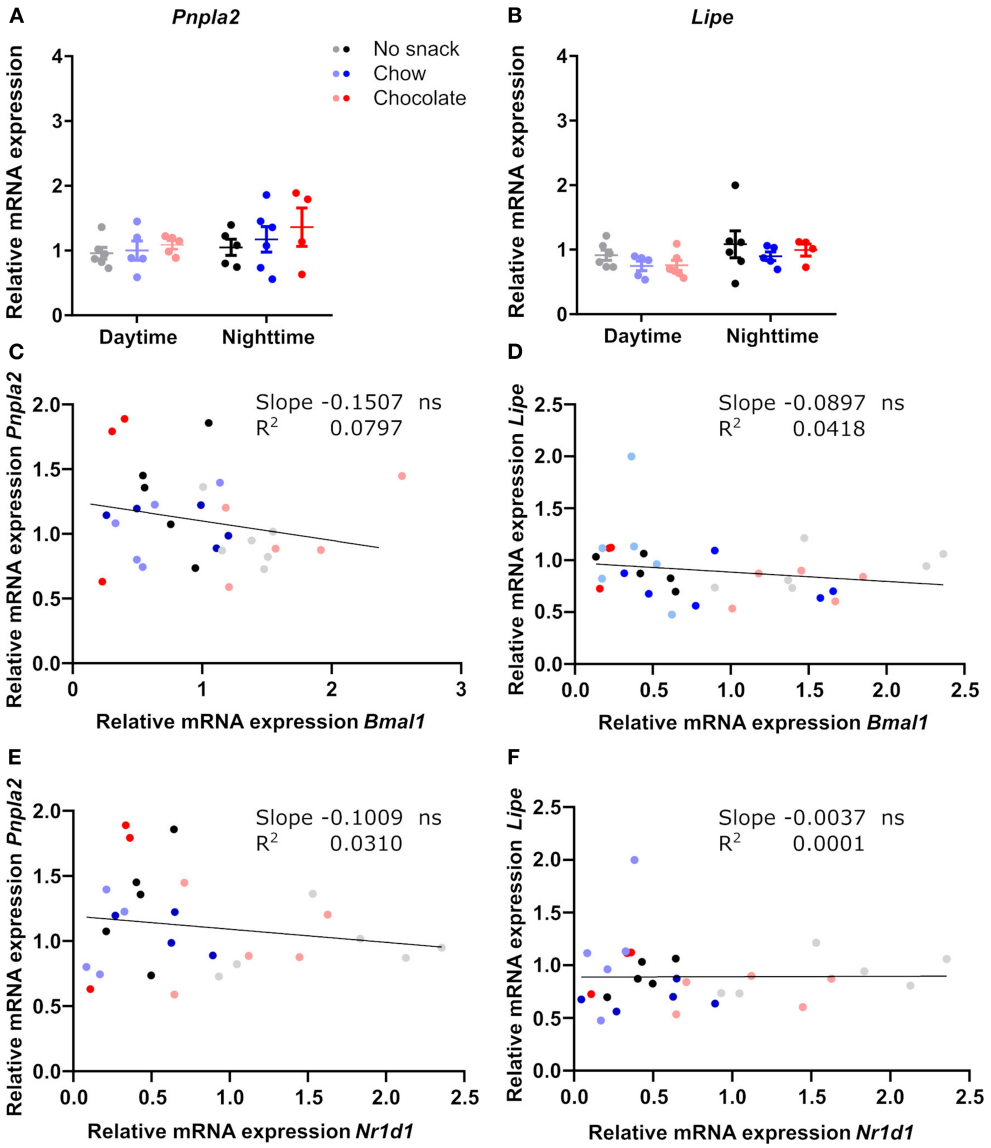

FIGURE 5
Genes of lipolysis key-enzymes in scWAT are not affected by snacking and do not correlate with clock gene expression. Relative mRNA expression of **(A)** *Pnpla2*, **(B)** *Lipe*. Linear regression of **(C)** *Bmal1* and *Pnpla2*, **(D)** *Bmal1* and *Lipe*, **(E)** *Nr1d1* and *Pnpla2*, **(F)** *Nr1d1* and *Lipe*. **(A,B)** Data are shown as mean ± SEM; $n = 4–6$ per group; 2-way ANOVA (time, group, interaction $p > 0.05$) with Bonferroni *post-hoc* test. **(C–F)** Simple linear regression analysis; $n = 3–6$ per group. ns, not significant.

compared to the control cohort (Supplementary Figure 3D). We observed similar effects for *Nr1d1* expression, but the time-of-day difference between the chocolate cohorts was lost due to a strong reduction in *Nr1d1* expression after chocolate/daytime snacking (Supplementary Figure 3D). Additionally, *Nr1d1* expression was reduced in the chocolate compared to the chow/daytime cohort (Supplementary Figure 3D). In contrast, we did not find any changes in genes encoding two lipolysis pacemaker enzymes, *Pnpla2* and *Lipe* (Figures 5A,B), nor a correlation between clock and lipolysis associated gene expression (Figures 5C–F). Thus, although FFA serum levels were reduced after daytime snacking (Figure 3A) and lipolysis genes have been reported to be under control of the circadian clock (19), our data would be in line with a snack-induced downregulation of adipose lipolysis *via* resetting of local tissue clocks.

Like FFAs, blood glucose levels were mainly affected by snack intake during daytime. As the small intestine, especially duodenum and jejunum, are important for glucose uptake (20), we investigated relative mRNA expression of glucose

transporter genes in comparison to clock genes in response to snack intake (Supplementary Figure 3, Figure 6). Expression of *Bmal1* and *Nr1d1* in the jejunum was reduced after snacking during daytime with little responses during nighttime snacking (Supplementary Figure 3B). The expression of both glucose uptake transporter genes, *Slc5a1* and *Slc2a2*, was decreased in the chocolate/nighttime cohort compared to the no snack/nighttime cohort leading to a loss of the time-of-day difference in chocolate cohorts (Figures 6A,B). As both, clock and glucose uptake transporter, transcript levels were affected by snacking, we investigated the relationship between both by linear regression analysis. Interestingly, we found a negative correlation between *Bmal1* and *Nr1d1*, respectively, with both glucose uptake transporter genes (Figures 6C–F).

Duodenal clock gene expression was reduced after chocolate/daytime snacking compared to the chow cohort (*Bmal1*) or the control and chow cohorts (*Nr1d1*), respectively (Supplementary Figure 3A). All cohorts had a higher expression at daytime compared to nighttime except of the chocolate cohort for *Nr1d1* (Supplementary Figure 3A). Interestingly, relative mRNA expression of glucose uptake transporters was decreased in the chocolate cohorts at both time points

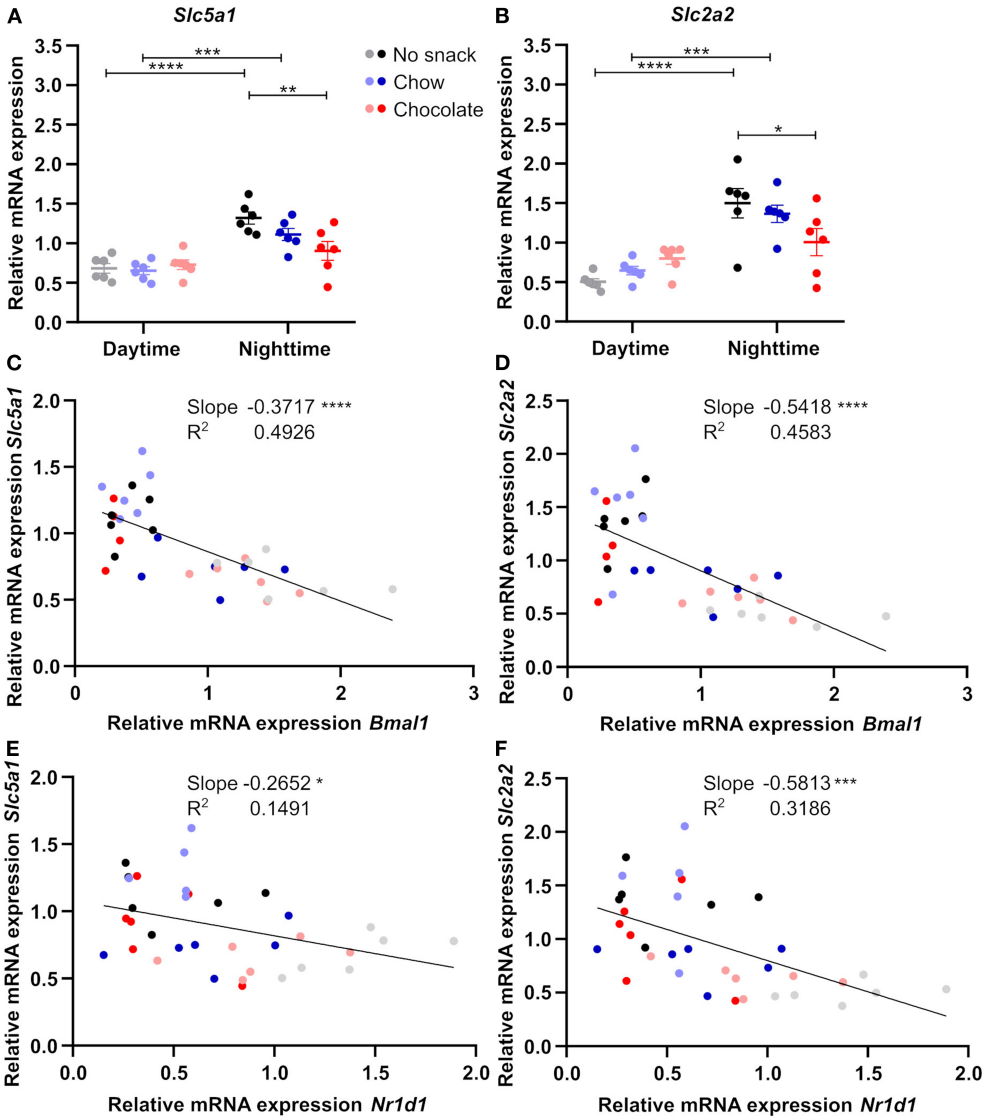

FIGURE 6
Jejunal glucose uptake transporter gene expression is influenced by snack type and negatively correlates with clock gene expression. Relative mRNA expression of **(A)** *Slc5a1*, **(B)** *Slc2a2*. Linear regression of **(C)** *Bmal1* and *Slc5a1*, **(D)** *Bmal1* and *Slc2a2*, **(E)** *Nr1d1* and *Slc5a1*, **(F)** *Nr1d1* and *Slc2a2*. **(A,B)** Data are shown as mean ± SEM; $n = 5–6$ per group; Bonferroni *post-hoc* test: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; 2-way ANOVA **(A)** time $p < 0.0001$, group $p > 0.05$, interaction $p < 0.05$, **(B)** time $p < 0.0001$, group $p > 0.05$, interaction $p < 0.01$. **(C–F)** Simple linear regression analysis; $n = 4–6$ per group. *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$.

compared to the control and chow cohorts except of control vs. chocolate/daytime for *Slc5a1* (Supplementary Figures 4A,B). However, unlike for jejunum, we did not find significant correlations between clock and glucose transporter gene expression in the duodenum (Supplementary Figures 4C–F).

The observed relationship of clock and glucose uptake transporter gene expression in the jejunum, but not in the duodenum, support the idea of a potential role of the jejunal circadian clock in mediating changes in intestinal glucose uptake upon snacking.

Discussion

In our study we investigated time-of-day dependent effects of snacking on tissue circadian clock function and physiological parameters. Our results indicate that a high-calorie snack such as chocolate at daytime has a stronger effect on physiological parameters as well as on peripheral clock gene expression than less energy-dense snack types or the same snack consumed during nighttime. This temporal regulation associates with similar effects on glucose regulation in line with clock and glucose uptake transporter gene regulation in the jejunum.

Snack type and time influence appetite and temperature/activity responses

In our snack experiment we observed an effect of snack type and snack time on behavioral and physiological responses. Chocolate consumption was always higher than chow consumption and chocolate/daytime mice consumed more than the respective nighttime cohort (Figure 1). These results go in line with data showing that overconsumption of chocolate peak in the early inactive phase in mice and time-of-day differences in the drive to eat in humans (7, 8). Likewise, we found a snack type dependent upregulation of core body temperature and locomotor activity mostly restricted to daytime (Figure 2). As body temperature shows a circadian rhythm with higher temperature throughout the active phase (21) and even on heat stress mice show little increase in body temperature throughout their active phase (22), a large body temperature increase upon snacking was not expected at nighttime. We observed a higher upregulation in body temperature after chocolate compared to chow/daytime snacking. As mice consumed more calories after a chocolate snack, these findings are in line with previous studies showing that body temperature is higher after high caloric meals (23). It has previously been shown that diet composition impacts body temperature (24). Considering the different nutrient composition of the snacks in this study (Supplementary Figure 1), an effect of snack type on body temperature change remains possible. However, we did not closer investigate the specific effects of individual macronutrients and therefore cannot distinguish which factor—snack type (i.e. snack composition) or calories—is mediating the observed effects. In line with the changes in core body temperature we found similar changes in locomotor activity after daytime snacking. Larger changes throughout nighttime snacking were not expected as the capacity for changes is higher throughout the usual inactive phase (i.e., daytime). Overall, we did not observe a mean change in activity in chow snacking mice. These animals first reduced their activity as they were eating the snack and then became more active afterwards. Our observation of upregulated locomotor activity after chocolate/daytime snacking is in line with human data showing that a daily chocolate snack for dinner leads to increased activity (25). However, one has to consider that subjects were monitored after a chronic chocolate snack showing overall activity (25). Additionally, our data of the chow/daytime cohort show that temperature increases do not merely follow upregulated locomotor activity.

Clock gene responses are influenced by snack time and type

Because of our findings of upregulated core body temperature and locomotor activity restricted to daytime snacking, we assumed a snack effect on peripheral clock gene expression. We found slightly more reduced relative mRNA expression of clock genes in the periphery after chocolate/daytime snacking hinting at a potential influence of snack type on clock gene expression (Figure 4). After nighttime snacking, we observed changes in the chow as well as in the chocolate cohorts (Figure 4). Clock gene expression is shifting and uncoupling from the SCN upon light-phase restricted feeding (5). But also fasting and re-feeding is changing peripheral tissue clock gene expression (26). Although our data do not determine full circadian profiles, they are in line with others showing downregulation of *Nr1d1* and *Per1* mRNA expression upon re-feeding (26). *Bmal1* mRNA expression was rather seen to be upregulated (26). The differences of those results and ours could be explained by different re-feeding times, durations and snack types. As we investigated only two time points, we cannot comment on circadian rhythm parameters such as period, phase or amplitude.

A potential role of jejunal circadian clocks in regulation of glucose metabolism and effects of snack type and timing

Our results revealed decreased FFA and increased glucose serum levels specifically in response to daytime snacking

(Figure 3) suggesting alterations in lipolysis and glucose uptake. Upon fasting, lipolysis is enhanced increasing FFA levels in the blood (27). After daytime re-feeding in rats, FFA concentrations in serum decreases again in line with our data (27). Another study found a fast reduction of FFA upon food intake in humans after breakfast and dinner (28). We did not observe decreased serum FFA levels after nighttime snacking in mice. However, these animals were only fasted for 12 h, so basically throughout daytime, the usual phase where they normally do not consume many calories. Potentially, the difference in FFA levels upon snacking was therefore more visible after daytime snacking because these cohorts were likely fasted even longer than 12 h due to the normal daily food intake pattern with low intake during the light phase. Our observation of unchanged TAG levels after daytime or nighttime snacking are in line with other data showing that plasma TAG levels in humans are unaltered after breakfast and dinner at least 1 h after the meal (28). The apparent absence of changes in leptin levels (Supplementary Figure 2) could be explained by different kinetics of leptin responses after snacking. We found altered serum glucose levels after daytime snacking and a time-of-day difference after chow snacking. These results are consistent with data showing that glucose tolerance varies throughout the day peaking in the morning (active phase) and that the postprandial glucose response is higher in the evening in humans (29). A higher increase in glucose concentration is also consistent with human data showing a higher glucose increase after dinner vs. breakfast (28). Unexpectedly, glucose levels after chocolate/daytime were lower compared to chow/daytime snacking. Mice consumed the chocolate within the very first min after the snack was provided which could indicate that the glucose levels were already dropping again, but we also cannot exclude an influence of snack type/food composition on serum parameters. Glucose uptake by peripheral tissues could be differently affected in the two daytime snacking cohorts. More glucose could be taken up by adipose tissue, by the liver for glycogen storage, or by the muscle and thereby reduce serum glucose levels in the chocolate/daytime cohort. As mice consume most of their food during the active phase, equal experimental fasting will likely be longer for the daytime than for the nighttime cohorts and, consequently, daytime cohorts might have been at a different fasting/metabolic state in the beginning of the snack time. Differences in insulin release upon chow or chocolate consumption could also explain the observed changes in the chow/daytime and chocolate/daytime cohorts. In our study insulin concentrations followed changes in glucose, however, they did not reach significance due to high variations within the cohorts. Lower insulin levels in the nighttime cohorts could indicate a higher insulin sensitivity at nighttime compared to daytime. A higher insulin sensitivity in the beginning of the active phase was also reported in humans (30). Additionally, changes in lipolysis are a possible explanation for the differences we observed in FFA serum concentrations after daytime snacking.

Due to the downregulation of *Bmal1* expression after chocolate, but not after chow/daytime snacking, we analyzed lipolysis related gene expression and the relationship of lipolysis and clock gene transcript levels. *Pnpla2* and *Lipe* are downregulated in *Bmal1* knockout mice (19). However, our data did not support a downregulation of lipolysis in scWAT upon daytime snacking (Figure 5). FFA are increased in the blood upon fasting and decrease after re-feeding (27). We already saw decreased FFA levels upon daytime snacking (Figure 3). However, the analyzed time point might be too early to observe changes upon lipolysis related genes or the effect of fasting might be stronger on mRNA expression. We also did not observe a relationship between clock and lipolysis related gene expression.

Our results indicate changes in clock and glucose uptake transporter gene expression upon snacking in the small intestine (Figure 6, Supplementary Figures 3, 4). Studies in cells revealed a regulation of BMAL1 on glucose uptake and *Slc5a1* mRNA expression (31). Additionally, BMAL1 can directly bind to the promotor region of glucose uptake transporter genes (32). In line with these data, we found an association between clock and glucose uptake transporter gene expression in the jejunum. Importantly, we mainly observed changes in glucose uptake transporter gene expression upon chocolate snacking. Even though the chocolate contains less carbohydrates compared to chow (Supplementary Figure 1), it contains more disaccharides (carbohydrates 54 g from which 47.6 g sugar vs. 5% dissaccharides but 35% polysaccharides in normal chow). Our data, thus, are in agreement with a potential role of the jejunal circadian clock on glucose uptake upon snacking.

In conclusion, we here show that distinct snack types have different effects on tissue circadian clocks whereby the effect is strongest after snacking during daytime. Our data suggest that an acute snack could influence the circadian clock and modulate glucose uptake in the small intestine. With our experimental setup we analyzed acute snack effects on gene expression, but we cannot make conclusions about how these effects would alter circadian rhythm parameters. As our study is limited to male mice it would be interesting to investigate whether the same effect is observed in females. These observations in mice could be interesting for shift workers with an irregular eating pattern. Although our data suggest a potential role of the jejunal clock in snack associated glucose uptake, further confirmatory work is needed to, e.g., investigate whether an acute snack at the "wrong" time of the day could lead to a larger shift in the internal clock network.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by the Ethics Committee of the Ministry of Energy, Agriculture, Environment, Nature, and Digitalization (MELUND) of the State of Schleswig-Holstein, Germany.

Author contributions

KB and HO designed the experiments, wrote the paper, revised, and approved the submitted version. KB performed experiments and analyzed data. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.956641/full#supplementary-material

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A matter of time: A systematic scoping review on a potential role of the circadian system in binge eating behavior

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Background: Emerging research suggests that food intake timing, eating behavior and food preference are associated with aspects of the circadian system function but the role that the circadian system may play in binge eating (BE) behavior in humans remains unclear.

Objective: To systematically evaluate the evidence for circadian system involvement in BE behavior.

Methods: Systematic searches of PubMed, EMBASE, and Scopus were performed for reports published from inception until May 2020 (PROSPERO Registration CRD42020186325). Searches were conducted by combining Medical Subject Headings related to the circadian system, BE behavior, and/or interventions. Observational and interventional studies in humans with BE behavior published in peer-review journals in the English language were included. Studies were assessed using quality and risk of bias tools (AXIS, ROB 2.0, or ROBINS).

Results: The search produced 660 articles, 51 of which were included in this review. Of these articles, 46 were observational studies and 5 were interventional trials. Evidence from these studies suggests that individuals with BE behavior tend to have more food intake, more binge cravings, and more BE episodes later in the day. Hormonal and day/night locomotor activity rhythm disturbances may be associated with BE behavior. Furthermore, late diurnal preference ("eveningness") was associated with BE behavior and chronobiological interventions that shift the circadian clock earlier (e.g., morning bright light therapy) were found to possibly decrease BE behavior. Substantive clinical overlap exists between BE and night eating behavior. However, there is a significant knowledge gap regarding their potential

relationship with the circadian system. Limitations include the lack of studies that use best-established techniques to assess the chronobiology of BE behavior, heterogeneity of participants, diagnostic criteria, and study design, which preclude a meta-analytic approach.

Conclusion: Current evidence, although limited, suggests that the circadian system may play a role in the etiology of BE behavior. Further mechanistic studies are needed to fully characterize a potential role of the circadian system in BE behavior. A chronobiological approach to studying BE behavior may lead to identification of its neurobiological components and development of novel therapeutic interventions.

Systematic review registration: [https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42020186325], identifier [CRD42020186325].

KEYWORDS

binge eating, circadian, chronobiology, actigraphy, obesity, chronotype, light, night eating

Introduction

Binge eating (BE) behavior is a form of disordered eating characterized by consuming an objectively large amount of food in a short amount of time with a sense of loss of control over eating (1). BE behavior is a core feature of the eating disorders (EDs) binge-eating disorder (BED) and bulimia nervosa (BN), and may occur in anorexia nervosa (AN). BE behavior may also occur in night eating syndrome (NES), classified as an Other Specified Feeding or Eating Disorder in the *DSM-5*, as well as in non-ED-psychiatric diagnoses like mood and attention-deficit/hyperactivity disorder (ADHD) (2–5). Currently, BE behavior is often identified as a symptom associated with deficits in impulse-control but its neurobiology remains poorly understood (3).

The circadian system is a robust multi-oscillator circadian network influencing most physiological and behavioral processes, including metabolism, hunger, food intake timing, and eating behavior (6–8). The circadian system consists of the suprachiasmatic nucleus (also called the master pacemaker) located in the hypothalamus and of peripheral oscillators or "clocks" located in other regions of the brain and most tissues of the body (8, 9). Inadequate or mistimed interactions among components of the circadian system with the environmental and/or behavioral cycle can have profound physiological consequences and are associated with multiple adverse health outcomes, which may include disordered eating (9–14). Early studies suggest that time-of-day clinical features are associated with BE behavior (15, 16) and that targeted chronobiological interventions may have therapeutic potential in EDs (17), suggesting a possible circadian system involvement in BE behavior not yet thoroughly explored. Moreover, variability across the day and night in a physiological variable can be the result of changes in behavior or environmental conditions cycling in parallel and do not necessarily reflect involvement of the circadian system.

Best-established techniques like constant routine (CR) or forced desynchrony (FD) are typically used to discern a change in the circadian rhythm in physiology and behavior by minimizing the influence of behavioral and environmental effects or by evenly distributing them during the circadian cycle (18). However, these techniques are typically conducted in well-controlled experimental conditions and have not been applied to study the role of the circadian system in BE behavior.

Emerging research supports that food intake timing (10, 19), eating behavior and food preference (12, 14, 20) are associated with aspects of the circadian system function in humans but the role that the circadian system may play in BE behavior remains unclear. Given the absence of published studies using best-established methods to study the circadian system in BE behavior, we aimed to systematically review current evidence on circadian-related or proxy measures that can inform the potential role of the circadian system in the etiology, phenomenology, and treatment of BE behavior.

Method

Initially, a systematic review and meta-analysis approach was conducted following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklist guidelines (**Supplementary Table 1**) (21). The study protocol

was registered with the International Prospective Register of Systematic Reviews (PROSPERO); registration number CRD42020186325. The limitations on available evidence precluded a quantitative analysis on each of the specific sub-categories initially planned to conduct the systematic review and meta-analysis approach. In this context, the complex and heterogeneous nature of the current body of evidence gave way to a systematic scoping review on the existing literature in this topic (22).

Database searches

Searches for relevant scientific articles were conducted in PubMed, EMBASE, and Scopus from inception to May 1st, 2020. Observational and interventional studies in humans published in peer-review journals in the English language were included. References from reviews, systematic reviews, and meta-analyses were also evaluated to identify additional original studies.

Observational articles included were those reporting on human BE behavior and some aspect of a circadian-related function. Studies included individuals with BE behavior and/or a diagnosis of BED, BN, AN, or NES. We included night eating because of its clinical overlap with BE behavior (23, 24). Aspects of circadian-related function included: (1) timing of BE behavior; (2) timing of food intake; (3) a behavioral assessment of chronotype (e.g., dimensional or categorical); or (4) objective evaluations of a circadian-related function (e.g., dim light melatonin onset, actigraphy or sleep/wake cycle).

Interventional trials included were those evaluating chronotherapeutic strategies in individuals with BE behavior and reporting on BE behavior as an outcome. Interventions of chronobiological relevance included light therapy, melatonergic agents, or scheduled food intake. Other interventions that may influence the circadian system via changes in sleep/wake cycles or the timing of light exposure targeting the circadian system in conditions with BE behavior (e.g., interpersonal and social rhythms therapy) were considered if their effect on a circadian-related assessment (e.g., circadian phase, actigraphy) was reported.

Specific search terms used for circadian-related factors were: suprachiasmatic nuclei or nucleus, biological clock, circadian, chronotype, morningness-eveningness, morning, evening, night eating, nocturnal binge, and skipping meals (e.g., breakfast). The following specific search terms for BE behavior and food intake timing were used: anorexia nervosa, bulimia nervosa, night eating, night eating syndrome, BE, and food intake timing or food intake time (or temporal pattern). Specific terms for chronological interventions were the following: bright light, light therapy, melatonin, melatonin agonist, melatonergic, and scheduled food intake.

Data extraction

All potentially relevant studies were screened by two authors (FR-N, AG, or NM) after independent evaluation of titles and abstracts. A third author (SM, FR-N, AG, or NM) made a final decision when there was disagreement between reviewers regarding the eligibility of a study. Upon completion of the screening process, a list of studies was compiled and reviewed for repetitions. Full articles were then reviewed to assess eligibility. Additional studies were selected when identified after reviewing a study. Two authors discussed the findings (FR-N, AG, or NM) and a third author (FR-N, AG, NM, or SM) helped make a final decision when there was disagreement. Reviewer agreement was substantial (Fleiss' kappa = 0.72). A critical appraisal of selected observational studies was conducted using the Appraisal tool for Cross-Sectional Studies (AXIS tool) (25) and is presented separately for each section (**Supplementary Tables 2–4**). For the interventional studies risk of bias was assessed using the COCHRANE ROBINS for non-randomized trials or Rob 2.0 tools for randomized trials (see **Supplementary Figures 1**, **2**, respectively) (26, 27). The PRISMA flowchart was followed for two categories of studies: observational and interventional. A data extraction form developed by the authors was used to systematically collect data from identified studies.

Strategy for data synthesis

Although considered initially, a meta-analytic approach for data synthesis was not possible due to the small number of studies meeting criteria for each subsection and their considerable methodological differences (heterogeneity). We therefore classified the 51 identified studies into two categories: observational and interventional, and conducted narrative syntheses of each category. Potential links between the categories and sections were addressed in the discussion.

Results

Observational studies

Forty-six observational studies reported relevant information on circadian-related features of BE behavior and/or food intake patterns, circadian-related outcome measures, or night eating among individuals with BE behavior (see **Figure 1**). Because these studies had heterogeneous methods, we elected to summarize them in three main sections: (1) patterns of food intake and/or timing of BE behavior (N = 16); (2) assessment of the circadian-related measures among individuals with BE behavior (N = 12); and (3) the co-occurrence of BE behavior and night eating (N = 18).

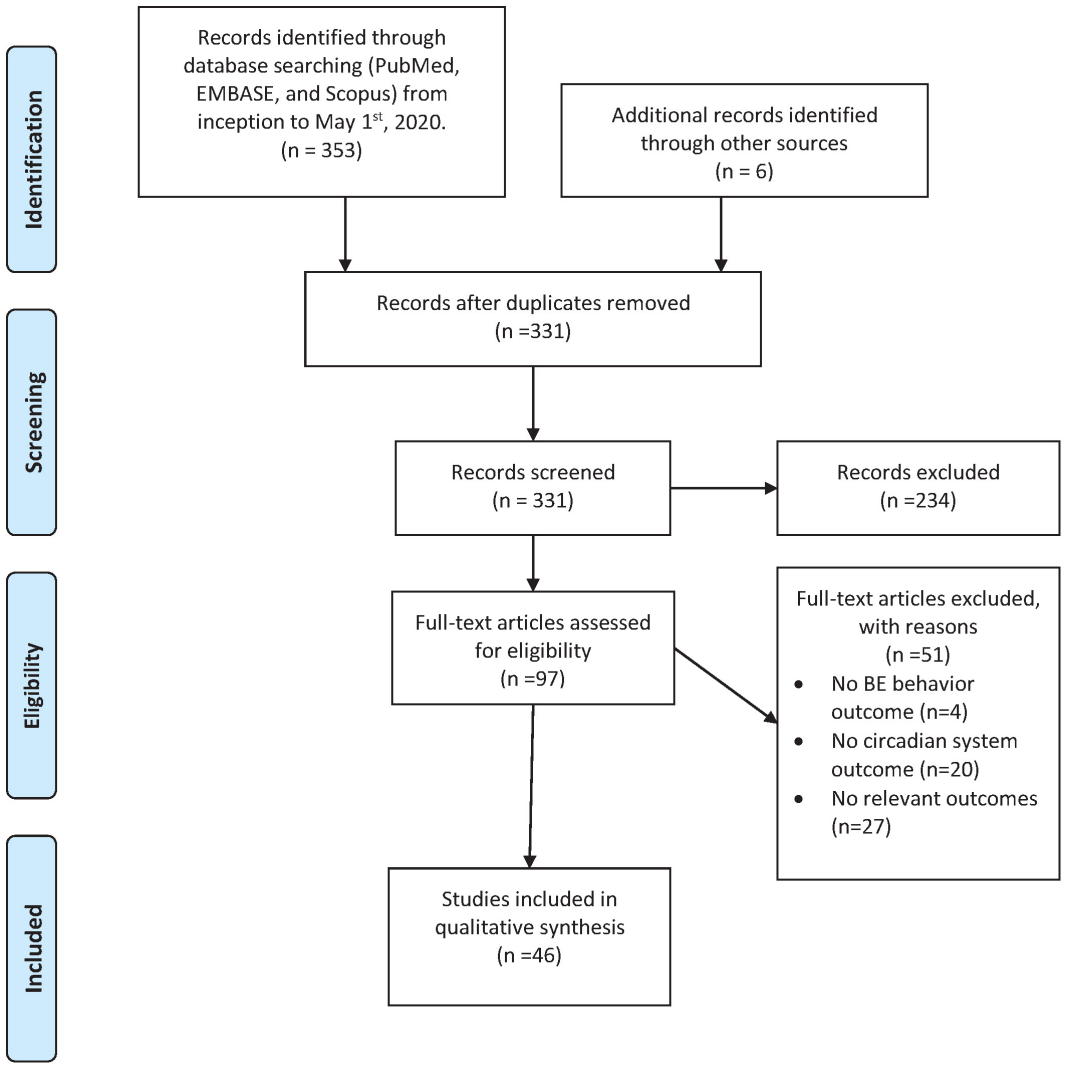

FIGURE 1
PRISMA flowchart for observational studies.

Patterns of food intake and/or timing of binge eating behavior

Sixteen studies reported information on time-of-day related features of BE behavior and/or food intake among individuals with BE behavior (**Table 1** and **Supplementary Table 2**). Nine of these studies explored food intake patterns throughout the day among individuals with BE behavior (15, 28–35); five explored timing of BE behavior or of the urge to binge eat (16, 36–39); one study evaluated the relationship between BE behavior and photoperiod (40); and one study evaluated food intake patterns in youth with BE behavior (41).

Daily food intake patterns

The first of nine studies exploring daily food intake patterns were conducted under laboratory conditions across a 24-h period (divided in four 6-h periods) in female patients with BN (n = 54) and showed a disturbed eating pattern characterized by the absence of the typical peaks in meal intake patterns during breakfast, lunch, and dinner that were observed in the control group (n = 11). In addition, 30% of the meals consumed between the afternoon (18% from 12 to 6 pm) and midnight (12% from 6 pm to 12 am) periods were considered "large" (i.e., a > 1000 kcal meal) in a subset of BN patients (n = 24) cataloged as "overeaters," with a 24-h caloric intake of more than two standard deviations higher than the mean of the control group (28). The combined percentage of meals that were considered "large" from midnight to noon was only 8%. Unfortunately, it was not reported whether the overeating episodes met criteria for BE behavior. In the second study, calorie intake in women with BED (*DSM-IV* criteria) and obesity was lower in the mornings and midday, and higher in the evening, when compared to a BMI and age matched

TABLE 1 Studies on patterns of food intake and/or timing of BE behavior.

Author, year	Study design	Participants and age (*SD*)	Sample size/ Female%	Outcomes	Instruments	Results	Comments and limitations
Blouin et al. (40)	Study 1: CS Study 2: CS	NW women with BN vs. Ctrl Study 1: BN = 24.6(6.0), Ctrl = 24.7(6.2) Study 2: BN = 26	Study 1: BN = 31, Ctrl = 31 F = 100% Study 2: BN = 197/F = 98%	Seasonal patterns in BN (DSM III-R) Association between BE and photoperiod	Study 1: M-SPAQ Study 2: Self-report questionnaire	Study 1: Dark hours predicted% of subjects with BE likelihood each month. SAD in 35.5% of BN. Study 2: BE/week directly correlated with dark hours in the month of assessment in all subjects. Purging did not show this correlation	Dark hours included twilight hours at dusk and dawn. Retrospective and self-reported assessments. BE time of day not assessed. High% with psychiatric comorbidities
Cachelin et al. (35)	CS	Latina women with/o ED All = 26.9 (?)	BED = 65 BN = 22 No ED = 68 /F = 100%	Eating patterns in Latina women with BE	*DSM-5* criteria	BN had more nocturnal eating than the other two groups (BN: 18.2%; BED: 3.1%; no ED: 1.5%). BED (48%) and BN (68%) snacked more frequently during the evening (more than half of the past 28 days). In BED, BN and Ctrl group no association between breakfast or evening snacks and BE	Association between meal timing and BE was not analyzed. BE timing was not assessed
Ellison et al. (34)	P	BN or partial BN (purging + objective or subjective BE) All = 27.3 (9.6)	BN = 68/F = 90%	Association between evening meals and BE	EDE	Increased evening meal consumption during treatment predicted decreased BE after 4-month follow-up. Similar results with depression as covariate.	Subjects were enrolled in a RCT and receiving psychotherapy focusing on food.
Elran-Barak et al. (41)	CS	Children and adolescents with AN-R = 14.7(2.1) AN BE/P = 16.26(1.9)	AN-R = 120 AN-BE/P = 40 F = 93.8%	Meal patterns in youth with AN types	*DSM-5* criteria EDE	Youth with AN-BE/P consumed less breakfast, lunch, dinner, as well as mid-morning and mid-afternoon snacks compared to AN-R types. BE-P type that consumed dinner more often had less BE episodes.	
Ferrer-Garcia et al. (38)	CS	BED and BN patients All = 30.1 (8.02)	Total = 101 BED = 50, BN = 51 /F = 88%	Transcultural contexts and cues that elicit food craving	*DSM-5* criteria	BE craving was higher in the afternoon/early evening and late evening/night.	
Harvey et al. (31)	CS	Women with BED or RBE (1/week for 3 months) All = 33.9(7.4)	Total = 106 BED = 48, RBE = 58 F = 100%	Meal patterns in BED and RBE women	*DSM-IV* criteria EDE	Dinner was the most and breakfast the least common meal. Evening snacking was most frequent and associated with BE. Meal frequency not associated with BE, BMI, or ED pathology	BE timing not assessed. Correlations for meal patterns and BE was not provided separately for BED and RBE
Leblanc et al. (32)	P (3-days)	Premenopausal women with overweight or OB All = 42.6 (5.6)	Total = 143 /F = 100%	Association between eating behavior and eating patterns	TFEQ Self-report dietary intake	BES scores positively correlated with% energy intake after 5 pm. This correlation was not significant after removal of "underreporters." % of energy intake from snacks after 5 pm mediates the association between BES scores and self-reported energy intake	BE frequency and timing not assessed. Presence of ED in participants not reported. Self report
Masheb et al. (30)	CS	BED, BN, and Ctrl Community volunteers All = 36.3 (12.4)	Total = 311 BED = 69, BN = 39, Ctrl = 203 /F = 100%	Eating behavior and patterns in BED or BN, and controls	EDE-Q	BED > snacks/day and evening snacks than controls, but not compared to BN. BED and BN > nocturnal eating compared to controls More frequent breakfast related to < weight in BED and control, but not BN. In BED, BE inversely related to dinner frequency.	BE timing not assessed Dx based on self-report

(Continued)

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TABLE 1 (Continued)

Author, year	Study design	Participants and age (*SD*)	Sample size/ Female%	Outcomes	Instruments	Results	Comments and limitations
Masheb and Grilo (29)	CS	BED All = 45.2 (8.8)	Total = 173 /F = 73%	Meal patterns in BED	SCID-I/P (*DSM-IV*), EDE, TFEQ	No association between meal frequency and BE Evening snack directly correlated with BE days and episodes	BE timing not assessed
Mitchell et al. (36)	CS	BN All = 24.8 (?)	Total = 275 /F = 100%	Description of clinical variables in BN	*DSM-III*	Subjects that reported BE usually occurring late afternoon (46.2%), early evening (52.4%), late evening (54.2%), or after midnight (15.3%). BE was reported as usual early in the morning (17.1%) or late morning (17.5%). BE could occur at any time in (33.5%)	BMI not reported
Pla-Sanjuanelo et al. (39)	CS	BED and BN = 30.1 (8.0) Ctrl = 22.64 (6.06)	Total = 101 BED = 50, BN = 51 /F = 88% Ctrls = 63/F = 85%	Binge craving and specific cues	*DSM-5* EDE-3 BULIT-R PBEBI	Higher binge craving at dinner, snacking between meals (after dinner), during the afternoon/evening, and between 8 and 12 pm	
Raymond et al. (15)	P	Obese women with BED BED = 37.9(7.8) Control = 34.9(8.0)	BED = 12 Control = 8 /F = 100%	Energy intake patterns in obese women with BED	SCID-IIIR *DSM-IV* BED criteria EDE, Dietary recall interviews	DEC in BED = Ctrl group. BED group had a higher calory intake (50% of DEC) in the evening compared to Ctrl (39% of DEC). In BE days, BED consumed less calories in the morning and midday compared to Ctrl. Similar patterns of energy intake between BE days and No-BE days.	BE timing not assessed Small sample size
Shah et al. (33)	P	Women with BN BN = 28.7(7.2)	BN = 158/F = 100%	Association between eating patterns and BE	EDE	Highest BE behavior abstinence rates in those with more meals and more afternoon or less evening snacks.	Participants received CBT or IBT during study follow-up.
Schreiber-Gregory et al. (16)	Study 1: CS Study 2: P	BED subjects Study 1 = 47.5(10.8) Study 2 = 37.9(11.8)	Study 1: BED = 139/F = 87.7% Study 2: BED = 5 Subthreshold BED = 4/F = 100%	BE duration and temporal pattern in BED	Study 1:EDE Study 2: SCID-IV E-recording of BE and meals	Study 2: BE more frequent in the early afternoon (12–3 pm) and evening hours (6–9 pm) BE less frequent during the weekend days	Study 1: BE timing not assessed Study 2: Small sample size, included subthreshold BED
Waters et al. (37)	P (1-week)	Women with BN All = 24.8 (SE 0.7)	Total = 15/F = 100%	Factors preceding BE	BE diary and craving record	Time of day had an effect on BE after craving BE more frequent in the afternoon	Statistical model underpowered
Weltzin et al. (28)	P	Women BN BN = 24.8(6.3) Ctrl = 22.5(4.0)	BN = 54 Ctrl = 11 /F = 100%	Food intake patterns in BN	SCID-*DSM-III* criteria. Conducted in laboratory conditions	Total calories BN > Controls. # meals/day BN = Controls BN: majority of meals and calories between noon and midnight	Study conducted in laboratory conditions

ADHD-RS, attention deficit and hyperactivity disorder-rating scale; AN, anorexia nervosa; AN-B/P, anorexia nervosa-bingeing/purging type; AN-R, anorexia nervosa-restrictive type; BE, binge eating; BED, binge eating disorder; BES, binge eating scale; BMI, body mass index; BN, bulimia Nervosa; BULIT-R, bulimia test-revised; CBT, cognitive behavioral therapy; CS, cross-sectional; Ctrl, control; DEC, daily energy consumption; DSPS, delayed sleep phase syndrome; Dx, diagnosis; ED, eating disorder; EDE, eating disorder questionnaire; EDI-3, eating disorder inventory-3; F, female; IPT, interpersonal therapy; M-SPAQ, Modified (Binge-Purging) Seasonal Pattern Assessment Questionnaire; NW, normal weight; OB, obesity; PBEBI, precipitating binge eating behavior inventory; P, prospective; RBE, recurrent binge eating; RCT, randomized controlled trials; SCID III, structured clinical diagnostic interview for the DSM-III; SD, standard deviation; TFEQ, Three Factor Eating Questionnaire; VOA, Dutch version of a Morning/Evening type Questionnaire.

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control group with obesity without BE behavior (15). The third study found that among 173 male and female patients with BED (*DSM-IV* criteria) with co-existing obesity, the frequency of evening snacking was directly correlated with the frequency of BE behavior days and BE behavior episodes per week (29). This correlation was not observed with the frequency of other meals individually or with the overall meal frequency. In the fourth study, eating patterns were evaluated among women with BN ($n = 39$) and BED ($n = 69$) defined by modified-*DSM*-IV criteria from a community sample (30). It reported that BE behavior frequency was inversely correlated with the average number of meals per day and with dinner frequency only in the BED group. This was interpreted as indicating that BED individuals with less regular meals and those who consumed dinner less frequently experienced more BE behavior episodes. In the same study, more frequent breakfast consumption was associated with lower weight in the BED group.

In the fifth study, 106 individuals with *DSM*-IV BED or recurrent BE behavior (e.g., one episode/week for at least 3 months, similar to current *DSM*-5 BED criteria) reported that dinner and evening snacks were the most common meal and snacks consumed, respectively, and that the number of days with BE behavior was directly correlated with the frequency of evening snacks (31). The sixth study found that severity of BE behavior correlated directly with the proportion of energy intake from snacks after 5:00 pm among premenopausal women with overweight or obesity (32). Of note, a secondary analysis identified that the association between BE behavior severity and total self-reported energy intake was partially mediated by the percentage of energy intake from evening snacks after 5:00 pm. In the seventh study, a prospective evaluation of 158 patients with BN, an increase in regular meals and more afternoon or evening snacks were both associated with increased abstinence rates of BE and purging behavior (33). In the eight study, also using a prospective design, increased evening meal intake frequency was associated with a decrease in BE behavior frequency in 68 patients with BN or partial BN (purging + objective or subjective BE behavior) (34). The ninth study showed no correlation between meal intake frequency and BE behavior frequency among Latina women with BN ($n = 22$) or BED ($n = 65$) (35). A limitation in these studies was that BE behavior timing and its relationship with meal and snack timing was not analyzed. Nonetheless, taken together, these studies suggest more regular meal and snack intake during the later part of the day among individuals with BE behavior is associated with less BE behavior.

Binge eating behavior timing

Five studies examined BE behavior timing. In the first study, with 275 patients with BN, the early evening (52.4%) and late evening (54.2%) were reported as the time of day when BE behavior usually occurred for more than half of participants (36). The second study was a one-week prospective investigation of the internal and external factors that preceded BE behavior occurrence after an urge to binge eat in 15 women with BN (37). This study identified that both the urge to binge eat and BE behavior occurred more frequently during the afternoon. An effect of time of day of the urge to binge eat on BE behavior was also reported. However, the statistical model was underpowered and results difficult to interpret. The third study utilized an ecological moment assessment approach to prospectively evaluate the duration and timing of BE behavior episodes in nine subjects with threshold ($n = 5$) and subthreshold BED ($n = 4$) according to *DSM-IV* criteria (16). Frequency of BE behavior was higher in the early afternoon (12:00 to 3:00 pm) and evening (6:00 to 9:00 pm) than in the morning. Interestingly, BE behavior was less frequent during weekend days than on weekdays. The fourth and fifth studies reported only on the timing of the urge (or craving) to binge eat in subjects with BN or BED. In both studies, the urge to binge eat was heightened in the afternoon/early evening (4:00 pm to 8:00 pm), late evening/night (8:00 pm to 12:00 am), and was consistently increased during the weekend (38, 39). These observations further suggest that the more frequent occurrence of BE behavior during the evening or night is not just explained by an "increased opportunity" to binge eat when individuals are not busy or working (e.g., after work hours) even if the craving to binge eat is high during the weekends.

Photoperiod and binge eating behavior

One study evaluated the relationship between BE behavior and photoperiod, defined as the average number of light hours across 24-h. It was observed that among 31 patients with BN, a decreased number of light hours predicted an increased likelihood for BE behavior each month but did not predict the likelihood of purging behavior (40). It was concluded that photoperiod may exert an influence over BE behavior that is likely mediated by the circadian system and is independent from other compensatory behaviors (e.g., self-induced vomiting, misuse of laxatives, weight loss medications, or diuretics, food restriction or prolonged fasting, or excessive exercise), although—as recognized by the authors—a role independent of other highly correlated seasonal changes such as environmental temperature could not be determined.

Patterns of food intake in children and adolescents with binge eating behavior

One study explored food intake patterns in youth with BE behavior. This study evaluated 160 children and adolescents with restrictive AN (AN-R, $n = 120$) or binge eating/purgative AN (AN BE/P, $n = 40$) subtypes. Youth with AN-BE/P consumed breakfast, lunch, dinner, as well as mid-morning and mid-afternoon snacks less often as compared to those with AN-R types. There were no group differences in evening snack consumption. Interestingly, as has been described in adults with

TABLE 2 Assessment of circadian-related measures among individuals with BE behavior.

Author, year	Study design	Participants, Age	Sample size/Female%	Outcomes	Instruments	Results	Limitations and comments
Carnell et al. (42)	CS	Obesity w/and w/o BED BED = 35.9(8.1), OB = 36.8(9.2)	*N* = 32, BED = 16(62.5%), OB = 16(56.3%)	Time of day and between group differences in hunger/fullness, food intake, and ghrelin levels	IDS, PSS, TEFQ, DEBQ, ZUNG, EMAQ, PFS, BES, STAI, NESHI	PM eating associated w/↑ hunger and ↓ fullness in BED. Fasting ghrelin ↓ in AM and ↑ in PM in BED. Greater experience of LOC and BE resemblance at AM and PM in BED	Limited information on BE behavior frequency/severity for BED participants.
Galasso et al. (46)	P	BED with obesity, 56.8(16.7) Controls with obesity = 61.5(13.8)	*N* = 28(100%), BED with obesity = 14, Controls with obesity = 14	Relationship between RAR and BED diagnosis	Wrist actigraphy	BED showed ↓ MESOR and amplitude and poor sleep quality compared to controls. No difference in acrophase.	BED actigraphy recordings while on intensive cognitive-behavioral therapy and nutritional program (8–5 p.m). Controls did not receive intervention
Harb et al. (49)	CS	Patients with obesity seeking weight loss treatment 39.5(11.7)	*N* = 100 (77%)	Associations between chronotype, eating patterns and BMI	MEQ, NEQ, EAT	Strong association between eveningness and BE, weak association w/number of night eating behaviors	Low incidence of night eating
Mason et al. (47)	P	DSM-5 BN, DSM-5 BED 44.28 (12.54)	*N* = 212(85%)	Sleep disturbance in obesity. Association between ED severity and subjective sleep measures	13-CSM (used in place of MEQ), EDDS, SATED	BN and BED associated w/↑eveningness, ↓subjective sleep. ↑ED severity correlated with ↓ sleep quality	Patients seeking weight loss treatment, self-report measures
Monteleone et al. (52)	CS	Obesity w/and w/o BED. Age reported by Genotype	*N* = 298 (85.4%), OB = 99, OB-BED = 107	CLOCK polymorphism in obesity. Eveningness in homozygous T111C genotype	SCID-IP	3111T/C associated w/↑BMI in obesity, not w/BED	No circadian-related measures. Ctrls significantly younger.
Romo-Nava et al. (12)	CS	Non-evening BD = 40.8 (14.8); Evening type BD = 37.5 (13.4)	*N* = 783; non-evening = 575(66%) Evening = 208(60%)	Association between chronotype and unhealthy eating behaviors	EDDS, REAPS	Evening chronotype associated w/increased eating psychopathology (EDDS scores), higher BE behavior, BN, and nocturnal eating binges, and BMI. Evening types had worse dietary habits (REAPS scores), including skipping breakfast more often, eating less fruits and vegetables, consumed more fried foods and unhealthy snacks.	Retrospective survey. No control for effects of medications or BMI.
Roveda et al. (48)	P	OB with BED = 55.7(15.6), OB w/o BED = 60.0(12.4)	*N* = 16(100%), OB with BED = 8, OB w/o BED = 8	Relationship between RAR and BED diagnosis	Actigraphy	BED showed ↓ MESOR and amplitude and poor sleep quality compared to controls. No difference in acrophase or sleep quality.	BED participant actigraphy recorded during day hospital care with a "multidisciplinary treatment"
Taylor et al. (43)	P	NW females w/normal menstrual cycles = 28.1(3.0)	*N* = 7(100%)	Effects of BE-like dinner on metabolism	BITE (Edinburgh Bulimic investigation Test)	Time of eating drove diurnal leptin rhythm. BE-like dinner increased fasting glucose levels, and increased postprandial insulin without a change in postprandial glucose levels	Small sample size. Metabolic parameters measured only during 14 h. Participants fasted prior to the BE-like dinner

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TABLE 2 (Continued)

Author, year	Study design	Participants, Age	Sample size/Female%	Outcomes	Instruments	Results	Limitations and comments
Tzischinsky et al. (44)	P	Obesity with BED = 37.8(5.5), Obesity w/o BED = 38.0(6.7) NW = 22.1(3.1)	*N* = 47(100%), Obesity with BED = 18, Obesity w/o BED = 13, NW = 16	Characterization of sleep disturbance in BED and OB	1-week Actigraphy, MSQ, Std Technion Clinical Sleep Questionnaire, Sleep diary	Compared to NC, the BED and OB group showed sleep disturbance, ↓quality, and on actigraphic measures for SE, TST, Long, Zero, and WAKE	No assessment of mediators in sleep disturbance (e.g., OSA)
Tzinchinsky and Latzer (45)	P	Children with OB with and w/o BE. OB with BE = 9.7(2.0), OB w/o BE = 9.1(1.70), NW = 10.2(1.5),	*N* = 61, OB-BE = 13 (38.5%), OB w/o BE = 23 (73.9%), NW = 25 (56%)	BE in children. Sleep in children with obesity	DSM IV BED criteria 1-week Actigraphy, MSQ, Standard Technion Clinical Sleep Questionnaire, Sleep diary	SE% was lower in OB with BE compared to OB w/o BE, and NC groups. WAKE was higher in OB with BE group compared to OB without BE and NC group.	No analysis of association between BE behavior severity and actigraphy or diurnal preference or sleep parameters
Weltzin et al. (81)	CS	NW BN = 24.8 (6.3) NW w/o BN = 22.5 (4.0)	*N* = 65(100%) NW BN = 54, NW w/o BN = 11	Food intake patterns in BN. 24 h hormonal pattern during BE days.	DSM III-TR Cortisol, HGH, PL	Nocturnal prolactin blunted in BN. No significant effect of binging/purging on other hormones.	BE behavior timing and potential effect of compensatory behavior was not analyzed.
Vogel et al. (50)	CS	OB = 43.8(11.2) ADHD = 34.9 (10.6) Controls = 23.6 (3.1)	OB = 114 (86%) ADHD = 202 (47%) Controls = 154 (65%)	Circadian-related variables in ADHD and their relationship with obesity	Dutch version of the ADHD rating scale Morning/Evening type Questionnaire MCTQ	Extreme evening chronotype higher in ADHD vs. OB group. Unstable eating pattern (skipping breakfast + evening/night BE), skipping breakfast and BE behavior at night higher in ADHD vs. OB. Rate of BE behavior similar in ADHD vs. OB. Unstable eating pattern (BE at mediated BMI in ADHD.	No comparisons between ADHD and Control group. No specific analysis for BE behavior association with other clinical variables

ADHD, attention deficit and hyperactivity disorder; AN, anorexia nervosa; BDI, Beck depression inventory; BD, bipolar disorder; BE, binge eating; BED, binge eating disorder; BN, bulimia nervosa; BLT, bright light therapy; CD, circadian; CS, cross-sectional; EAT, eating attitudes test; EDDS, eating disorder diagnostic scale; ED, eating disorder; HDRS, Hamilton depression scale; HGH, growth hormone; Long, longest episode of continuous sleep; MEQ, morningness-eveningness questionnaire; MCTQ, Munich Chronotype Questionnaire; NEQ, night eating questionnaire; NW, normal weight; NE, night eating; OB, obesity; PL, prolactin; PSG, polysomnograph; P, prospective; REAPS, rapid eating assessment for patients; SE, sleep efficiency; TST, total sleep time; w/o, without; Zero, minutes of zero activity counts.

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BE behavior, youth with AN BE/P type with a less regular dinner consumption experienced more BE behavior episodes (41).

Assessment of circadian-related measures among individuals with binge eating behavior

Twelve studies provided evidence on objective or subjective circadian-related parameters in individuals with BE behavior (see **Table 2** and **Supplementary Table 3**). Assessment methods used to explore circadian-related function in these studies included hormonal levels (n = 3), actigraphy (n = 5), diurnal preference or chronotype (n = 3), and genetics (n = 1). These studies are summarized below.

Hormonal studies

Three studies have investigated the association between diurnal hormonal variations and BE or BE-like behavior (42, 43). In the first study, normal weight participants with and without BN were evaluated during 72 h in a laboratory setting. Cortisol, growth hormone, and prolactin plasma levels were obtained from blood samples taken every 20 min for 24 h on one of the experimental days. Participants with BN were asked to conduct a BE behavior during one of the days and hormone levels were compared to those without BN. Prolactin levels were decreased during the latter part of the night in patients with BN compared to those without BN. No other differences in 24-h hormonal patterns were observed. The time of BE behavior and presence of purging (e.g., self-induced vomit) or other compensatory behaviors (e.g., vigorous exercise, skipping meals) behavior, as well as sleep/wake cycles were not described. The potential effect of these variables on hormone levels was not analyzed.

In the second study, healthy-weight females (n = 7) without a history of an ED were evaluated under a strictly controlled laboratory setting. It was observed that the time of eating determined the diurnal variation of leptin secretion and that consumption of BE-like dinners induced increased fasting glucose levels. Having a BE-like dinner increased postprandial insulin secretion, as well as decreased postprandial leptin secretion without a postprandial difference in glucose levels (43). These findings suggest that consuming BE-like meals might induce profound metabolic changes that persist for hours, potentially altering hormone physiological diurnal variation.

In the third study, 32 individuals with obesity and with (n = 16) or without (n = 16) BED were evaluated for morning vs. evening variation in fullness and hunger ratings according to visual analog scale ratings, as well as hormonal variation differences in response to a standardized liquid meal or an *ad libitum* buffet-like meal in a controlled environment (42). BED participants showed decreased fullness and a trend toward increased hunger ratings during the evening when compared to control participants in response to the liquid meal. Compared to the control group, BED participants showed decreased fullness after the buffet-like meal in the evening than in the morning and reported an increased sense of loss of control and similarity to a BE behavior episode at both time-of-day conditions. Moreover, altered hormonal patterns were identified in the BED group, with decreased initial fasting ghrelin levels in the morning and increased initial fasting levels in the evening prior to the ingestion of the liquid meal. These findings are consistent with a heightened evening susceptibility to overeating in BED.

Actigraphy studies in individuals with binge eating behavior

Five studies employed wrist actigraphy to examine locomotor activity in individuals with BE behavior (44–48). Actigraphy records wrist acceleration and enables objective estimates of behavioral activity, sleep, and the sleep/wake cycle. The first of these studies compared 1-week actigraphy data across three adult groups: individuals with obesity and *DSM-IV* BED (n = 18), individuals with obesity and without BED (n = 13), and normal weight individuals without BED (n = 16) (44). The BED and non-BED obesity groups showed altered actigraphic parameters compared with the normal weight control group, including decreased sleep efficiency (percentage of time asleep while in bed), total sleep time, minutes of zero activity counts, longest episode of continuous sleep, as well as increased total minutes of wake during sleep. There were no differences between BED participants and the obesity control group. Unfortunately, the sample was relatively small and *post hoc* analyses were not clearly described. The second study, conducted in children with obesity (n = 36), found that participants with BE behavior (n = 13) had a lower sleep efficiency percentage and more minutes of wake during sleep compared with participants without BE behavior (n = 23) and a group of normal weight children without BE behavior (45).

The third and fourth studies utilized 5 days of actigraphic recordings to evaluate diurnal variations in adult women with obesity and with or without BED (46, 48). In the third study, BED subjects (n = 8) showed lower Midline Estimated Statistic of Rhythm (MESOR; a rhythm-adjusted mean) and absolute amplitude compared with controls (n = 8), but no difference in acrophase (time at which the peak of a rhythm occurs) was observed. The fourth actigraphy study (46) reported on a sample expansion from the third study (48). It replicated the MESOR and amplitude findings from the prior study. However, inter-daily stability, a measure of diurnal rhythm strength, was higher in BED (n = 14) compared to sex, age, and BMI matched control subjects (n = 14). Activity counts on L5 (average activity for the least 5 active hours) and M10 (average activity for the most active 10 h) were lower in BED compared to controls. The results of these two studies are difficult to interpret because BED participants were receiving an intensive (8-h per day) combination of cognitive behavioral therapy and a nutritional program during the 5 days of actigraphic recording that individuals on the control group apparently did not receive. Results may thus indicate a combination between the effects of the intervention inducing better sleep parameters in BED

compared with controls subjects and a blunted rest/activity rhythm due to biological differences in patients with BED vs. control, as shown by a decreased MESOR and amplitude.

The fifth study utilized a commercial accelerometer (Fitbit) among other circadian-related assessments (including chronotype and sleep diary and quality parameters) in 52 patients with threshold or sub-threshold *DSM-IV* BN (n = 22) or BED (n = 30) diagnosed according to the Eating Disorder Diagnostic Scale (EDDS) (47). Global eating psychopathology was not related to sleep duration, sleep efficiency, or sleep onset latency. Participants with BN showed greater 'eveningness' and decreased self-reported sleep health compared to the control group, but no difference was observed between the BED and the other groups. Among participants with an eating disorder, greater 'eveningness' was strongly associated with increased global eating disorder psychopathology. The main limitation of this study was the inclusion of threshold and sub-threshold BN or BED symptoms in the analysis without providing details about diagnostic criteria distribution. Other limitations included participation of patients in a weight loss program and use of a commercial accelerometer instead of research-designed actigraphy devices.

Chronotype (diurnal preference) and binge eating behavior

Three studies evaluated chronotype in individuals with BE behavior (12, 49, 50). In the first study, self-reported 'eveningness' was associated with increased severity of BE behavior when assessed with the BE scale among 100 participants seeking care at a nutrition clinic (49). The second and third studies evaluated BE behavior and self-reported diurnal preference in other psychiatric conditions, specifically ADHD (50) and bipolar disorder (12). In the first of these studies, 64% of adult ADHD patients (n = 202) had BE behavior, which was comparable to 72% in a group of individuals with obesity (72%, n = 114) and higher than a 34% in the control group (n = 154) (50). ADHD patients showed a higher rate of proxy markers of circadian phase delay, including self-reported measures of delayed sleep phase syndrome (25.8% vs. 6.9%) and extreme evening chronotype compared with obesity patients (15.5% vs. 4.4%). Of note, ADHD subjects skipped breakfast more often compared to the group of subjects with obesity. Unfortunately, the association between BE behavior and diurnal preference was not reported. However, a similar proportion of subjects with ADHD and obesity reported BE behavior in the evening, but a higher percentage of ADHD subjects reported nocturnal BE behavior compared to the group with obesity. In addition, participants were classified as having an "unstable eating pattern" if they skipped breakfast and had BE behavior at night/evening. Participants with ADHD had a higher rate of unstable eating pattern compared to the group with obesity, but the group with obesity did not differ from controls. It was also observed that the unstable eating pattern mediated body mass index (BMI) only in the ADHD group.

The next study evaluated correlates of self-reported chronotype among 783 patients with bipolar disorder. The 205 (27%) patients with evening chronotype had increased rates of BE behavior, BN, and nocturnal BE behavior, as well as higher EDDS global eating psychopathology scores and higher BMI compared with those with non-evening chronotypes (51). Interestingly, participants with evening chronotype also reported skipping breakfast more often, eating less fruit and vegetables, and consuming more fried foods, unhealthy snacks, and sugary drinks.

Circadian system genetics and binge eating behavior

We found one study that investigated circadian system genetics in participants with BE behavior (52). Specifically, the association between the 3111C CLOCK (Circadian locomotor output cycles kaput) allele genotype and obesity in participants with overweight/obesity with BED (n = 107) or without BED (n = 85) and a normal weight control group (n = 92) was evaluated. Secondarily, authors hypothesized that genotypes homozygous or heterozygous for the 3111C CLOCK allele would be associated with obesity compared with the homozygous genotype. Although an association between the 3111T/C genotype and a higher BMI was found, there was no association with BED. This study had several limitations, including the study of a single clock gene, the lack of intermediate or proxy measurements of circadian system function (e.g., chronotype or actigraphy), and lack of reporting on features of BE behavior (e.g., timing, frequency, or severity scales).

Overlap of binge eating and night eating behavior

Night eating syndrome is currently included as an example of an "Other Specified Feeding or Eating Disorder" in the *DSM-5* and is defined as recurrent episodes of night eating, manifested by eating after awakening from sleep or by excessive food consumption after the evening meal (1). The behavior causes distress, there is recall and awareness of the behavior, and the behavior is not better explained by another ED.

In contrast to the generally accepted definition of BE behavior, there have been various definitions of night eating behavior and diagnostic criteria for NES. Older studies used Stunkard's (53) criteria for NES (53), with one criterion being evening hyperphagia, described as consuming 25% or more of the total daily caloric after the evening meal (54). Several food intake and neuroendocrine circadian phase and amplitude disturbances have been documented in NES (55). In some cases, night eating episodes may meet criteria for BE behavior episodes if the amount of food consumed is unusually large and is associated with a sense of loss of control. Conversely, an episode

TABLE 3 Studies on the overlap of BE and night eating behavior.

Author/year	Study design	Participants age (*SD*)	Sample size/Female%	Outcomes	Instruments	Results	Limitations and comments
Adami et al. (60)	P	Bariatric surgery candidates 37.6	*N* = 63/76%	Frequency of BED and NES	*DSM IV* criteria NE defined as no appetite for breakfast, consuming > 50% of food after 7 p.m., and having trouble getting to sleep and/or staying asleep.	BED in 42.8% and NE in 7.9%. All NE patients met criteria for BED.	Standardized tools for BED and NES diagnosis were not used
Allison et al. (58)	CS	BED with OB NES with OB OB w/o BED or NES (Ctrl group) Age per group not provided	BED = 177/79% NES = 68/69% Ctrl = 45/66%	Eating patterns, disordered eating, clinical features, and measures of psychological distress	SCID *DSM IV* for BED EDE NES criteria ≥ 25% caloric intake after evening meal and/or awakenings to eat ≥ 3/week. TFEQ BDI	Higher BDI scores in BED and NES groups compared to Ctrl group. BE behavior frequency BED > NES > Ctrl Objective overeating episodes BED > NES = Ctrl. Breakfast and lunches in NES > BED and Ctrl. Afternoon snacks in NES < BED. Evening meals in BED > Ctrl but no difference with NES. Evening snacks NES > Ctrl. Nocturnal snacks in NES > BED and Ctrl groups. BED and NES groups reported higher dietary restraint and eating concerns than Ctrl. Shape and weight concerns in BED > NES and controls, and NES > controls. Disinhibition and hunger in BED > NES and control groups, and NES > control group.	Study did not include a group of patients with comorbid BED and NES. Depressive symptoms were included as covariate in the analysis of outcome measures.
Allison et al. (62)	CS	Bariatric surgery candidates 44.4 (10.7)	*N* = 215/82%	NES and BED in bariatric pre-surgery patients	*DSM IV*- criteria NEQ	BED in 15% and NES in 3.9% of the total sample. NES in 15% of BED patients.	Homogenous sample. Evening hyperphagia or Nocturnal Eating criteria were not reported separately in BED patients.
Colles et al. (4)	CS	Community sample 41.3 (13.5) OB sample 55.1 (12.4) Bariatric surgery candidate 44.8(11.2)	Total sample = 431 Community *n* = 158/78.5% OB *n* = 93/91.4% Bariatric surgery candidate *n* = 180/78.3%	Clinical significance on NES and nocturnal snacking	Combination *DSM IV* and *DSM 5* criteria for BED Self-report NES survey	BED in 12%, NES in 11%. BED and NES in 4%. NES in 37% of BED. BED in 40% of NES. NES is associated with high BMI and BED. BED seven times more likely to have NES. Significant differences in BED and NES prevalence among sub-samples.	Self-report weight; varying recruitment methods Heterogeneous population
de Zwaan et al. (23)	CS	Community sample reporting Night-time eating 49.2 (?)	N106/64.2%	NE in a community sample	Phone interview. SCID *DSM IV* criteria. NEQ, Night eating syndrome history and inventory, sleep disorder questionnaire, Eating Disorder Questionnaire.	NES criteria 29.2% Evening hyperphagia ≥ 25% or ≥50% calorie intake after evening meal in 76.4 and 45.3% of the sample. No control over NE food ingestion in 44.5% of NES participants. 14.2% had BED or BN	All participants had self-reported night eating. Clinical features of NE episodes among BED or BN with NES participants was not described.
Greeno et al. (57)	P	Women with OB seeking treatment 39.47 for BED 39.07 for Ctrls	DSM III BED *N* = 39/100% Ctrls *N* = 40/100%	Behavioral and psychological correlates of NE	Food intake diary for 5 to 10 days. BED diagnosed with BES score > 17. NE defined as "getting up out of bed during the night to eat."	NE in 7.5% of participants. All subjects with NE had BED. BED with NE was 15% 5/7 NE episodes described with low perceived control and 4/7 were described as likely BE behavior. NE episode average 639 calories and 41% fat content.	Homogenous sample; women only. Small sample size

(Continued)

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TABLE 3 (Continued)

Author/year	Study design	Participants age (*SD*)	Sample size/Female%	Outcomes	Instruments	Results	Limitations and comments
Grilo and Masheb (61)	CS	DSM IV BED seeking treatment 44.4 (9.3)	*N* = 207/78.3%	Comparison of BED vs. BED + Night-time eating	SCID *DSM IV* criteria Night-time eating based on EDE (eating after going to sleep ≥ 4 days/past 28 days), EDE, TFEQ, BDI, RSE	28% Night-time eating in BED. More men than women had NE in BED. Women with BED and NE had greater eating, weight and shape concerns than men with BED and NE.	Homogenous sample
Grilo et al. (24)	CS	BED with OB Hispanic/Latino seeking treatment; 46.32 (9.68)	*N* = 79, 81%	NE in obese Hispanic population	SCID DSM IV criteria Night-time eating based on EDE (eating after going to sleep at least once in the past 28 days) EDE, BDI	NE in 53% of BED participants and 23% of participants without BED. BED present in 70% of those without NE and 18% of those without NE. Frequency of BE behavior and NE episodes were correlated NE associated with higher levels of psychopathology	Homogeneous sample. Comparison of clinical characteristics between BED with and without NE groups were not explored.
Harb et al. (49)	CS	Outpatient nutrition clinic 39.5 (11.7)	*N* = 100, 77%	Correlation between chronotype and eating behavior	NEQ, MEQ, BES BE behavior classified as BES ≥ 18	BE behavior in 18% and NE behavior in 18%. BES and NEQ scores were inversely correlated with MEQ; BES and NEQ were also associated	Self-report instruments. Co-occurrence of BE and NE behaviors not reported. BED criteria not used for analysis.
Latzer et al. (5)	P	Women seeking treatment with BE behavior (BN or BED) with NES or without NES	*N* = 59/100% BE behavior with NES = 25 BE behavior without NES = 34	Dietary pattern differences between those with NES and without NES	*DSM IV* criteria 24 hour food diary (7 days) BDI	Participants with NES had higher BDI scores, more BE behavior days and episodes per week, calories ingested per day, and higher evening consumption of calories, than those without NES	BE group included BN and BED patients. Characteristics of NE episodes and timing of BE behavior was not reported.
Meule et al. (69)	CS	German college students 23.55 (3.89)	*N* = 729/77%	Correlation between NE, BMI, emotional eating and binge eating	Online NEQ, MES, EDE	NE, BE, emotional eating and BMI are positively correlated. NE severity related to more frequent BE	Self-report; normal weight sample. Diagnostic for BE, BED or NES prevalence not reported
Napolitano et al. (67)	CS	Weight loss program participants 48.1	*N* = 83, 52.5%	Psychological and behavioral characteristics associated with both NES and BED	*DSM IV* criteria QEWP-R, TFEQ, RSE, ESES, CES-D, NESI, IDED-IV	No ED = 27%; BED = 15%; NES 27%; BED + NES = 15% NES scored lower on disinhibition than BED. BED + NES scored higher on state and trait anxiety and disinhibition, than NES alone	Homogenous sample of participants planning a 4 week stay at weight loss program. Small sample size.
Rand et al. (59)	CS	General sample 52.8 (19.8) Post bariatric restriction surgery patients 44.6 (10.4)	Non-clinical sample (*n* = 2115)/57.5% Post bariatric surgery (*n* = 111)/93.1%	Prevalence of NES	Self-report for NE symptoms. NES: presence in the past 2 months of all the followings: morning anorexia, delay of eating after awakening for several hours, excessive evening eating, evening tension and/or feeling upset, and insomnia.	1.5% NES in general sample; 27%NES in patient sample; 26% NES patients reported BE behavior	Self-report.

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TABLE 3 (Continued)

Author/year	Study design	Participants age (*SD*)	Sample size/Female%	Outcomes	Instruments	Results	Limitations and comments
Root et al. (65)	CS	Swedish twin study registry	Twins *N* = 11,604 BE = 427/92% NE = 419/54%	Heritability of BE and NE behavior	Online survey with DSM-IV criteria for BE and two independent questions for NE.	Heritability estimates for BE were 0.74 and for NE were 0.35. Genetic correlation between BE and NE behavior was 0.66.	Self-report online questionnaires. Low male BE prevalence.
Runfola et al. (66)	CS	University Students 20.9 (1.7)	Total = 1,636/59.5%	NES prevalence and characteristics in University Students	BE based on EDE DSM-5 criteria and ≥4/past month NES based on NEQ ≥ 25 (broad) or ≥ 30	NES in 4.2% 32.8% on the NES group had BE Participants with NES had 4x BE behavior episodes compared to those without NES (4.4 vs. 1.4 episodes/past month)	Self-reported evaluation 60% were competitive athletes
Sassaroli et al. (68)	CS	Patients with obesity previously admitted to ED unit 48.5 (12.9)	Total *N* = 202/80% BED = 54/? NES = 13/? BED + NES = 16/?	Nocturnal anxiety severity in BED, NES or both	DSM IV TR criteria for BED NES Dx established by eating ≥ 25% daily food intake after dinner, to be affected by morning anorexia and nocturnal awakenings followed by nocturnal ingestions at least 2/week for 3 months. NEQ, SAS, SDQ	Severe anxiety in BED + NES; Correlation between SAS and nocturnal ingestions in BED; Evening hyperphagia correlated to nocturnal mental anxiety in NES and with daytime mental anxiety in BED	Self-reported evaluation with SAS and SDQ
Schenck et al. (56)	CS	Sleep-related eating Disordered Adults 38.8 (9.8)	*N* = 38, 65.8%	Eating behavior and clinical characteristics of sleep-related eating	Clinical interview SCID DSM-III-R Daytime eating disorders questionnaire	68% reported high caloric nocturnal binging 84% reported nightly sleep-related binge eating (without hunger or purging).	Small sample size; self-report. Nightly sleep-related binge eating data only reported in abstract and not clearly described in results section.
Striegel-Moore et al. (63)	CS	Community sample of insured women age range 18–35	*N* = 259 BED *N* = 89 Ctrls	Clinical correlation of NE in BED vs. no BED	BED defined as episodes of overeating with LOC at least once/week in the past 3 months. NE based on EDE (at least 1 NE episode in the past 28 days). Survey questions, BDI, RSE, WSAS	NE in 12.5% of the total sample. % of NE participants with at least 1 BE behavior and with recurrent BE behavior (1/week for the past 3 months) was higher compared to no NE group (39% vs. 20.8% and 26.8% vs. 11.1%, respectively).	Homogenous sample of white female
Tholin et al. (64)	CS	Population based sample of Swedish twins 37.4 (7.5)	*N* = 741/55%	NE in twin samples	Survey questions	NE is associated with BE, sleep disturbance and obesity	LOC not assessed for NE

BDI, Beck Depression Inventory; BE, binge eating; BED, binge-eating disorder; BES, binge eating scale; BMI, body mass index; bn, bulimia nervosa; CES-D, center for epidemiological studies depression scale; ctrl, control; CS, cross-sectional; DSM, diagnostic and statistical manual of mental disorders; ED, eating disorder; EDE, eating disorder questionnaire; F, female; IDED-IV, 4th ed. of the interview for diagnosing eating disorders; MEQ, morningness-eveningness questionnaire; LOC, loss of control; MES, mood eating scale; NE, night eating; NEQ, night eating questionnaire; NES, night eating syndrome; NESI, night eating syndrome interview; OB, obesity; P, prospective; QEWP-R, questionnaire on eating and weight patterns revised; RSE, rosenberg self-esteem scale; SAS, self rating anxiety scale; SCID, structured clinical interview for DSM; SD, standard deviation SDQ, self rating anxiety scale; TFEQ, three factor eating questionnaire; WSAS, work and social adjustment scale.

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of BE behavior during the night can also meet criteria for a night eating episode.

Eighteen studies evaluated the overlap of BE and night eating behavior. We subdivided these studies into those evaluating the clinical similarities between BE and night eating behavior (n = 3), those reporting prevalence estimates of the occurrence of BE and night eating behavior (n = 11), and those evaluating clinical correlates of the co-occurrence of these conditions (n = 5) (**Table 3** and **Supplementary Table 4**).

Clinical overlap between binge eating and night eating behavior

The first of three studies exploring clinical similarities between BE and night eating behavior evaluated 38 patients with sleep related eating conditions (56). Although no patient was diagnosed with daytime BE behavior, 26 (68.4%) reported "high caloric nocturnal binging" and 27 (71%) reported night sleep-related BE (without hunger or purging). The authors noted that sleep-related eating episodes were characterized by the immediate and "compulsive" urge to eat as well as the tendency to eat high calorie foods (e.g., milkshakes, pies, brownies, and candy). These foods were not typically consumed during the day. In the second study, the presence and characteristics of night eating episodes were analyzed in 40 patients with BED and obesity during a 1-week follow-up period (57). Six (15%) patients had at least one night eating episode with an average of 639 calories with 41% fat content. Four out of the seven documented night eating episodes were described as "definitely or sort of a BE episode," and five out of seven were described as being associated with lack of control during food ingestion. Perceived low control over eating during night eating episodes was highly correlated with describing the night eating episode as being a BE behavior episode.

A third study evaluated the clinical features of participants with BED (n = 177), NES (n = 68), and a control group of individuals with overweight or obesity without BED or NES (n = 45) (58). The BED and NES groups had elevated depressive symptom scores compared to the control group and therefore depression scores were used as a covariate, along with BMI, in analysis comparing eating-related behaviors. As expected, BE behavior frequency was higher in the BED group compared to the other two groups and BE behavior frequency was also higher in the NES group compared to the control group; unfortunately, timing of BE behavior was not reported. The BED group reported more objective overeating episodes and more frequent eating episodes during the day than both the NES and control groups. The three groups differed significantly in several food intake patterns. NES participants reported fewer breakfast and lunches compared to the BED and control groups, whereas the BED group reported more afternoon snacks compared to the NES group. The BED and NES groups had more evening meals and evening snacks compared to the control group but did not differ from each other. The NES group reported more nocturnal snacks compared to BED and control groups. BED and NES groups reported higher dietary restraint and eating concerns than the control group. Disinhibition and hunger were higher in BED than in the NES and control groups and were higher in NES compared to the control group.

Prevalence estimates of co-occurrence of binge eating and night eating behavior

Eleven studies reported estimates of the co-occurrence of BE and night eating behavior among clinical (n = 7) and non-clinical (n = 4) samples. The first clinical study evaluated 111 bariatric surgery patients and found NES present in 30 (27%) of the sample. Eight (27%) of those patients with NES also had BE behavior (59). The second study evaluated patients eligible for bariatric surgery for both *DSM-IV* BED and NES. Of 63 patients, 27 (42.8%) met BED criteria and 5 (7.9%) met criteria for NES (60). Importantly, all NES patients met criteria for BED, while none of those without a history of BE behavior had a history of night eating behavior. In the third study, which evaluated 106 participants with self-reported night eating behavior, 31 (29.2%) met full criteria for NES [Stunkaard's (53) criteria], and 13 (12%) and 21 (20%), respectively, met criteria for "current" or "lifetime" BED or BN by *DSM-IV* criteria (23).

The fourth study evaluated 207 treatment-seeking patients with *DSM-IV* BED and found that 58 (28%) had at least one episode of "night-time eating," defined as eating after having gone to sleep, in the past 28 days (61). Night eating episodes occurring as frequently as half of the past 28 days was reported by 19 (9.2%) of BED participants. BED participants with NE showed a higher current and lifetime BMI compared to those without night eating. Both groups were similar regarding BED age of onset and other eating behavior and mood related clinical characteristics. Rate of night eating behavior was higher in men (42%) than women (24%), but women with night eating had higher eating, body shape, and weight concerns scores on the Eating Disorders Examination subscales than their male counterparts.

A fifth study with 215 bariatric surgery eligible participants with obesity, reported 15% had BED (*DSM-IV* criteria) but only 3.9% of all participants met criteria for NES based on the night eating questionnaire (NEQ) that included both evening hyperphagia and nocturnal food ingestion (62). However, 23.7% showed evening hyperphagia only and 7.2% reported nocturnal food ingestion only. The authors also conducted a clinical interview evaluating eating behavior in the past 2 days and found that NES prevalence was reduced to 1.9%. Among 27 patients that met criteria for NES or BED according to a clinical interview, 18.5% (n = 5) met criteria for both.

The sixth study evaluated 431 individuals, including 93 from a weight loss program, 180 eligible for bariatric surgery, and 158 from the community (4). BED criteria (*DSM IV* or *DSM 5* criteria equivalent) were met by 12% of the total sample. NES criteria (based on a past 3-month self-report survey) were met

by 11% of participants. In total, 4% met criteria for BED and NES. Overall, 37% of participants with BED met NES criteria. On the other hand, 40% of NES participants also met criteria for BED. Of note, participants with BED were seven times more likely to meet criteria for NES. The seventh study investigated 79 treatment-seeking Hispanic patients with obesity with BED (n = 40) or without BED (n = 39) and reported that 38% of the total sample met criteria for regular night eating episodes (≥4 days/month) (24). Night eating was present in 53% of participants with BED, and only in 23% of those without BED. Conversely, BED criteria were met by 70% of participants with night eating versus 18% of those without night eating. Frequency of BE behavior and night eating episodes was directly correlated.

In the first non-clinical study, 41 (14.3%) of 285 women from the community reported night eating episodes (63). Having at least one BE episode or having recurrent BE behavior episodes in the past 28 days was reported by 39 and 26.8% of participants with night eating, which was higher than the 20.5 and 11.1% of those without night eating, respectively. The second study evaluated a population-based sample of 21,741 twins (zygosity not reported) and found the prevalence of night eating was 4.6% among men and 3.4% among women (64). Men and women with night eating had 3.4- and 3.6-times higher risk of BE behavior, respectively, compared to those without night eating. In addition, night eating was 2.5 and 2.8 times higher in men and women with obesity, respectively, compared with normal weight men and women. No twin-pair data was reported.

The third study evaluated heritability of BE and night eating behavior in 11,604 Swedish twins (mono and dizygotic pairs) (65). Heritability estimates were 0.74 for BE behavior and 0.35 for night eating. The study also reported a genetic correlation of 0.66 between BE and night eating behaviors, suggesting significant genetic overlap.

The fourth study evaluated NES and BE behavior among 1,636 university students (66). NES criteria were met by 67 (4.2%) participants, of which 22 (32%) also reported BE behavior. Compared to those without NES, individuals with NES had almost four times more BE behavior episodes per month.

Clinical features of co-occurrence of binge eating and night eating behavior

Five studies evaluated the associated clinical features in patients with both BE and night eating behavior. The first study evaluated 83 patients seeking weight loss treatment. Thirteen (15%) had *DSM-IV* BED, 23 (27%) had NES (based on two questions), 13 (15%) had BED and NES, and 27% did not meet criteria for an ED (n = 34). Participants with BED and NES showed greater behavioral disinhibition and trait and state anxiety as compared to those with NES alone (67). The second study found that self-reported anxiety was higher in patients with BED and NES as compared to those with only BED and those with only NES (68).

The third study examined chronotype in relation to BE and night eating behavior among 100 patients attending a nutrition clinic (49). Significant correlations were found between BE and night eating severity scores and self-reported diurnal preference assessments, suggesting that eveningness was associated with higher severity of both BE and night eating behavior. Moreover, BE and night eating severity scores were significantly correlated. The fourth study identified a positive correlation between BE behavior frequency and severity of night eating behavior among 729 college students (69). The fifth study investigated dietary pattern differences between patients with *DSM-IV* BE behavior with NES (n = 25) or without NES (n = 34) during a 1-week prospective evaluation (5). Participants with NES had higher scores on depressive symptoms, more BE behavior days and episodes per week, ingested more calories per day, and had a higher consumption of calories after the evening meal compared to those without NES.

Interventional studies

Five relevant interventional studies were identified (see **Figure 2**, **Table 4** and **Supplementary Figures 1**, **2**). All studies evaluated bright light therapy (BLT) in patients with BN and reported outcomes on BE behavior. No studies examining melatonin, melatonergic agents, or scheduled food intake were identified.

In four studies, including one non-RCT with a parallel group design (70), one RCT with a crossover design (71), and two open-label trials (72, 73), BLT was administered in the morning (between 6 and 9 AM) at 10,000 lux for 30–60 min. In the parallel-group clinical trial, a statistically significant decrease in BE behavior frequency was reported after three weeks in the active BLT group compared with the sham BLT group (70). However, there was no significant group difference in compensatory behavior. In the crossover RCT, the active BLT group showed a statistically significant decrease in the frequencies of both BE and compensatory behaviors after two weeks as compared to the sham group (71). In a 4-week open label study in participants with BN and a comorbid seasonal affective disorder, BLT decreased BE and compensatory behavior frequency (72). More recently, a 2-week open label study reported a decrease in BE-behavior frequency with BLT but with no change in compensatory behaviors (73). Of note, none of these studies described the distance between the subject and the light source, which is helpful to ensure the intensity of light to which each subject is actually exposed remains constant throughout light sessions.

In the only study that administered BLT in the evening, 18 patients were randomized to receive 2 weeks of bright (2,500 lux, n = 9) or moderate (500 lux, n = 9) light, each given at 17:00 h

TABLE 4 Interventional studies.

Author, year	Study design	Participants, Age	Sample size/Female%	Intervention	Outcomes	Instruments	Results	Limitations and comments
Blouin et al. (74)	1- week Double-Blinded RCT	BN, 27.9 (8.0)	BN = 18/100% BLT = 9, Dim light = 9	Laboratory sessions: Fluorescent "BLT" (2,500 lux) or "Dim light" (500 lux) at 3 ft. from source at 17:00 h. 2 h/session, 6 days	BE behavior episodes Compensatory behavior Depressive symptoms	NIMH-DIS-R BDI SIGH-SAD	No group difference in BE episodes, compensatory behavior, perceived control of food intake, or perceived control of food intake. BLT group decreased BDI scores compared to Dim light group.	Short study duration. BLT administered in the evening, at low intensity, and large distance from source (3 ft).
Braun et al. (70)	3- week Double-Blinded non-randomized controlled trial	BN, BLT = 30.0 (7.3) Dim light = 30.5 (8.6)	BN = 34/100% BLT = 16, Dim light = 18	BLT (10,000 lux) or red dim light (50 lux) for 30 min, administered between 6-9 am, for 3-weeks	Assess change in: BE episodes Compensatory behavior Depressive symptoms	SCID-*DSM IV* Eating behavior Diaries DEBQ HAM-D	Greater BE/week decrease in BLT vs. Dim light group. No group difference at follow-up. No group differences in compensatory behavior, meal or snack frequency, or urge to binge carbohydrates, or HAM-D scores.	Short study duration. MDD in 22-25% of participants Distance from light source during sessions not reported
De Young et al. (73)	2- week open-label	BN = 23.58 (4.52)	BN = 9/100%	BLT (10,000 lux) for 30 min, between 7-8 AM	Assess relationship between change in BE episodes, Compensatory behavior, and negative affect	SCID- *DSM IV*-TR EDE CHEDS CESD-R	CHEDS BE scale decreased during intervention. 27% decrease in BE days. Negative affect does not account for BE change. No significant decrease in compensatory behavior.	Short study duration BE days/week not reported BE/week change not reported Distance from light source during sessions not reported
Lam et al. (71)	2-weeks double blinded, randomized controlled crossover design	BN, 31.6 (6.5)	BN = 17/100%	Early morning (7:00 to 8:00 am) BLT (10,000 lux) or Dim red light (500 lux) for 30 min	BE episodes Compensatory behavior Depressive symptoms	*DSM-III* criteria Eating disorders diaries Eating Attitudes Test HAM-D	BLT decreased BE and compensatory behavior compared to dim light intervention. Difference in Eating Attitudes Test approached significance. BLT decreased depressive symptoms according to HAM-D, BDI.	Short study duration, small sample. Distance from light source not described. Fixed sleep schedules (10:00 pm to 7:00 am) and early morning session timing could induce phase advance through forced wake up time. Quick "relapse" of BE after BLT crossover to dim light.
Lam et al. (72)	4-week open-label	BN + MDD (SAD) = 30.2 (5.5)	BN + MDD (SAD) = 22/100%	BLT (10,000 lux) for 30-60 min, between 7–9 am, for 4 weeks	BE episodes Compensatory behavior Depressive symptoms	Eating behavior diaries HAM-D BDI	Decrease in BE and compensatory behaviors Decrease in depressive symptoms	45% taking antidepressant medication for at least 4 weeks Distance from light source during sessions not reported

BDI, Beck depression inventory; BE, binge eating; BLT, bright light therapy; BN, bulimia nervosa; CESD-R, Center for Epidemiological Studies Depression Scale – Revised; CHEDS, Change in Eating Disorder Symptoms Scale; DEBQ, Dutch Eating Behavior Questionnaire; DSM, Diagnostic and Statistical Manual of Mental Disorders; HAM-D, Hamilton Depression Rating Scale; MDD, Major Depressive Disorder; NIMH-DIS-R, National Institute of Mental Health Diagnostic Interview Schedule-Revised; RCT, randomized controlled trial; SAD, seasonal affective disorder; SCID, structured clinical diagnostic interview for the DSM; SIGH-SAD, Structured Interview Guide for the Hamilton Depression Rating Scale-Seasonal Affective Disorder Version.

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for 2 h and at three feet from the light source (74). There were no significant differences between the active or sham groups regarding frequencies of BE or compensatory behaviors, or in perceived control of food intake.

Taken together, these findings suggest that morning BLT (e.g., at 10,000 lux) might reduce BE behavior. However, these data must be considered preliminary due to small sample sizes and heterogeneity in study design and BLT characteristics.

Discussion

Binge eating behavior is a well-defined trans-diagnostic psychopathological phenomenon with clinical features that suggest a potential circadian system involvement. Although little systematic research has directly assessed the chronobiological aspects of BE behavior, we identified four broad categories of studies: (1) studies of patterns of food intake and/or timing of BE behavior (N = 16); (2) studies of circadian-related measures among individuals with BE behavior (N = 11); (3) studies of the co-occurrence of BE behavior and night eating (N = 18); and (4) studies evaluating BLT in the treatment of BN patients and reporting outcomes on BE behavior (n = 5). Studies of food intake timing typically found that BE behavior, as well as higher caloric intake in individuals with BE behavior, occurred later during the day. Studies on circadian-related measures found that BE behavior is associated with diurnal hormone and locomotor activity disturbances, as well as a late diurnal preference or more "eveningness." Studies of BE and night eating behavior found substantial overlap between these constructs. Interventional studies, though limited, suggest that morning BLT might reduce BE behavior. Below, we summarize and discuss the primary findings of the studies and identify research gaps in each category.

Patterns of food intake and timing of binge eating behavior

Evidence from studies in this category suggest a delayed food intake pattern and BE behavior occurrence throughout the day in individuals with BE behavior. A lower caloric consumption in the morning and a higher caloric consumption later in the day, may be a common trait among individuals with BE behavior that is stable during BE and non-BE behavior days (15, 28). These observations are consistent with the reports indicating that BE behavior and the urge to binge eat occur predominantly in the later part of the day (16, 36–39). In addition, irregularity and quantity of evening meal or snack consumption may be associated with increased frequency of BE behavior (29–34), and this association may be influenced by several factors including photoperiod (40) and cultural differences (35). However, it is also important to consider that the observed food intake patterns and timing of BE behavior could result from an interaction between chronobiological and non-chronobiological factors, or merely non-chronobiological factors. For example, the effect of environmental factors such as societal and work-related schedule restrictions, the associated homeostatic pressure on hunger and appetite regulatory mechanisms and how these may affect the timing and patterns of food intake is not known. Moreover, inverted sleep/wake cycles (e.g., shift work) or nighttime exposure to light may be associated with disturbances in circadian system function (75–77) that impact eating behavior in unknown ways and warrant further study (76, 78–80).

Assessment of circadian system function among individuals with binge eating behavior

This category was characterized by a limited number of studies with objective circadian-related assessments and no studies utilizing best-established techniques to assess circadian system function, and an involvement of the circadian system in BE behavior is not yet unequivocally confirmed. However, metabolic changes after binge-like food ingestion or BE behavior (42, 43, 81), actigraphic changes in subjects with BE behavior (44–47), as well as the potential association between evening chronotype and BE behavior (12, 49) support that aspects of the circadian system may be potentially compromised in affected individuals. Of note, studies reviewed have important limitations. For example, interpretation of earlier studies is challenging because subjects with BED were not typically included or BE behavior was often not examined independently from compensatory behaviors. Additionally, most studies are small with a preponderance of female participants and rely on retrospective self-report measures subject to response bias (e.g., underreporting of BE behavior and disordered eating symptoms). Early studies also had limited means of measuring circadian-related parameters. More recently and despite its limitations (e.g., inaccurate sleep onset time assessment), wrist actigraphy has become a useful circadian-related parameter. However, more studies with longer actigraphic recordings that prospectively document food intake pattern and BE behavior timing are needed to characterize locomotor activity in BE behavior. Future studies will be needed to address these limitations as well as to take advantage of the improved availability of objective measures of circadian system function (e.g., dim light melatonin onset) to prospectively study the chronobiology of BE behavior.

Of note, genetic research on the topic is extremely limited (52, 82), and we did not find genome-wide association studies on BE behavior and the circadian system. Whether the potential association between BE behavior and circadian system function is mediated by genetic factors is currently unknown. In

addition, studies on the circadian system function and BE behavior in youth are lacking. This is a relevant knowledge gap because BE behavior may occur early in life when the circadian system is still maturing. Understanding if BE behavior is associated with anomalies in the developmental trajectory of the circadian system may provide valuable insight into the normative-pathological spectrum of eating behavior and should be addressed.

Overlap of binge eating and night eating behavior

As observed in individuals with BE behavior, night eating and NES are characterized by a delayed pattern of food intake, suggestive of circadian system involvement which adds to their substantial clinical overlap (4, 23, 56, 57, 59, 60, 62–64, 67) and potential shared heritability (65). Moreover, individuals with both BED and NES appear to have higher BMI or levels of psychopathology compared to individuals with BED without night eating (4, 24, 61, 64, 68). However, there is a significant knowledge gap regarding the co-occurrence of BE behavior in night eating episodes and how to differentiate the two conditions as the timing of BE behavior, amount of food intake, and loss of control in night eating is not systematically documented in most studies. In addition, most BE behavior episodes occur in the later part of the day but BE is not usually investigated in night eating or NES, or considered as a possible comorbidity.

It remains unclear if BE behavior during the day differs from BE behavior during the night, how often night eating episodes meet strict criteria for BE behavior, and whether the circadian system might be implicated and how is not known. Presently, NES criteria do not consider the absence or presence of BE behavior during a night eating episode. Moreover, NES DSM-5 criteria under "Other Specified Feeding or Eating Disorder" limits its diagnosis if "the disordered pattern of eating is not better explained" by BED or another mental disorder. . ." (1). This perpetuates a notion of BE behavior and night eating as mutually exclusive phenomena, which has no

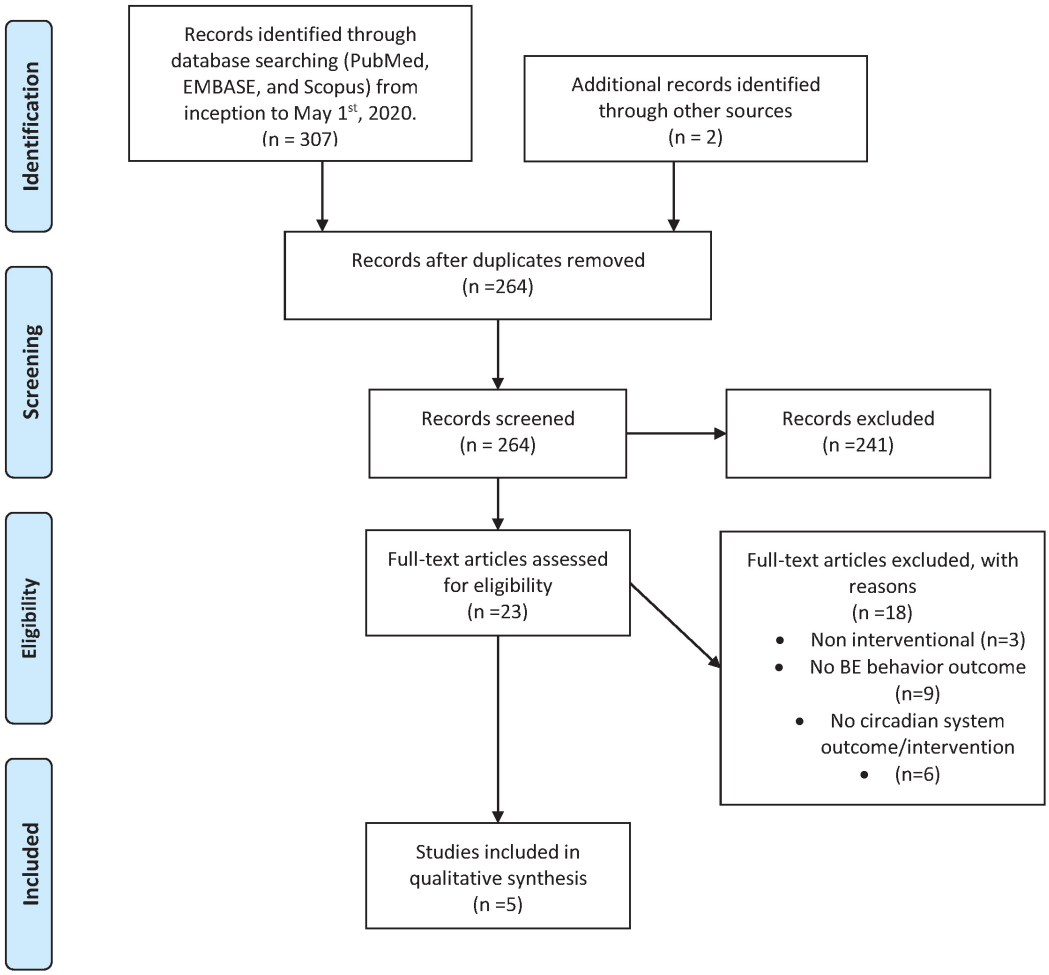

FIGURE 2
PRISMA flowchart for interventional studies.

clinical or scientific basis. Moreover, this may interfere with the identification and study of the association between BE behavior and night eating in the clinical and research settings that hampers the understanding of the potential role of the circadian system in both.

Interventional studies

This category included five studies evaluating BLT in patients with BN. BLT administered in the morning appeared to decrease BE behavior (70–73), an effect that was not observed in the one study administering BLT in the evening (74). BLT directly targets the circadian system, and these results suggest that a phase advance (circadian shift to occur earlier) or entrainment of the circadian system may influence BE behavior. These studies had a number of methodological limitations that curtail their clinical applicability, and their findings must be considered preliminary. In addition, it is not possible to discard a publication bias on this field. Nonetheless, BLT and other chronobiological interventions, such as nightly administration of exogenous melatonin or melatonin analogs and scheduled sleep/wake cycles, meals, or exercise, may require further consideration in the management of individuals with BE behavior (83, 84).

Potential future areas of research

Despite recent advances in the understanding on how the circadian system may be involved in appetite/hunger and metabolic regulation, knowledge of its potential role in EDs lags behind (8, 10, 85–87). Virtually none of the assessments that have been performed to date can distinguish involvement of the circadian system vs. influences of the sleep/wake, rest/activity, dark/light, fasting/eating cycle, mood/impulse control rhythms, and/or social/work/school schedules in causing daily rhythms related to BE behavior. It is thus relevant to highlight additional knowledge gaps and areas of opportunity in this field.

First, the concept of BE behavior as a discrete phenomenon has only recently gained the attention of clinicians, researchers, and the pharmaceutical industry. Exploring stand-alone BE behavior time-of-day occurrence and associated food intake patterns might serve to provide valuable insight into its

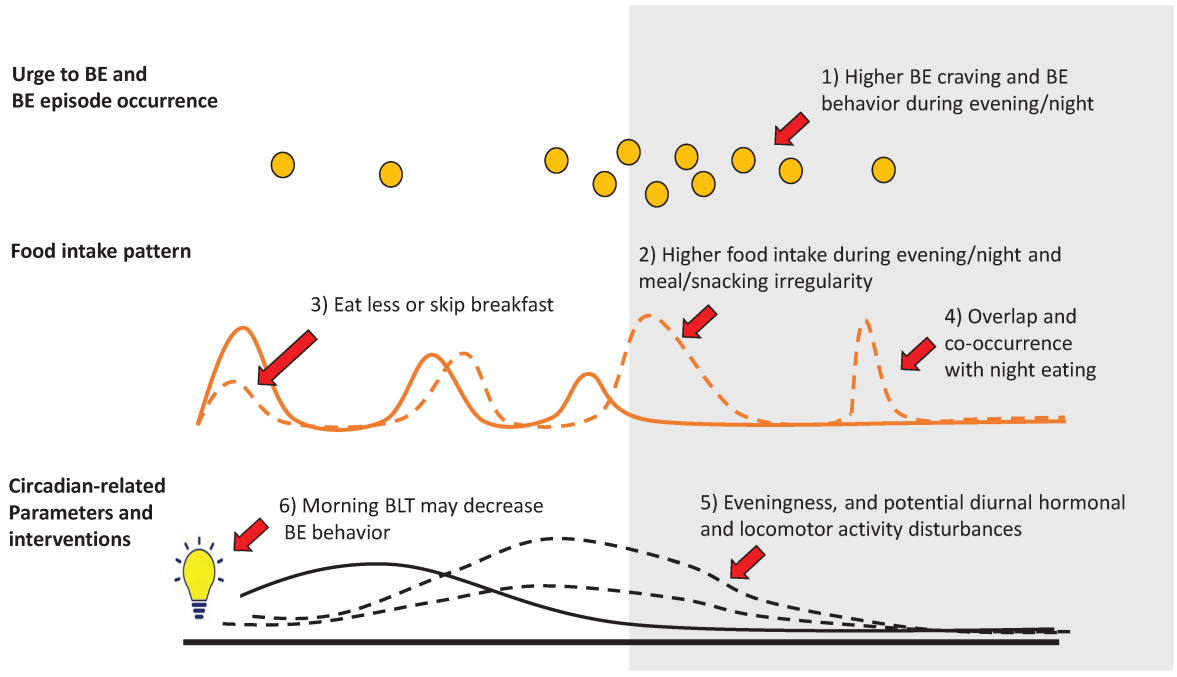

FIGURE 3
Circadian-related features in binge eating (BE) behavior. Illustrates six key circadian-related features that support a potential circadian system involvement in BE behavior: (1) Although both, the urge to binge eat and BE behavior (orange circles) may occur at any time of day (e.g., during a one-month period), they occur predominantly during the late afternoon/evening and night periods. Compared to individuals without BE behavior (solid lines), food intake in individuals with BE behavior (dashed lines) is characterized by; (2) higher calorie intake during the evening/night, and BE behavior frequency/severity is associated with late meal/snack irregularity, (3) less food intake in the morning or skipping breakfast more often, and (4) show clinical overlap and co-occurrence with night eating behavior. Circadian-related parameters associated with BE behavior include: (5) late diurnal preference (eveningness), as well as potentially disturbed diurnal hormonal and locomotor activity parameters, and (6) a potential therapeutic effect of morning bright light therapy (BLT) in BE behavior. Background colors illustrates day to the right (white) or evening/night periods to the left (gray).

relationship with the circadian system in clinical studies and preclinical research models (7, 88). Second, the current approach to studying the co-occurrence of BE behavior and night eating requires further attention. A step forward could be the recognition that a night eating episode may or may not meet criteria for BE behavior, and that this form of "night BE behavior" episodes require proper assessment, documentation, and analysis. Greater understanding of these issues might challenge the notion that NES is limited by the presence of other EDs as currently conceptualized in the DSM-5 (1). Third, the use of diaries to assess BE behavior and food intake patterns, combined with the use of best-established techniques (e.g., CR or FD) and objective tools (e.g., dim light melatonin onset) to assess circadian system function in BE behavior could contribute to address the indirectness (use of proxy measures) and enhance certainty of published evidence to close the knowledge gap on the association between circadian system function and BE behavior. Fourth, as supported by the available interventional studies (70–73), chronobiological interventions (e.g. morning BLT, evening melatonin) to induce circadian phase advances to assist with realignment in subjects with BE behavior may provide a mechanistic tool to further understand the potential influence of the circadian system in BE behavior, and to evaluate whether the circadian system represents a plausible therapeutic target.

Finally, understanding the mechanisms by which certain medications that affect BE behavior may also act on the circadian system may provide important clues. For example, antipsychotics may have a deleterious effect on BE behavior but their effect on the circadian system is poorly understood (89, 90). Likewise, there are medications used to reduce BE behavior for which an effect on the circadian system is plausible but currently unexplored, for example, lisdexamfetamine and topiramate (91). However, BE behavior clinical trials seldom include the assessment of outcome measures that inform on circadian system function. Perhaps it is time for that to change.

Summary

We identified six key findings that support the need to further explore the circadian system in BE behavior and its therapeutic potential. These are: (1) BE craving and behavior tend to occur in the later part of the day; (2) infrequent food intake and/or snacking during the evening/night are associated with increased BE behavior; (3) individuals with BE behavior tend to eat less early in the day or to skip breakfast; (4) BE behavior frequently co-occurs with night eating with remarkable clinical overlap; (5) eveningness, as well as diurnal hormonal and locomotor activity abnormalities, may be associated with BE behavior; and (6) interventions that specifically target the circadian system may decrease BE behavior (**Figure 3**). A continued effort to study the chronobiological aspects of BE behavior will be necessary to advance this re-emerging and relevant topic.

Author contributions

FR-N, AG, NM, and SM conceived and/or designed the work. FR-N, AG, NM, FL, CG, and SM conducted the primary and/or secondary literature search, data extraction, synthesis, and interpretation. FR-N, AG, NM, FS, HB, RM, JW, CG, and SM played an important role in interpreting the results. All authors contributed to drafting and revising the manuscript, and approved the final version.

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Conflict of interest

FR-N receives grant support from the National Institute of Mental Health K23 Award (K23MH120503) and from a 2017 NARSAD Young Investigator Award from the Brain and Behavior Research Foundation; is the inventor on a U.S. Patent and Trademark Office patent # 10,857,356; and has received non-financial research support from Soterix Medical. FS served on the Board of Directors for the Sleep Research Society and has received consulting fees from the University of Alabama at Birmingham. FS interests were reviewed and managed by Brigham and Women's Hospital and Partners HealthCare in accordance with their conflict-of-interest policies. FS consultancies are not related to the current work. HB served on the scientific advisory board for Natrol, LLC, and Moving Mindz, Pty Ltd, and is a consultant for F. Hoffmann-La Roche Ltd. CG

reports no competing interests but reports several broader interests which did not influence this manuscript including honoraria for lectures, CME activities, and presentations at scientific conferences and Royalties from Guilford Press and Taylor & Francis Publishers for academic books. SM is or has been a consultant to or member of the scientific advisory boards of F. Hoffmann-La Roche Ltd. Idorsia, Myriad, Novo Nordisk, Otsuka, Sipnose, Sunovion, and Takeda. She is or has been a principal or co-investigator on studies sponsored by Brainsway, Idorsia, Janssen, Marriott Foundation, Myriad, National Institute of Mental Health, Novo Nordisk, Otsuka, and Sunovion. She is also an inventor on United States Patent No. 6,323,236 B2, Use of Sulfamate Derivatives for Treating Impulse Control Disorders, and along with the patent's assignee, University of Cincinnati, Cincinnati, OH, United States has received payments from Johnson & Johnson, which has exclusive rights under the patent. AG is a paid consultant for Signant Health.

The remaining 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.978412/full#supplementary-material

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Role of sympathetic pathway in light-phase time-restricted feeding-induced blood pressure circadian rhythm alteration

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Disruption of blood pressure (BP) circadian rhythm, independent of hypertension, is emerging as an index for future target organ damage and is associated with a higher risk of cardiovascular events. Previous studies showed that changing food availability time alters BP rhythm in several mammalian species. However, the underlying mechanisms remain largely unknown. To address this, the current study specifically investigates (1) the relationship between rhythms of food intake and BP in wild-type mice; (2) effects of light-phase time-restricted feeding (TRF, food only available during light-phase) on BP circadian rhythm in wild-type and diabetic *db/db* mice; (3) the roles of the autonomic system and clock gene in light-phase TRF induced changes in BP circadian rhythm. Food intake and BP of C57BL/6J and *db/db* mice were simultaneously and continuously recorded using BioDAQ and telemetry systems under *ad libitum* or light-phase TRF. Per2 protein daily oscillation was recorded *in vivo* by IVIS spectrum in mPer2^{Luc} mice. Autonomic nerve activity was evaluated by heart rate variability, baroreflex, urinary norepinephrine (NE) and epinephrine (Epi) excretion, and mRNA expressions of catecholamines biosynthetic and catabolic enzymes, and alpha-adrenergic receptors in mesenteric resistance arteries. We found that in wild-type mice, the BP level was correlated with the food intake temporally across the 24 h. Reversing the feeding time by imposing light-phase TRF resulted in reverse or inverted BP dipping. Interestingly, the net changes in food intake were correlated with the net alteration in BP temporally under light-phase TRF. In *db/db* mice, light-phase TRF worsened the existing non-dipping BP. The food intake and BP circadian rhythm changes were associated with alterations in Per2 protein daily oscillation and the time-of-day variations in heart rate variability, baroreflex, and urinary excretion of NE and Epi, and increased mRNA expression of *Slc6a2* (encoding NE transporter) and *Adra1d* (encoding alpha-adrenergic receptor 1d) in the mesenteric resistance arteries, indicating the sympathetic nervous system (SNS) was modulated after light-phase TRF. Collectively, our results demonstrated that light-phase

TRF results in reverse dipping of BP in wild-type and diabetic *db/db* mice and revealed the potential role of the sympathetic pathway in light-phase TRF-induced BP circadian rhythm alteration.

KEYWORDS

blood pressure circadian rhythm, time-restricted feeding, sympathetic nervous system, heart rate variability, baroreflex, norepinephrine, alpha-adrenergic

Introduction

Normal blood pressure (BP) exhibits a circadian rhythm that rises in the morning and decreases 10–20% during the night, known as dipping BP. Disruptions of BP circadian rhythm, which often manifest as non-dipping BP (less than 10% decrease of nighttime BP from daytime BP) or reverse or inverted dipping BP (higher nighttime than daytime BP), are highly prevalent in patients with hypertension, type 2 diabetes, chronic kidney disease, and sleep apnea syndrome (1, 2). Clinical studies demonstrate that non-dipping and reverse dipping BP is associated with target organ damages and increased detrimental cardiovascular events (1, 3, 4). Thus, understanding the mechanisms that control normal BP circadian rhythm has high clinical relevance.

Experimentally limiting food availability only to the inactive phase alters BP rhythm markedly in several mammalian species (5–8), suggesting that the timing of food intake plays a critical role in regulating BP circadian rhythm. However, these studies did not simultaneously monitor the food intake and BP circadian rhythm. Hence, whether or not food intake temporally correlates with BP circadian rhythm and directly triggers BP change under altered feeding schedule remains unclear. Nocturnal animals consume most food during the dark phase when they are active under normal conditions. The current study alters the feeding schedule to the light phase, the inactive phase of nocturnal mice, termed as light-phase time-restricted feeding (light-phase TRF) and aims to address these important issues and investigate whether food intake temporarily correlates with BP alteration by simultaneously monitoring the episodic feeding activity by the BioDAQ system and the beat-to-beat BP by telemetry in singly housed mice under *ad libitum* feeding (ALF) or light-phase TRF.

Among the multiple mechanisms regulating BP homeostasis, the critical role of the sympathetic nervous system (SNS) is well recognized. SNS activity, measured by plasma or urine catecholamine levels, exhibits circadian rhythms that parallel BP rhythm (9, 10). Previous studies demonstrated that SNS activity is suppressed during fasting and enhanced after feeding (11–15). Consistent with these studies, we recently reported that the non-dipping BP is associated with diminished rhythms of food intake and SNS activity in diabetic *db/db* mice (16). Importantly, we demonstrated that aligning food availability with the standard light-dark cycle by dark-phase TRF, which limits food availability only to the active dark phase, restores BP and SNS activity rhythms in *db/db* mice (16). However, whether SNS activity rhythm serves as the mechanistic linkage between light-phase TRF and BP circadian rhythm alteration has not been investigated. The current study tested if the modulation of SNS activity mediates, at least in part, the light-phase TRF-induced BP circadian rhythm alterations in wild-type mice. In addition, we recently reported that dark-phase TRF protects BP circadian rhythm in diabetic *db/db* mice (16). However, the effect of light-phase TRF on BP circadian rhythm in diabetic *db/db* mice remains to be illustrated. The current study also investigated whether, in contrast to the dark-phase TRF-induced protection of BP circadian rhythm (16), light-phase TRF worsens the non-dipping BP in diabetic *db/db* mice.

The molecular basis of circadian rhythm is the endogenous autonomous clocks present in the central suprachiasmatic nucleus and nearly all peripheral tissues. The intrinsic clocks, comprised of a group of transcription factors that form a feedback loop, are entrained by the environment and thus maintained in a ∼24-h rhythm (17). While light is the most potent environmental cue to entrain the central clock, accumulating evidence indicates that food intake is a major factor in entraining peripheral clocks (17). Mouse models harboring clock gene mutation or knockout suggest that clocks play a critical role in regulating BP circadian rhythms (18). To investigate the potential involvement of clocks in light-phase TRF-induced alteration of BP circadian rhythm, we, therefore, examined the oscillation of Period 2 (per2), one of the core clock genes, in response to light-phase TRF using luciferase knock-in mice ($Per2^{luc}$) (19).

Collectively, the current study aims to elucidate (1) the temporal relationships between food intake and BP circadian rhythm in wild-type mice fed *ad libitum* and light-phase TRF; (2) the responses of the SNS and Per2 oscillation to light-phase TRF in wild-type mice; (3) the effects of light-phase TRF on BP circadian rhythm in *db/db* mice.

Materials and methods

Animals

C57BL/6J (Stock No.: 000664), homozygous mPer2^{Luc} (Stock No: 006852), and *db/db* (Stock No.: 000642) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, United States). Only male mice were used in the current study. The mice were housed under a 12:12 light: dark cycle in a light-tight box and fed with a standard rodent diet with free water access. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

Time-restricted feeding and food intake monitoring

Food intake of 17-week-old wild-type mice (n = 7) or 21-week-old *db/db* mice (n = 10) were recorded under ALF (*ad libitum*) for 3 days, followed by 7 days' of light-phase TRF (10 h of food access from zeitgeber time (ZT) 2 to ZT12). The time of food availability and the amount of food consumed were recorded by a BioDAQ system (Research Diet, New Brunswick, NJ) (16). The BioDAQ system has a feeding module fitted with an electronic sensor and an automated gate controller, allowing food access at a designated time and continuous accurate monitoring of food intake. Mice were acclimated to the system for at least 7 days before the experiment, and food intake at different time intervals was analyzed as indicated in the figures.

Measurement of blood pressure, heart rate, and locomotor activity with telemetry

The same wild-type and *db/db* mice used in the food intake monitoring experiment were implanted with a telemetry probe (TA11PA-C10, Data Sciences International, St. Paul, MN, United States) into the left common carotid artery to continuously record BP, heart rate, and locomotor activity in free-moving mice at a sampling rate of 1,000 Hz (16, 20, 21). Mice were allowed 7–10 days of recovery from surgery before measurement.

In vivo imaging of mPer2 time-of-day variation in the kidney and liver

Seventeen-week-old mPer2^{luc} mice (n = 17) under ALF were imaged at zeitgeber time ZT5, ZT11, ZT17, and ZT23 and then subjected to light-phase TRF and imaged again on day 3 and day 7 using the IVIS system (IVIS Spectrum *in vivo* imaging system, PerkinElmer, Waltham, MA, United States) as previously described (22). Briefly, mice were anesthetized with 2.5–4% isoflurane and subcutaneously injected with D-luciferin (15 mg/kg body weight in PBS). The mice were imaged 7 min later for dorsal side up and 10 min later for ventral side up for 5 s using the IVIS spectrum. Total bioluminescence (photon/s/cm2/sr) was quantified by setting the region of interest to the same shape and size using Living Image software (IVIS Imaging System). To eliminate individual mouse and measurement variation and to better quantify the phase of Per2 oscillation, relative bioluminescence intensity was calculated by normalizing absolute bioluminescence to the average bioluminescence of the four-time points (22).

Heart rate variability analysis

Heart rate variability was analyzed using frequency and time domain methods by Ponemah Software (Data Sciences International; St. Paul, MN) as previously described (16). Briefly, for frequency domain determination, 2-min artifacts-free beat-by-beat BP waveform segments were selected from every 20 min across the 72 h of recordings for the final analysis. Each segment was then interpolated to 20Hz using the quadratic method, followed by Fast Fourier Transformation using hanning window method. The cut-off frequency ranges for low-frequency (LF) and high-frequency (HF) were 0.15–0.6 Hz and 1.5–4 Hz, respectively. For time-domain analysis, 5-min beat-by-beat BP waveform segments over 72 h were calculated, and the root-mean-square successive beat-to-beat difference (rMSSD) was plotted as the parasympathetic heart rate control marker. Systolic pressure was used as the trigger for frequency domain and time domain analysis. The heart rate variability was averaged in each correspondent hour over 3 days for both the frequency and time domain data to generate a 24-h heart rate variability profile.

Cardiac baroreflex sensitivity analysis

Cardiac baroreflex sensitivity (referred to as baroreflex sensitivity in the manuscript for simplicity) was analyzed using sequence techniques by Hemolab software[1] as previously described (16, 21). For each hour, the software searches sequences in which the systolic arterial pressure and pulse interval were positively correlated ($r^2 > 0.80$) to identify valid sequences with at least four consecutive changes as an effective Baroreflex. The average slope of the systolic pressure-pulse interval relationships is calculated as baroreflex sensitivity. For each mouse, 72 hourly baroreflex sensitivity data points were

1 http://www.haraldstauss.com/HemoLab/HemoLab.html

calculated from the three consecutive days of BP data and then averaged to generate one 24-h profile.

Urine collection and catecholamines measurement

Urine was collected using metabolic cages (Tecniplast, West Chester, PA). To prevent mice from dehydration and urine contamination by food crumbs, the mice were fed a gel diet (DietGel76A, ClearH_2O, Portland, ME) during the urine collection period. The mice were acclimated to the metabolic cage and gel diet for 3 days before actual urine collection. The 12-h light- and dark-phase urine samples were first collected in 15- to 16-week-old mPer2^{Luc} mice (n = 11) under ALF. Then the mice were subjected to light-phase TRF for 7 days, and the 12-h light- and dark-phase urine samples were collected again on the last day of light-phase TRF. We used mPer2^{Luc} mice because mPer2^{Luc} mice have been backcrossed to C57BL/6J inbred mice for 11 generations, and mPer2^{Luc} mice exhibited a similar normal food intake and BP rhythm as C57BL/6J mice (22). Urinary norepinephrine (NE) and epinephrine (Epi) were determined by the ELISA kits (Abnova, Taipei, Taiwan). Total contents of NE and Epi were calculated by concentrations × urine volumes (16).

Quantitative analysis of mRNA expression

Twenty-week-old male Per2^{Luc} mice were randomly divided into ALF (n = 5) or light-phase TRF (n = 6) groups.7 days later, mice were euthanized between ZT9 and ZT11, and mesenteric arteries were collected in RNAlater solution (ThermoFisher Scientific, United States). The fat surrounding the mesenteric arteries was carefully removed under a dissecting microscope. As previously described (16, 21, 23–25), the mRNA levels of various genes were quantified by real-time PCR. The real-time PCR primers for each gene were described in **Supplementary Table 1**.

Statistical analysis

The sample size was determined based on previous publications (16, 26). All data were expressed as mean ± standard error of the mean (SEM). For comparison of 1 parameter in the same mice, a paired t-test was performed. For analysis of 1 parameter in different mice, an unpaired t-test was used. Repeated two-way ANOVA with matching conditions between light vs. dark phase and between ALF vs. light-phase TRF with Tukey's post-test was conducted to compare two parameters in the same mice. The main factors for two-way ANOVA were feeding (ALF vs. light-phase TRF) and time (light phase vs. dark phase or feeding vs. fasting). Correlations between food intake and BP and between Δfood intake/Δlocomotor activity and ΔBP were calculated using linear regression. An ANCOVA was performed where feeding method (ALF vs. light-phase TRF) and light condition (light vs. dark) were used as factors, and BP and locomotor activity under ALF or light-phase TRF were used as dependent variable and covariant respectively. All statistical analyses, expect ANCOVA, were performed by Prism 9 software (GraphPad Software, San Diego, CA). ANCOVA was performed by IBM SPSS Statistics. $P < 0.05$ was defined as statistically significant.

Results

Food intake rhythm correlates with blood pressure rhythm in mice under *ad libitum* feeding

To determine the temporal relationship between food intake and BP under ALF and light-phase TRF, 16-week-old male wild-type C57BL/6J mice were implanted with telemetry and acclimated in BioDAQ cages under ALF for 10 days, followed by light-phase TRF for 7 days. During light-phase TRF, food was available from ZT2 to ZT12 (ZT0: lights-on and ZT12: lights-off). Food intake and BP were monitored simultaneously and continuously in the same single-housed mouse using BioDAQ and telemetry systems during the last 3 days of ALF and 7 days of light-phase TRF.

Under ALF, wild-type mice consumed more food during the dark phase than during the light phase (**Figure 1A**), and their mean arterial pressure (MAP) was higher during the dark phase than during the light phase (**Figure 1B**). The two parameters showed a highly similar pattern when 2 h-average MAP was plotted with 2 h-sum food intake during the corresponding time on the same graph (**Figure 1C**). There was a significant correlation between food intake and MAP, with a linear correlation coefficient of r = 0.75 (**Figure 1D**).

Light-phase time-restricted feeding rapidly alters blood pressure rhythm and results in reverse dipping

As shown in **Figure 2A**, light-phase TRF started with food withdrawal on day 4 during the dark phase, leading to the shift of food consumption mostly during the dark phase (day 1-3) to exclusively during the light phase (day 4-10). In parallel with the shift in food intake, a rapid decrease in the dark-phase MAP occurred during the first day of light-phase TRF (day 4; **Figure 2B**) and nearly reached a steady state by the second day

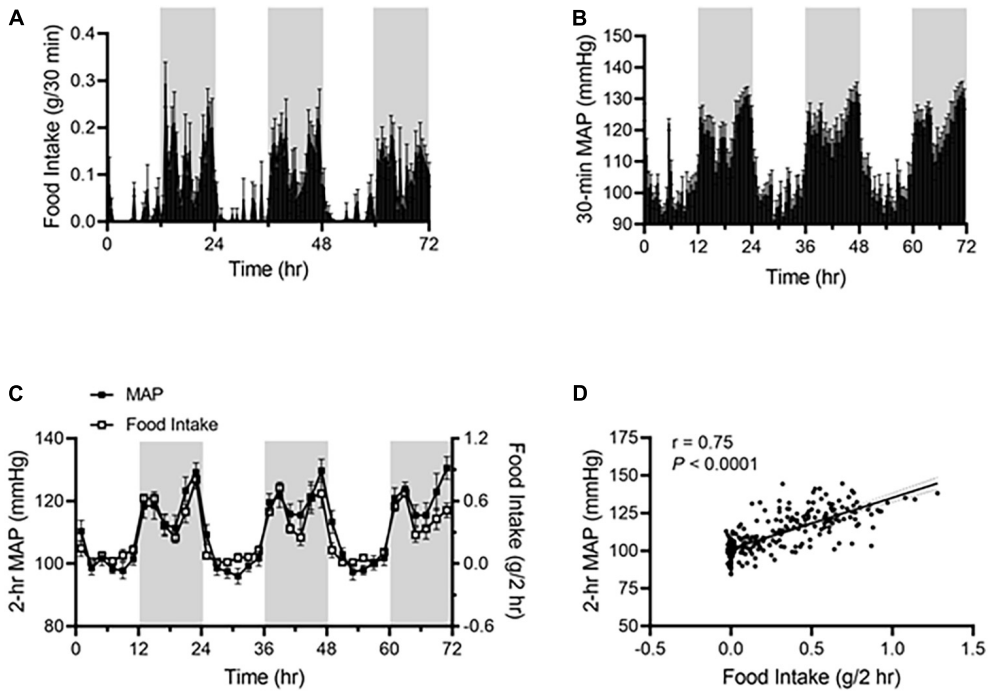

FIGURE 1
Food intake correlates with BP rhythm in *ad libitum-fed* mice. Food intake and BP were recorded by BioDAQ and telemetry in 17-week-old male C57BL/6J mice (N = 7). **(A,B)** Daily profiles of accumulated food intake **(A)** and average mean arterial pressure [MAP; **(B)**] in 30-min (min) intervals over 72-h during the light and dark phases, shown in white and gray, respectively. **(C)** Average MAP (left axis) and accumulated food intake (right axis) in 2-h intervals over 72-h during the light and dark phases. **(D)** Linear regression analysis of average MAP and accumulated food intake in 2-h intervals. Data were expressed as the mean ± standard error of the mean (SEM).

of light-phase TRF (day 5; Figure 2B). In contrast, an increase in the light-phase food intake and MAP was observed on the second day of light-phase TRF and reached a steady state on the fourth day of light-phase TRF (day 7; Figures 2A,B).

Light-phase TRF induced a phase shift of food intake and MAP from the dark phase to the light phase (Figures 2C,F). Cosinor analysis showed that the acrophase of MAP was shifted from ZT19.2 ± 0.2 with ALF to ZT9.7 ± 0.3 after light-phase TRF (Supplementary Table 2). Consequently, there was a significant increase in the food intake and MAP during the light phase and a significant decrease in the food intake and MAP during the dark phase, respectively (Figures 2D,G). As a result, the light-dark phase MAP difference under ALF was reversed under light-phase TRF (Figure 2G) and the amplitude of MAP oscillation was decreased after light-phase TRF compared to ALF (Supplementary Table 2). When analyzing the dipping status, light-phase TRF resulted in reverse-dipping of BP (Figure 2I). In contrast, light-phase TRF did not affect 24-h average MAP (Figure 2H). Interestingly, the 24-h daily food intake was comparable between ALF and light-phase TRF (Figure 2E).

To further define whether changes in MAP during light-phase TRF temporally correlate with the changes in food intake, we calculated the net differences in food intake and MAP between ALF and light-phase TRF by subtracting food intake or MAP during ALF from light-phase TRF. We found that the net differences in food intake were almost completely parallel to the net difference in MAP (Figure 2J), indicating a change in food intake can timely trigger a change in MAP. Linear regression analysis revealed a significant correlation between Δfood intake and ΔMAP (Figure 2K). Light-phase TRF also induced similar changes in SBP (Supplementary Figures 1A–D) and DBP (Supplementary Figures 1E–H).

Telemetry also recorded heart rate and locomotor activity in mice under ALF or light-phase TRF. Similar to its effects on BP, light-phase TRF also induced similar changes in locomotor activity (Supplementary Figures 2A-C) and heart rate (Supplementary Figures 2D-F). Notebly, the net changes in locomotor activity also parallel to the net differences in MAP (Supplementary Figure 2G) and linear regression analysis showed a significant correlation between Δlocomotor activity and ΔMAP (r = 0.79, Supplementary Figure 2H). To further evaluate whether alterations in locomoter activity mediates light-phase TRF induced BP changes, we analyzed the effects of light-phase TRF on MAP after adjusting for locomotor activity using ANCOVA. The result showed that locomotor activity is a covariate [F (1,23) = 6.618, p = 0.017], however, the feeding

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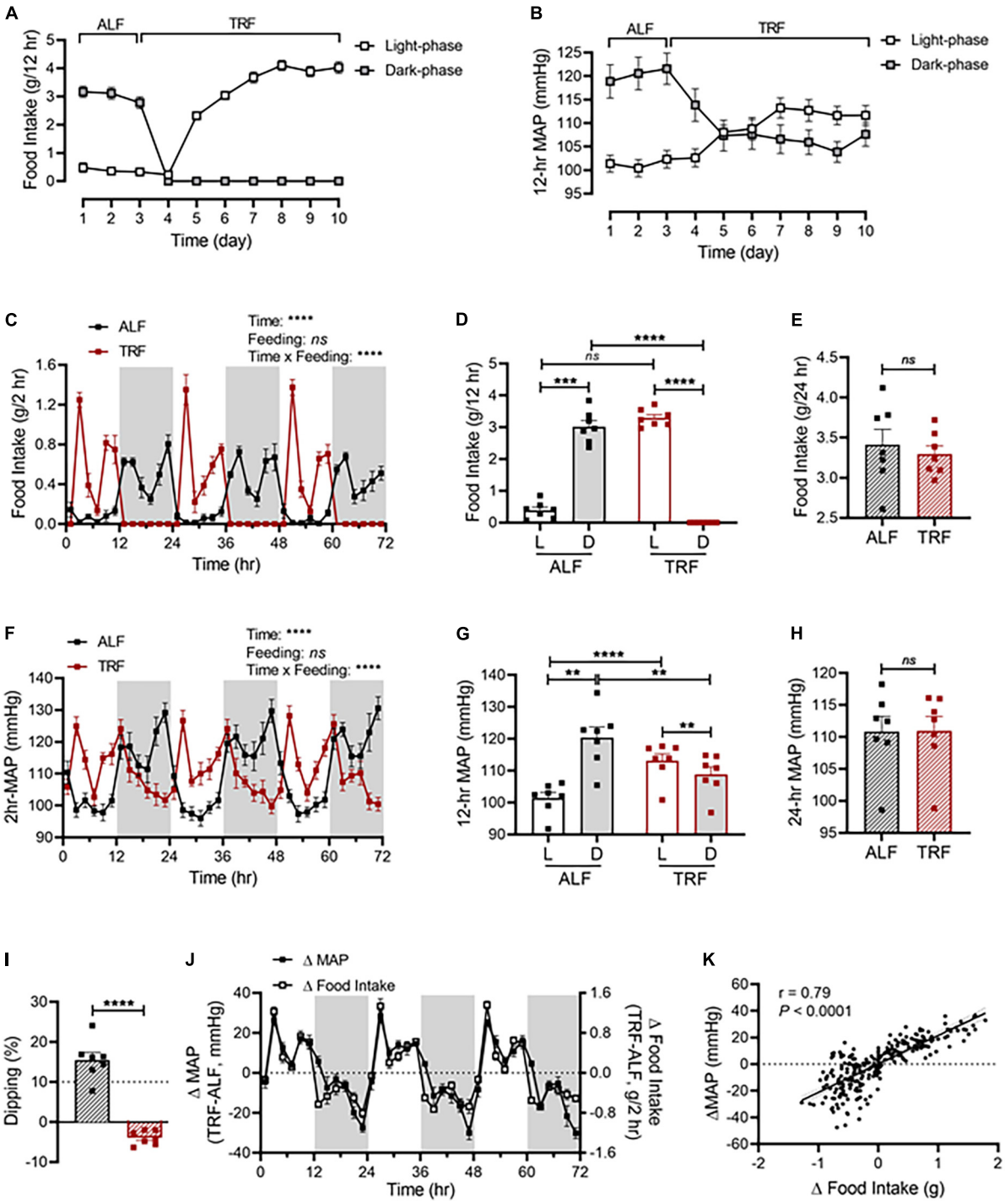

FIGURE 2

Light-phase TRF rapidly alters BP rhythm. **(A,B)** Accumulated food intake **(A)** and average MAP **(B)** during the light and dark phase in 17-week-old male C57BL/6J mice (N = 7) under 3 days of ALF, followed by 7 days of TRF. **(C–E)** Accumulated food intake in 2-h **(C)**, 12-h **(D)**, and 24-h **(E)** intervals over 3 days under ALF and during the last 3 days under light-phase TRF. **(F–H)** Average MAP in 2-h **(F)**, 12-h **(G)**, and 24-h **(H)** intervals over 3 days of ALF and the last 3 days under light-phase TRF. **(I)** MAP dipping with ALF and reverse dipping with light-phase TRF. **(J)** Net changes in accumulated food intake (Δfood intake) and average MAP (ΔMAP) in 2-h intervals over 72 h. **(K)** Linear regression of Δfood intake and ΔMAP. Data were expressed as the mean ± SEM and analyzed by repeated two-way ANOVA with matching conditions between light vs. dark phase and between ALF vs. light-phase TRF with Tucky's *post-hoc* analysis **(C,D,F,G)**, paired *t*-test **(E,H,I)**, and simple linear regression **(K)**.$^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; ns, not significant.

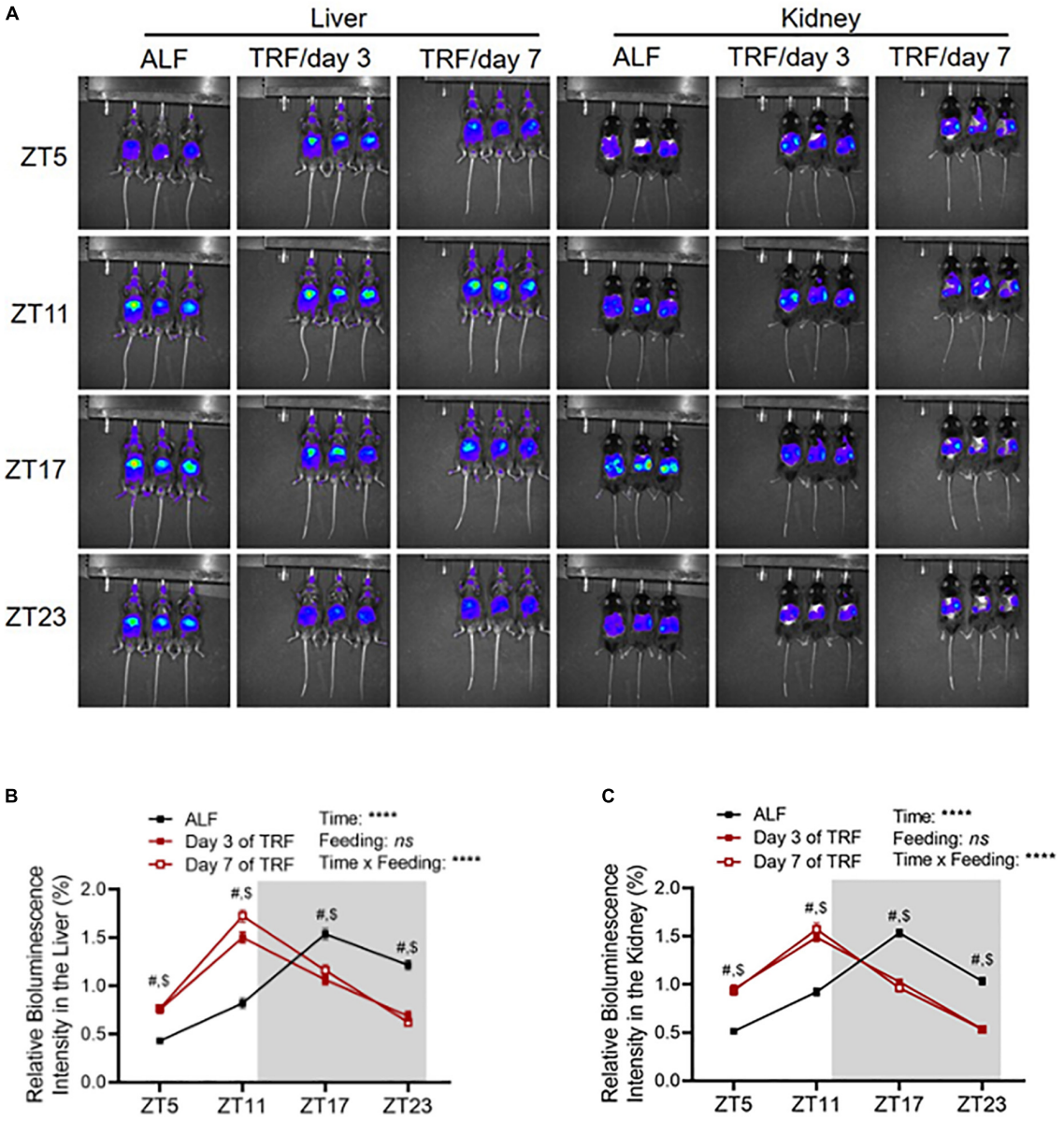

FIGURE 3
Light-phase TRF shifts Per2 protein daily oscillation. **(A)** Representative images of Per2 bioluminescence in the liver and kidney in 17-week-old male mPer2^{Luc} mice under ALF and at day 3 and day 7 after light-phase TRF. **(B,C)** Relative Per2 bioluminescence intensity in the liver **(B)** and kidney **(C)** under ALF and day 3 and day 7 after light-phase TRF. Data were expressed as the mean ± SEM (N = 17) and analyzed by repeated two-way ANOVA with matching conditions between ZT and between ALF vs. light-phase TRF with Tucky's *post–hoc* analysis **(B,C)**. #, ALF *vs.* day 3 after TRF, $P < 0.0001$. $, ALF *vs.* day 7 after TRF, $P < 0.0001$.

method (ALF vs. TRF) still has a significant effect on BP even after adjusted for locomotor activity [F (1,23) = 4.64, p = 0.042].

Light-phase time-restricted feeding advances Per2 protein daily oscillations

To explore whether clocks are involved in light-phase TRF-induced BP circadian rhythm alteration, we determined Per2 protein expression by *in vivo* imaging in the liver and kidney in 17-week-old male mPer2^{Luc} mice at ZT5, ZT11, ZT17, and ZT23 under ALF and on day 3 and day 7 of light-phase TRF using IVIS Spectrum. Under ALF, Per2 bioluminescence peaked at ZT17 during the dark phase in the liver and kidney (Figures 3A-C). Light-phase TRF for 3 days advanced Per2 bioluminescence for 6 h in the liver and kidney, with the highest at ZT11 during the light phase (Figures 3A-C). Prolonged light-phase TRF from 3 to 7 days did not further shift the Per2 bioluminescence phase

in the liver and kidney since no difference was observed in Per2 protein diurnal variations between day 3 and day 7 under light-phase TRF (Figures 3A-C).

Light-phase time-restricted feeding alters the sympathetic pathway

To explore whether light-phase TRF might modify the sympathetic nervous system (SNS), thus altering BP circadian rhythm in wild-type mice, we first calculated the heart rate variability (HRV) in the same groups of ALF- and light-phase TRF-fed 17-week-old wild-type C57BL/6J mice described above. HRV was calculated by frequency and time domain measurements as the low-frequency spectral power (LFSP), high-frequency spectral power (HFSP), and root means square of successive RR interval differences (rMSSD). Under ALF, LFSP was lower in the dark phase than in the light phase (Figures 4A,B). Light-phase TRF did not change the average LFSP during the 12-h light phase but significantly increased LFSP during the 12-h dark phase (Figure 4B). Because food was available only for 10 h during the 12-h light phase under light-phase TRF, we also analyzed the HRV during the fasting (ZT2 to ZT12) *vs.* feeding (ZT13 to ZT2) period in mice under ALF and light-phase TRF to depict food intake-induced LFSP changes accurately. Interestingly, light-phase TRF *vs.* ALF significantly decreased the LFSP during the feeding period but increased the LFSP during the fasting period (Figure 4C). A similar result was also obtained from the HRV analysis of HFSP and rMSSD during the dark/fasting phase but not during the light/feeding period (Figures 4D-I).

We next determined spontaneous cardiac baroreflex sensitivity (BRS) in the same groups of ALF- and light-phase TRF-fed 17-week-old wild-type C57BL/6J mice described above. Results showed that mice have higher BRS during the light phase than during the dark phase under ALF (Figures 4J,K). Light-phase TRF significantly decreased BRS during the light phase (Figure 4K) or the feeding period (Figure 4L) but increased BRS during the dark phase (Figure 4K) or the fasting period (Figure 4L).

To more directly evaluate the role of the SNS in light-phase TRF-induced BP circadian rhythm alteration, we analyzed urinary norepinephrine (NE) and epinephrine (Epi) excretion during the light and dark phases in 15- to 16-week-old male mPer2^{Luc} mice under ALF and light-phase TRF. The 12-h light- and dark-phase urine samples were collected in metabolic cages on day 4 of ALF and day 7 of light-phase TRF. Results showed that urinary NE and Epi exhibited a diurnal variation that is significantly lower during the light phase than during the dark phase in mice under ALF (Figures 5A,B), which coincided with BP rhythm (i.e., Figure 2G). Also, in line with its effect on BP circadian rhythm alteration, light-phase TRF significantly suppressed urinary NE and Epi during the dark phase (Figures 5A,B). In addition, light-phase TRF also had a trend of increased urinary NE and a significant increased urinary Epi excretion during the light phase (Figures 5A,B). As a result, diurnal variations of urinary NE and Epi excretion were abolished in mice under light-phase TRF (Figures 5A,B).

To investigate the molecular mechanism by which light-phase TRF induces BP circadian rhythm alteration, we determined mRNA expressions of the genes responsible for catecholamine biosynthesis, catabolism, and function in mesenteric resistant arteries in 20-week-old male mPer2^{Luc} mice under ALF and 7 days after light-phase TRF. To rule out the potential effect of the timing on the gene expression, mice were euthanized at the same time (ZT9-ZT11). Interestingly, light-phase TRF significantly increased the expression of *carrier family 6 member 2 (Slc6a2),* a NE transporter (NET) gene, but did not affect expressions of *tyrosine hydroxylase (Th)* and *dopa decarboxylase (Ddc)*, two NE biosynthesis enzyme genes, and *phenylethanolamine N-methyltransferase* (*Pnmt*), *catechol-O-methyltransferase* (*Comt*), and *monoamine oxidase A and B (MaoA and MaoB)*, three NE catabolism genes (Figures 5C–I). Also of interest is the light-phase TRF upregulated alpha *1d adrenergic receptor* (*Adra1d) gene* but not other subtype adrenergic receptors, including alpha *1a adrenergic receptor* (*Adra1a),* alpha *1b adrenergic receptor* (*Adra1b),* alpha *2a adrenergic receptor* (*Adra2a),* alpha *2b adrenergic receptor* (*Adra2b),* and alpha *2c adrenergic receptor* (*Adra2c)* (Figures 5J–O).

Light-phase time-restricted feeding worsens blood pressure rhythm and blood glucose in diabetic *db/db* mice

Given that light-phase TRF reverses BP circadian rhythm in non-diabetic C57BL/6J mice (Figure 2), we hypothesized that light-phaseTRF was detrimental rather than beneficial to BP circadian rhythm in *db/db* mice. To test this hypothesis, 21-week-old wild-type male *db/db* mice were implanted with telemetry and acclimated in BioDAQ cages under ALF for 10 days, followed by light-phase TRF for 7 days. Food intake and BP were monitored simultaneously and continuously during the last 3 days of ALF and 7 days of light-phase TRF.

Compared with non-diabetic C57BL/6J mice, diabetic *db/db* mice under ALF exhibit dampened circadian rhythms in food intake (Figures 6A vs. 2C) and MAP (Figures 6D vs. 2F), lost the diurnal difference in food intake (Figures 6B vs. 2D) and MAP (Figures 6E vs. 2G), and were non-dippers (Figures 6G vs. 2I). Importantly, light-phase TRF significantly increased the food intake (Figures 6A,B) and MAP (Figures 6D,E) during the light phase while decreasing them during the dark phase in *db/db* mice (Figures 6A,B,D,E), resulting in reverse dipping BP (Figure 6G). Similar to its effect on MAP, light-phase TRF also induced a similar detrimental impact on

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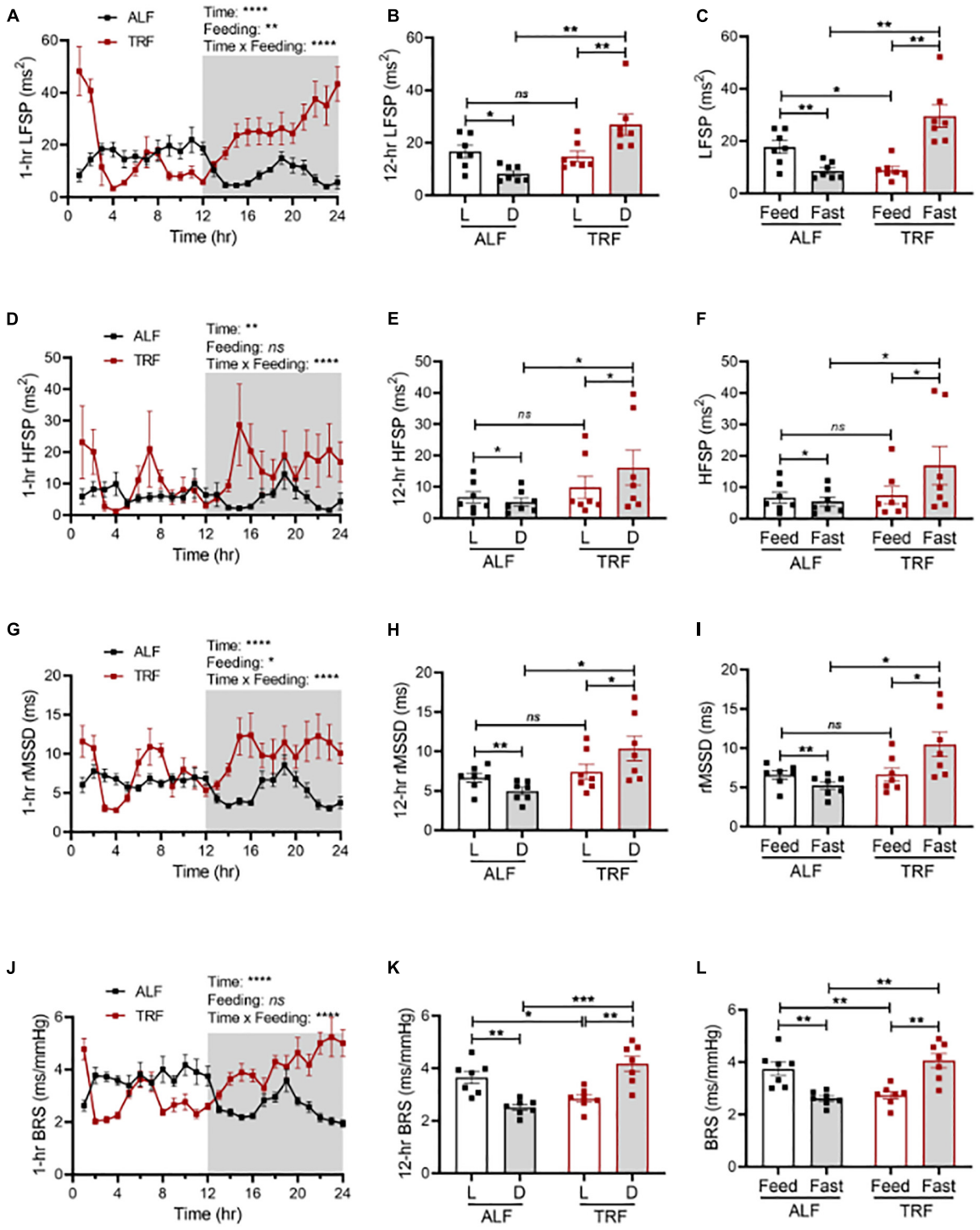

FIGURE 4
Light-phase TRF modulates autonomic nervous activity. Heart rate variability and cardiac baroreflex sensitivity (BRS) were calculated from BP data recorded 3 days under ALF and the last 3 days of TRF. $N = 7$. **(A,B)** Low-frequency spectral power (LFSP) in 1-h **(A)** and 12-h **(B)** intervals. **(C)** LFSP during feeding and fasting period. **(D,E)** High-frequency spectral power (HFSP) in 1-h **(D)** and 12-h **(E)** intervals. **(F)** HFSP during feeding and fasting period. **(G,H)** Root mean square of successive differences between normal heartbeats (rMSSD) in 1-h **(G)** and 12-h **(H)** intervals. **(I)** The rMSSD during the feeding and fasting period. **(J,K)** BRS in 1-h **(J)** and 12-h **(K)** intervals. **(L)** BRS during feeding and fasting period. Data were expressed as the mean ± SEM and analyzed by repeated two-way ANOVA with matching conditions between light vs. dark phase or feed vs. fast and between ALF vs. light-phase TRF with Tucky's *post–hoc* analysis **(A–L)**. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$; ns, not significant.

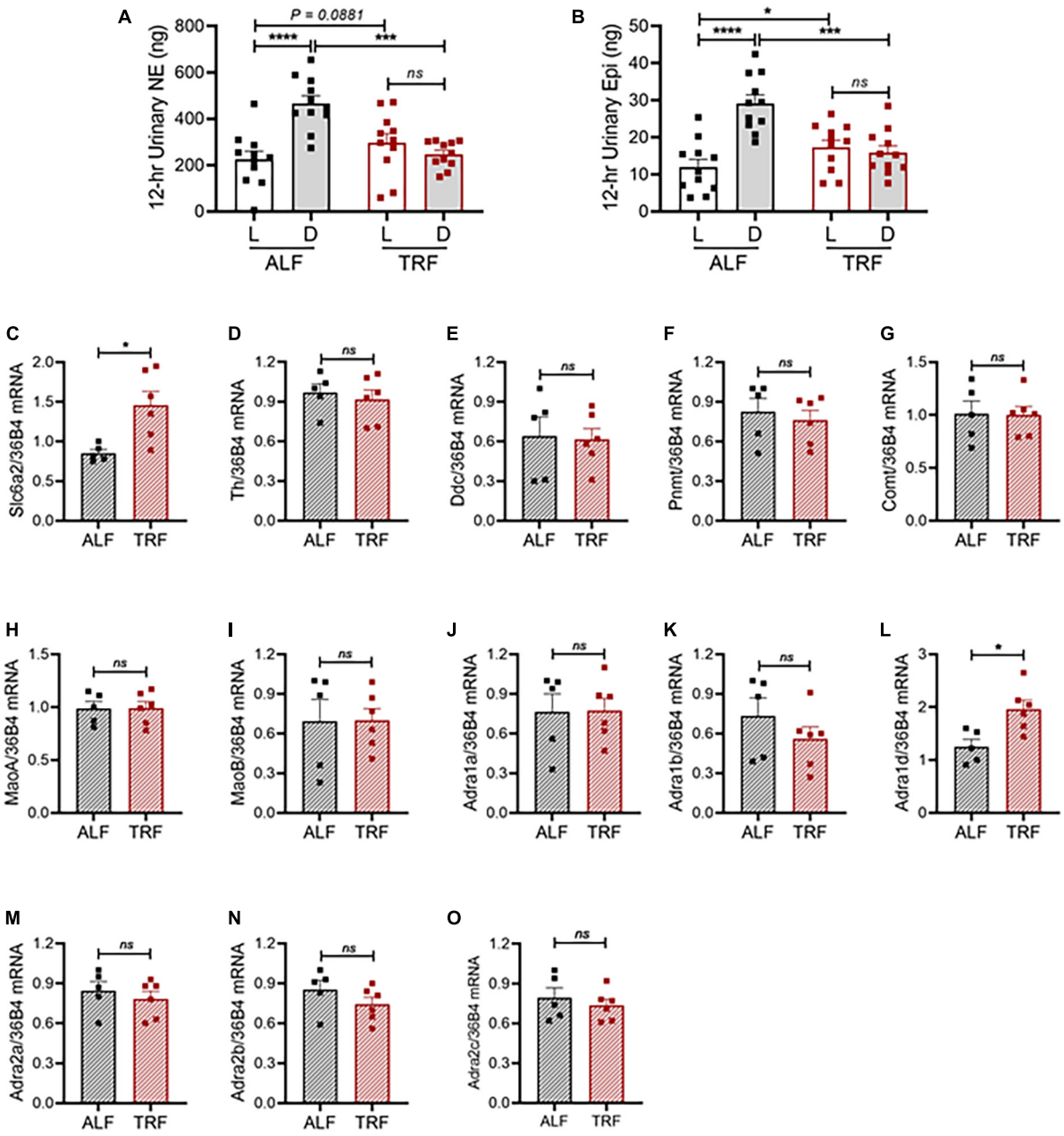

FIGURE 5
Light-phase TRF alters the sympathetic pathway. **(A,B)** Urinary norepinephrine [NE; **(A)**] and epinephrine [Epi; **(B)**] during the light and dark phases in 15- to 16-week-old male mPer2^{Luc} mice (N = 11) under ALF and the 7th day of light-phase TRF. **(C–O)** mRNA expression of solute carrier family 6 member 2 [(*Slc6a2*, **(C)**], tyrosine hydroxylase [*Th*, **(D)**], dopa decarboxylase [*Ddc*, **(E)**], phenylethanolamine *N*-methyltransferase [*Pnmt*, **(F)**], catechol-O-methyltransferase [*Comt*, **(G)**], monoamine oxidase A [*MaoA*, **(H)**], monoamine oxidase B [*MaoB*, **(I)**], alpha-1a adrenergic receptor [*Adra1a*, **(J)**], alpha-1b adrenergic receptor [*Adra1b*, **(K)**], alpha-1d adrenergic receptor [*Adra1d*, **(L)**], alpha-2a adrenergic receptor [*Adra2a*, **(M)**], alpha-2b adrenergic receptor [*Adra2b*, **(N)**], and alpha-2c adrenergic receptor [*Adra2c*, **(O)**] in mesenteric arteries isolated during ZT9 to ZT11 from 20-week-old male mPer2^{Luc} mice under ALF and 7 days after light-phase TRF. N = 5 or 6 in each group. Data were expressed as the mean ± SEM and analyzed by repeated two-way ANOVA with matching conditions between light vs. dark phase and between ALF vs. light-phase TRF with Tucky's *post hoc* analysis **(A,B)** or unpaired t-test **(C–O)**. *P < 0.05; ***P < 0.001; ****P < 0.0001; ns, not significant.

SBP (Supplementary Figures 3A-D), DBP (Supplementary Figures 3E-H), locomotor activity (Supplementary Figures 4A-C), and heart rate (Supplementary Figures 4D-F). In contrast to its little effect on 24-h total food intake and average MAP in non-diabetic C57BL/6J mice (Figures 2E,H), light-phase TRF significantly reduced 24-h total food intake and average MAP in diabetic *db/db* mice (Figures 6C,F). Interestingly, despite its reduced food intake and daily MAP, light-phase TRF did

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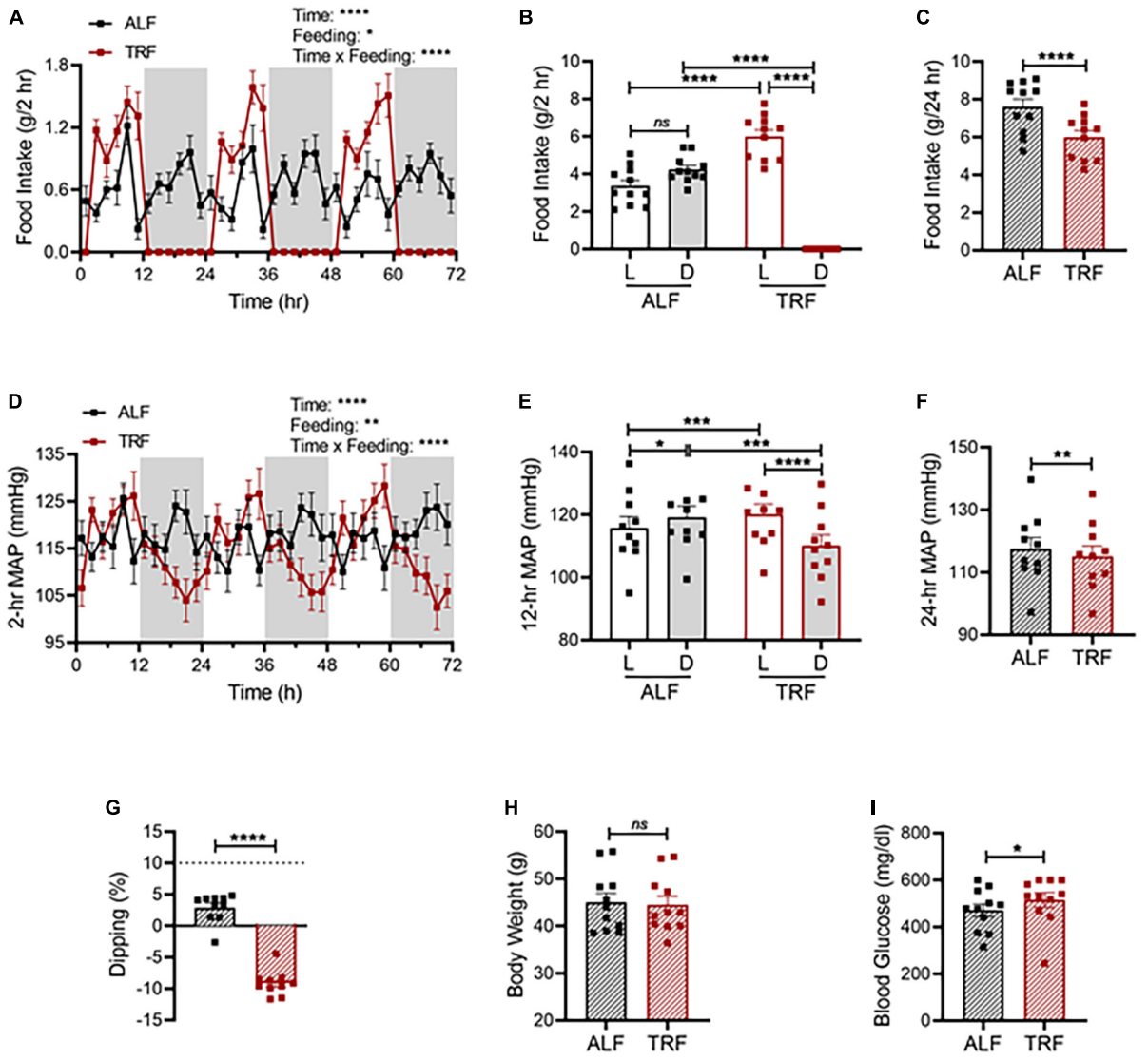

FIGURE 6
Light-phase TRF worsens BP rhythm and blood glucose in *db/db* mice. **(A–C)** Accumulated food intake in 2-h **(A)**, 12-h **(B)**, and 24-h **(C)** intervals in 21-week-old male *db/db* mice (N = 10) over 3 days of ALF and during the last 3 days of TRF. **(D–F)** Average MAP in 2-h **(D)**, 12-h **(E)**, and 24-h **(F)** intervals over 3 days with ALF or during the last 3 days of TRF. **(G)** Non-dipping MAP (less than 10%) with ALF and reverse dipping with light-phase TRF. **(H,I)** Body weight **(H)** and blood glucose **(I)** were determined at ZT1 under ALF and after 7 days of TRF. Data were expressed as the mean ± SEM and analyzed by repeated two-way ANOVA with matching conditions between light vs. dark phase and between ALF vs. light-phase TRF with Tucky's *post hoc* analysis **(A,B,D,E)** or paired *t*-test **(C,F,G–I)**. *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$; ns, not significant.

not affect the body weight of *db/db* mice (Figure 6H), but significantly elevated blood glucose level in diabetic *db/db* mice (Figure 6I).

Discussion

The current study demonstrated that (1) the BP level correlated with the food intake temporally across the 24 h in C57BL/6J mice under ALF; (2) light-phase TRF rapidly reverted BP circadian rhythm, leading to reverse dipping; (3) light-phase TRF-induced net changes in food intake temporally correlated with the net changes in BP; (4) light-phase TRF-induced BP rhythm alteration was associated with alterations in the time-of-day variations in Per2 protein expression, heart rate variability, baroreflex sensitivity, and urinary excretion of NE and Epi; (5) light-phase TRF increased mRNA expression of *Slc6a2* and *Adra1d* during the light phase in mesenteric arteries; and (6) light-phase TRF worsened BP dipping and increased blood glucose in diabetic *db/db* mice.

In contrast to the extensive studies linking TRF to metabolic health, there are limited studies that examined the effect of TRF

on BP circadian rhythm. A few publications on this topic suggest the timing of food intake is critical for BP circadian rhythm. In nocturnal rats and mice, a higher BP during the dark phase than during the light phase parallels higher food consumption during the dark phase. Experimentally restricting food availability to a period during the normal fasting/inactive phase is associated with an increase in BP during the fasting/inactive phase in rabbits (5), dogs (6), rats (7), and mice (8). However, these studies measured the total food intake during the entire feeding period or the 12-h light and dark phase. Thus, whether food intake temporally correlates with BP change is unclear. In the current study, we simultaneously monitored food intake every 1-min using BioDAQ and BP continuously using telemetry in a single housed mouse. These state-of-art technologies allow us to define the temporal relationship between food intake and BP in a single-housed mouse. Our results demonstrated for the first time that the BP level correlated with the food intake temporally across the 24 h in C57BL/6J mice under ALF (**Figure 1**).

We recently reported restricting food availability to 8 or 12 h during the dark/active phase (dark-phase TRF) protected the BP circadian rhythm in diabetic *db/db* mice (16). However, whether the light-phase TRF has similar protection for diabetic *db/db* mice has not been investigated. In contrast to dark-phase TRF (16), the current study illustrated that light-phaseTRF rapidly exacerbated rather than protected *db/db* mice from disruption of BP circadian rhythm (**Figure 6** and **Supplementary Figures 3**, **4**). Also, in contrast to the dark-phase TRF that did not influence BP circadian rhythm in non-diabetic wild-type mice (16), the current study demonstrated that light-phase TRF rapidly reversed BP circadian rhythm in C57BL/6J mice, leading to reverse or inverted dipping (**Figure 2** and **Supplementary Figure 1**). Moreover, the current study also showed light-phase TRF-induced net changes in food intake are temporally correlated with net changes in BP in C57BL/6J mice (**Figure 2K**), indicating a causal role of food intake in regulating BP circadian rhythm in mice. In line with the previous reports (5–8, 16), these results suggest that TRF could be beneficial or detrimental to BP circadian rhythm depending upon the timing of food intake.

The potent influence of the timing of food intake on BP circadian rhythm could have significant relevance to human health. In modern society, more and more people consume food during the entire 16 h of awake time, including night hours (27). This erratic eating rhythm is associated with increased metabolic syndrome/diabetes and non-dipping or reversed dipping BP (28). In these patients, the reverse dipping BP is often caused by an increase in dark-phase/nighttime BP (29–39). However, some patients have a decline in active-phase/daytime BP in addition to a rise in inactive-phase/nighttime BP (40–46). The reverse dipping BP caused by light-phase TRF in wild-type mice described in the current study seems to resemble more of the latter group of patients. Further investigation is warranted to verify this. In addition, postprandial hypotension, a common cause of falls, syncope, and stroke is common among the elderly, reaching a prevalence of 25–67% in institutionalized elders (47). Impaired regulation of eating associated BP changes likely contribute to the cause. It will be interesting to test whether old mice would develop postprandial hypotension and serve as a model to dissect the underlying mechanism.

How does TRF trigger alterations in BP circadian rhythm? While complex mechanisms are likely underlying light-phase TRF-induced alteration in BP circadian rhythm, the current study provides several lines of evidence for a potentially important role of the SNS. The results are in line with our previous study showed that dark-phase TRF protects BP circadian rhythm via suppressing sympathetic nervous activity during fasting in diabetic *db/db* mice (16).

Firstly, our power spectral analysis of HRV, an index of the autonomic activity (48, 49), demonstrated that light-phase TRF increased LFSP, HFSP, and rMSSD during the dark/fasting phase but decreased LFSP or did not affect HFSP and rMSSD during the light/feeding period (**Figures 4A-I**). It is generally accepted that HFSP and rMSSD reflect parasympathetic activity (50). In contrast, the interpretation of LFSP is inconsistent. Some considered it a sympathetic modulation marker, whereas others considered it a parameter of sympathetic and parasympathetic activity (51). There were also studies claiming that during resting conditions, LFSP primarily reflects baroreflex activity but not sympathetic innervation (50, 52, 53). Regardless of these different interpretations, the current study is consistent with our recent report (16), light- or dark-phase TRF modulates BP circadian rhythm, at least in part, through the auto autonomic nervous system, including the SNS.

Secondly, the baroreceptor reflex system plays a predominant role in preventing BP fluctuations by modulating both SNS and PNS activities and therefore reflects the overall integrity of the autonomic nervous system (54). Under physiological conditions, baroreceptors are constantly active and continuously inhibit SNS activity. Under pathological conditions, such as hypertension, coronary artery disease, myocardial infarction, and heart failure, baroreflex control is impaired, with an imbalance of sympathetic—vagal outflow (55). Our results that BRS decreased during the light/feeding phase but increased during the dark/fasting phase after light-phase TRF (**Figures 4J-L**) indicates feeding, in addition to light, modulate sympathetic-vagal outflow thus can contribute to the changes in BP rhythm.

Thirdly, the current study showed that urinary NE and Epi were increased during the light phase but decreased during the dark phase after light-phase TRF, abolishing the day-to-night NE and Epi variations (**Figures 5A,B**). These results are commensurate with our recent report in *db/db* mice (16) and other studies that SNS activity, measured by sympathetic firing rate in brown adipose tissue in rats fed a high-fat diet (11), NE turnover in the heart in normal rats (12), renal sympathetic nerve activity in cats (14), urinary catecholamines and their

metabolites excretion in humans (13), indicating that eating or feeding enhances SNS activity whereas fasting reduces it.

Fourthly, the current study revealed that light-phase TRF increased the mRNA expression of *Slc6a2* without affecting NE synthesis or catabolic enzymes in mesenteric resistant arteries (**Figures 5C-I**). *Slc6a2* encodes a NET protein responsible for removing NE from synapses by uptaking NE back into the presynaptic nerve terminal, the rate-limiting step to terminate NE function (56). It has been shown that increasing catecholamines by chronic depolarization (57) or stimulating TH activity (58) are associated with NET mRNA upregulation. Thus, enhanced mRNA expression of NET after light-phase TRF suggests that catecholamine synthesis/release may be increased. Nevertheless, the lack of change in NE metabolic enzymes at the mRNA level does not exclude possible alterations in catecholamine synthesis because acute stimulation of NE synthesis is mostly achieved by regulating TH activity through post-translational phosphorylation (59). Further experiments are needed to test these possibilities.

Lastly, one of the intriguing findings from the current study is that light-phase TRF selectively increased the mRNA expression of *Adra1d* among six subtypes of αARs (α_{1A}-, α_{1B}-, α_{1D}-, α_{2A}-, α_{2B}-, and α_{2C}-AR) in mesenteric resistant arteries (**Figures 5J-O**). *Adra1d* encodes an αAR protein responsible for regulating physiopathological responses mediated by NE and Epi, particularly in cardiovascular diseases, including hypertension (60). Mice deficient in *Adra1a* and *Adra1d* are hypotensive (61, 62), whereas mice deficient in *Adra1b* are normotensive (63). All three subtypes of α_1ARs knockout mice have decreased vasopressor response (61–63), but only *Adra1a* or *Adra1d* knockout mice have reduced aortic vascular contractility to NE and Epi (61, 62). Consistent with these genetic studies, pharmacological studies showed that α_{1D}-AR selective antagonist BMY7378 inhibits phenylephrine-induced vasoconstriction in rat aorta (64–66) and decreases BP (64). Therefore, it is tempting to speculate that the finding that increased *Adra1d* mRNA expression in mesenteric resistant arteries in response to light-phase TRF may indicate that light-phase TRF alters BP circadian rhythm, at least in part, through *Adra1d*. Further experiments are also needed to explore this possibility.

In addition to the SNS, other mechanisms may also participate in light-phase TRF-induced BP circadian rhythm alteration. Locomotor activity is a well-known factor for BP regulation. Our data showed that light-phase TRF promoted significant alterations in locomotor activity, which is highly correlated with the net changes in BP and locomotor activity acts as a covariant in light-phase TRF-induced BP alteration. Clocks, including period circadian regulator 2 (Per2), are well recognized for regulating BP homeostasis and circadian rhythms (16, 20, 67–69). To determine the timeline changes of Per2 expression, *in vivo* $Per2^{Luc}$ bioluminescence was determined in $mPer2^{Luc}$ mice because $mPer2^{Luc}$ mice have a luciferase reporter gene fused to the endogenous mPer2 gene, thus allowing *in vivo* monitoring of the Per2 clock protein oscillation in response to light-phase TRF (19). A previous study showed that $mPer2^{Luc}$ mice are viable and fertile, with no developmental or morphological differences compared to wild-type littermates (19). We found that light-phase TRF-induced BP circadian rhythm alteration was associated with $Per2^{Luc}$ bioluminescence in the liver and kidney (**Figure 3**). Consistent with this notion, it has been reported that mice with Per2 mutation exhibited impaired endothelium-dependent relaxations in the aorta and non-dipping BP in mice with stimulated renin-angiotensin signaling by Ang II infusion (70–72). However, it is worth mentioning that a recent study showed that light-phase TRF altered BP circadian rhythm in mice independent of Bmal1 (8), indicating that the clock gene is not involved. Thus, whether Per2 is involved in light-phase TRF-induced BP alteration remains elusive.

In conclusion, the current study demonstrated that light-phase TRF results in reverse dipping of BP in both wild-type and diabetic *db/db* mice, and the SNS pathway plays a potential role in mediating light-phase TRF-induced BP circadian rhythm alterations.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of the University of Kentucky.

Author contributions

TH, AC, and WS performed the experiments. TH analyzed the data. YK evaluated statistical analysis of the data. TH, ZG, and MG contributed to the idea, experimental design, and writing and editing of the manuscript. AC contributed to the editing of the manuscript. All authors contributed to the article and approved the submitted version.

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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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Supplementary material

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Timed restricted feeding cycles drive daily rhythms in female rats maintained in constant light but only partially restore the estrous cycle

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Light at night is an emergent problem for modern society. Rodents exposed to light at night develop a loss of circadian rhythms, which leads to increased adiposity, altered immune response, and increased growth of tumors. In female rats, constant light (LL) eliminates the estrous cycle leading to a state of persistent estrus. The suprachiasmatic nucleus (SCN) drives circadian rhythms, and it interacts with the neuroendocrine network necessary for reproductive function. Timed restricted feeding (RF) exerts a powerful entraining influence on the circadian system, and it can influence the SCN activity and can restore rhythmicity or accelerate re-entrainment in experimental conditions of shift work or jet lag. The present study explored RF in female rats exposed to LL, with the hypothesis that this cyclic condition can rescue or prevent the loss of daily rhythms and benefit the expression of the estrous cycle. Two different feeding schedules were explored: 1. A 12-h food/12-h fasting schedule applied to arrhythmic rats after 3 weeks in LL, visualized as a rescue strategy (LL + RFR, 3 weeks), or applied simultaneously with the first day of LL as a preventive strategy (LL + RFP, 6 weeks). 2. A 12-h window of food intake with food given in four distributed pulses (every 3 h), applied after 3 weeks in LL, as a rescue strategy (LL + PR, 3 weeks) or applied simultaneously with the first day of LL as a preventive strategy (LL + PP, 6 weeks). Here, we present evidence that scheduled feeding can drive daily rhythms of activity and temperature in rats exposed to LL. However, the protocol of distributed feeding pulses was more efficient to restore the day–night activity and core temperature as well as the

c-Fos day–night change in the SCN. Likewise, the distributed feeding partially restored the estrous cycle and the ovary morphology under LL condition. Data here provided indicate that the 12-h feeding/12-h fasting window determines the rest-activity cycle and can benefit directly the circadian and reproductive function. Moreover, this effect is stronger when food is distributed along the 12 h of subjective night.

KEYWORDS

circadian rhythms, artificial light at night (ALAN), estrous cycle, restricted feeding schedules, reproductive function

Introduction

Artificial light at night (ALAN) (1) has brought enormous benefits to human society by changing the outdoor, home, and work environment (2), which enables people to work, walk safely in the streets, and engage in social and leisure activities during the night. However, exposure to ALAN has caused unexpected emerging problems for human and animal health (3, 4). Clinical observations and experimental studies point out that ALAN is a factor leading to disruption of circadian function, which is associated with health disturbances including gastrointestinal, immune, and menstrual irregularities (5).

Experimental studies have provided consistent evidence of the adverse effects caused by ALAN. The results show that constant bright light (LL) or dim light at night can cause arhythmicity by altering directly the activation in the SCN, observed as reduced expression of c-Fos, loss of clock genes rhythms, and the main neuropeptides, all necessary for transmitting circadian rhythms to the body (1, 6–8). The loss of circadian regulation is observed in activity, core temperature, and hormonal regulation like cortisol, melatonin, prolactin, testosterone, or estrogens. Some hormones exhibit either constant high or constant low levels, associated with LL (9–12). Furthermore, LL promotes tumor growth (13), dyslipidemia, and overweight (14, 15). Importantly, early studies reported that LL induces a condition of persistent estrus, in which follicles continue maturing to a preovulatory stage but the ovulation does not occur, resulting in anovulation, continuous high serum estradiol levels, vaginal cornification, and continuous sexual receptivity (9). Moreover, this persistent estrus leads to a polycystic ovary condition (16).

The estrous cycle in rodents is organized by the interaction of at least two oscillatory systems, one driven by the reproductive axis that includes the cyclic secretion of reproductive hormones, and the other is driven by the participation of regulatory cells in hypothalamic nuclei and the ovaries. The second oscillatory system is the circadian system, orchestrated by the suprachiasmatic nucleus (SCN) (17). The interaction of the SCN with the arcuate nucleus (ARC), the anteroventral medial periventricular nucleus (AVPV), and the ovary is necessary for hormonal regulation of the estrous cycle (18) and provides timing to the hypothalamic–pituitary–gonadal axis for the LH surge for ovulation (9) as well as the timing for the sensitivity in the ovary to hormones (19). The production and release of the gonadotropin-releasing hormone (GnRH) are controlled by kisspeptin-producing neurons in the ARC and in the AVPV. The coordinated timing of these hypothalamic nuclei is required for ovulation (17); thus, disruption of circadian rhythms may affect this fine-tuned oscillatory interaction.

Early studies reported that SCN lesions in female rats resulted in anovulation and persistent estrus evaluated by persistent vaginal cornification (20, 21). In a similar way, LL, which results in arrhythmic behavioral patterns, disrupts the estrous cycle (22). Under LL, persistent estrus occurs, characterized by the maturation of follicles to a preovulatory stage without ovulation, and it is associated with continuous high serum estradiol levels, vaginal cornification, and continuous sexual receptivity (9, 23).

In experimental and clinical studies, diverse strategies have been tested to prevent or revert circadian disruption, and this includes scheduled melatonin administration (24), scheduled dexamethasone administration (25), exercise (26), or scheduled feeding (27). Scheduled feeding has shown to be a strong entraining signal when it is coupled to the activity phase. In experimental studies, restricted food access (RF) coinciding with the active phase accelerated resynchronization in a jet-lag model (27, 28) and prevented circadian desynchrony, depressive-like and anxiety-like behaviors, as well as an exacerbated immune response in an experimental model of shift work (29–31). Moreover, in mice exposed to a high-fat diet, RF prevented deleterious effects on metabolism (32–34). In contrast, scheduled feeding that is in conflict with the light–dark cycle exerts detrimental effects on the circadian function and metabolism (35).

In this study, we hypothesized that in female rats exposed to LL, scheduled feeding based on a 12-h feeding/fasting cycle would impose 24 h daily rhythms and consequently would

restore the estrous cycle. We tested scheduled feeding as a re-entrainment (rescue) strategy in arrhythmic rats after 3 weeks in LL, or as a preventive strategy by exposing rats to the 12-h feeding schedule simultaneously to the onset of LL. The present study explored RF in female rats exposed to LL, with the hypothesis that this cyclic condition can restore or prevent the loss of daily rhythms and the estrous cycle. The effect of food given in distributed pulses as a stronger entraining rescue or preventive signal was also investigated.

Daily rhythms of general activity and core temperature were monitored, as well as c-Fos day–night patterns in the SCN, in the ARC, and in the AVPV. To test the direct impact on the estrous cycle, vaginal smears were obtained and, at the end of the study, blood samples and ovaries were extracted for hormonal determinations and histological analysis.

Materials and methods

Animals and housing

Female Wistar rats weighing 120–150 g were housed in individual acrylic cages (45 cm × 30 cm × 20 cm) placed on tilt sensors, in soundproof ventilated lockers. Rats were maintained under controlled temperature (22 ± 1°C), and they were given free access to water and regular chow (Rodent Laboratory Chow 5001, Purina, Minnetonka, MN, USA), except in the experimental stage of food restriction. During baseline, rats were kept in a controlled 12:12-h light/dark (LD) cycle, lights on at 08:00 h (ZT0). The committee for ethical evaluation at the Facultad de Medicina UNAM approved experiments (FM/DI/140/2019) according to international guidelines for the ethical use of animals. Procedures were aimed at minimizing the number of animals and their suffering.

Experimental design

Experiment 1

The first experiment was explored in female rats exposed to constant light (LL), the potentiality of a timed feeding schedule of 12-h feeding/12-h fasting on activity and temperature circadian rhythms induced or prevented the loss of regular estrous cycles. All rats were first monitored for 1 week of baseline (BL) in a 12:12 LD cycle. For the restitution protocol, a group of rats (LL + RFR; N = 9–10) was made arrhythmic by exposing them for 3 weeks to LL (250 lx at cage level). Arhythmicity was defined as the absence of a circadian peak of general activity in the periodogram (**Supplementary Figure 1**). This was followed by 3 more weeks in LL combined with a 12-h feeding/12-h fasting restricted feeding schedule, which was expected to restore the daily rhythms (LL + RFR). For convenience, the external LD cycle was used as a reference for setting the 12-h food window starting food access at 07:00 am external time.

For the preventive strategy, rats were exposed to the 12-h feeding/12-h fasting restricted feeding schedule simultaneously to the first day of constant light (LL + RFP; N = 9–10) for 6 weeks. Rats were exposed to LL, and simultaneously the 12-h feeding/12-h fasting schedule was imposed for 6 weeks. The 12-h feeding started at the previous ZT12 and ended at the previous ZT0.

Two different groups of rats were used. One group was employed for the activity and temperature monitoring and the end for obtaining ovaries and brains. The second group of rats was used for performing vaginal smears, blood samples, and obtaining ovaries and brains.

Experiment 2

In the first experiment, the 12-h feeding/12-h fasting schedule partially induced a general activation in the expected active phase. General activation and food intake were mainly seen in the first half of the activity phase (see **Supplementary Figure 2**). Therefore, in this second experiment, food was provided in four pulses distributed every 3 h during the 12-h feeding window. The hypothesis was that keeping rats active and feeding for 12 h, due to distributed feeding, would provide a stronger entraining signal for the SCN and the circadian function and this would promote regular estrous cycles.

After 1 week in BL, rats were randomly assigned to one of two conditions. The first group of rats (N = 9–10) was made arrhythmic by exposing them for 3 weeks to LL. Arhythmicity was confirmed as in experiment 1. This group of rats continued for 3 more weeks in LL combined with a distributed 12-h timed restricted feeding with food distributed in four feeding pulses (LL + PR). Similar to experiment 1, the external LDL cycle was used as a reference for setting the 12-h window starting food access at 07:00 am external time. Thus, it was termed ZT12, and then, food was provided every 3 h to complete the 12 h feeding phase. Meal events were at ZT12, ZT15, ZT18, and ZT21. The second group of rats (N = 9–10) was exposed to LL, and simultaneously on the first day of LL, and food was given in four feeding pulses (LL + PP) for 6 weeks as a preventive strategy. To define the amount of food to be delivered every 3 h, the same rats were monitored during their BL to assess the amount of food consumed for 24 h. This amount was divided into four portions of 3.5 g of regular chow pellets for rats distributed during the 12 h of subjective night. Similar to experiment 1, the 12-h food window started at the previous ZT12 and ended at the previous ZT0.

The effects on circadian function were evaluated in all rats, by monitoring general activity and core temperature during the baseline and the 6 weeks of the experimental condition. At the end of the experimental protocol, the day–night c-Fos activation was determined for the SCN, ARC, and AVPV.

To determine the effects on the reproductive function, the second series of rats (N = 6–7) was exposed to the same two experimental conditions. Vaginal smears were obtained for 9 consecutive days, three times along the experiment: during BL, after 3 weeks in LL, and after 3 weeks in the restricted feeding schedule. At the end of the experimental protocols, rats were euthanized, and a blood sample, ovaries, and brains were extracted. As control groups, we added two more conditions: (1) rats maintained in 12:12 LD control conditions (N = 7) and (2) rats maintained in constant light (LL; N = 7) for 3 weeks. Tissues and blood were incorporated for the analysis.

Activity recording and analysis

General activity in the home cage was continuously monitored with the tilt sensors placed under the cages, and behavioral events were collected with a digitized system and automatically stored every minute in a PC for further analysis with the program SPAD9 (Data processing system, 1.1.1 version; Omnialva SA. De CV. Mexico City, Mexico) based on MATLAB. Double-plotted actograms were obtained by collecting the sum of activity for 15-min intervals. For each experimental stage, 24h mean activity profiles were obtained using data from the last week in the condition. To evidence the day–night activity patterns for each experimental stage, the % of day and night activity counts in 24 h was estimated. Due to the statistical difference between nocturnal activity in LL, LL + RFR, and LL + RFP suggesting a different distribution of the nocturnal activity, the activity counts of the first half were compared with the second half of the night.

Monitoring of core temperature

One week before starting BL, rats underwent a brief surgery to receive an intra-abdominal temperature sensor (iButton Sensor-Temperature Logger; Maxim integrated products, USA), programmed to store samples every 30 min as previously described. Briefly, rats were anesthetized with an intramuscular dose of xylazine (Procin 8 mg/kg) and ketamine (Inoketam 40 mg/kg). Under anesthesia, a small incision was performed to the dorsal abdominal cavity, and the temperature sensor, previously sterilized, was introduced in the peritoneal region. Muscles were sutured with absorbable catgut (000), and skin was sutured with surgical suture (000 Atramat, International Farmacéutica, SA. de CV. Mexico). Rats were left for 1 week to recover before starting the BL. For each experimental stage, heatmaps were elaborated with the mean temperature of all subjects from the corresponding group. For each experimental stage, 24 h mean temperature profiles were obtained using data from the last week in the condition. To evidence the day–night temperature patterns for each experimental stage, the mean temperature of the day and night was estimated.

Preparation and analysis of vaginal smears

For each experimental stage, vaginal smears were obtained for 10 days between 10:00 and 11:00 h, representing ZT2-ZT3 of the rat cycle. Vaginal swabs were collected by careful and gentle pipetting of 60 μl of sterile saline solution (0.9% NaCl), approximately 3–5 mm into the rat vagina. Smears were collected on gelatin-coated glass slides and counterstained with hematoxylin–eosin. The cell types contained within each vaginal smear were determined using a light microscope under 40X magnification. The stage of the estrous cycle was determined by observing the predominant cells in the sample as previously reported (36, 37). Briefly, estrus is characterized by the absence of nucleated cells and the presence of cornified squamous epithelial cells, diestrus is characterized by the predominance of leukocytes cells, and proestrus is indicated by the predominance of nucleated epithelial cells. Rats were scored as having regular estrous cycles if they exhibited 4- to 5-day cycles throughout the monitoring period. Conversely, if rats exhibited continuous days in the same stage or did not follow the order of the progression of estrous states, they were scored as having irregular estrous cycles.

The number of days occupied by each stage was represented as % of the 9 sampled days, and the proportion of days occupied by an estrous phase was compared between groups.

Brain and ovary extraction

At the end of each experiment, all rats were euthanized at either one of two time points, at ZT2 or ZT14. For rats under LL condition, ZTs were based on the time of food intake, considering ZT12 as the time of food access and ZT0 as the time of food removal. After an overdose with pentobarbital (Pisabental, sodic pentobarbital. Euthanasia dose: 65 mg/kg), rats were perfused with 200 ml saline (0.9%), followed by 200 ml of 4% paraformaldehyde in 0.1 mM phosphate buffer (pH 7.2). Brains and ovaries were extracted and were postfixed in 4% paraformaldehyde. After 48 h, brains were cryoprotected in 30% sucrose solution for 5 days and then cut with a cryostat at −18°C in sections of 40 μm and organized in four series.

Brain immunohistochemistry

One series from each brain was incubated for the c-Fos protein for 72 h (4°C) with the primary antibody anti-c-Fos made in rabbit (1:3000, Millipore) diluted in phosphate-buffered saline (PBS, 0.1 Mmol pH 7.2), 0.25% nutritive gelatin, and 0.5% triton (PBSGT). Brains were processed according to the avidin-biotin method (ABC kit, Vector) and were reacted with 0.05%

diaminobenzidine and nickel sulfate, which produced a brown-blue precipitate. After incubations, tissue was rinsed three times in PBS (10 min each). Sections were mounted on gelatin-coated slides, dehydrated in a series of alcohols, cleared with xylene, and coverslipped with Entellan (Merck, Darmstadt, Germany).

The arcuate nucleus, SCN, and AVPV nuclei were identified in the atlas of Paxinos and Watson (38). Three sections were chosen for each region and were counted bilaterally. Microphotographs were obtained under 20X magnification (**Supplementary Figures 3, 5**) with an optical microscope (LEICA DM500) and a digital camera (LEICA ICC50 HD). Immunopositive c-Fos neurons were counted with the Image J software setting an automatic color and size threshold.

Histology for ovarian morphometry

Follicular presence was assessed in paraffin sections using one ovary at random from each animal. Ovaries were fixed overnight in 4% paraformaldehyde in PBS, dehydrated in ethanol, and embedded in paraffin. The tissue was serially sectioned into 5 μm thick sections, every fifth section was collected on glass slides, and a total of 5–7 sections per ovary were obtained and analyzed.

The tissue sections were deparaffined and stained with hematoxylin and eosin for follicle counting. Microphotographs were obtained in a Primo Star upright light microscope, using a 10X magnification and an Axiocam ERc 5s digital camera (Zeiss, Oberkochen, Germany). Follicles were classified according to the previously reported criteria (22). Briefly, primary follicles displayed a single layer of cuboidal granulosa cells, and follicles were classified as secondary when more than one layer of granulosa cells was observed without a visible antrum. Finally, Graafian or tertiary follicles were those with an evident antral cavity with a ring of cumulus cells surrounding the oocyte. Only follicles with a visible nucleus in the oocyte in each section were counted, to avoid counting the same follicle more than once. The number of follicles per ovary was calculated as the sum of each follicle category in every section quantified. In addition, the number of cysts for each ovary was determined. Briefly, cysts were identified as those follicles displaying a large antral space lacking an oocyte, surrounded by a dense thecal cell layer with little to none viable granulosa cells. The absence of an oocyte in every section was necessary to classify a follicle as a cyst. The number of cysts is reported as the mean number of cysts per ovarian section.

Hormonal determinations

At the end of each experimental stage, blood samples (300 μl) were collected from tail puncture at ZT2 (9:30 h) in Microvette tubes (SARSTEDT AG & Co.) containing an anticoagulant agent (EDTA). The blood was then centrifuged at 7000 rpm for 7 min, and aliquots of 60 μl of plasma were frozen at −45°C for subsequent analysis. The levels of 17 beta-estradiol and progesterone in the plasma were determined using an enzyme-linked immunosorbent assay kit (IBL International GmbH, Hamburg, Germany). The E2/P ratio was estimated for each experimental stage.

Statistical analysis

Comparison of activity and temperature profiles was compared with a two-way ANOVA for repeated measures (RM). Day–night values for activity and temperature, 1st half and 2nd half of the night data, c-Fos cell count, and the number of follicle types were compared among groups with a two-way ANOVA. The effects on time spent in estrus, ovary weight and the total number of follicles, and estradiol/progesterone ratio were compared among groups with a one-way ANOVA. All ANOVAs were followed by a Tukey or Sidak multicomparison *post hoc* test with $\alpha = 0.05$.

Results

Experiment 1

Activity and temperature daily patterns

During BL, all rats exhibited 24 h daily rhythms adjusted to the LD cycle with low activity counts during the day and high activity counts during the night as can be seen in the representative actogram (**Figure 1A** top and bottom) and in the mean daily activity profile (**Figure 1B** top, blue lines). Constant light-induced initially a free-running pattern in all rats that resulted in arhythmicity after 3 weeks of LL exposure (**Figures 1A** top,**B**, green line), and by imposing a 12-h feeding schedule (LL + RFR), the general activity acquired a daily activity-rest pattern with increased activation during the 12 h of food access (**Figure 1B** top, red line). The two-way ANOVA for repeated measures indicated a significant effect for the interaction of time × groups [$F_{(96,1248)} = 11.25$; $P < 0.0001$].

The restricted feeding schedule also prevented an arrhythmic pattern when it was imposed simultaneously with the LL protocol (LL + RFP; **Figures 1A** bottom, **B** bottom, black line).

The two-way ANOVA for RM indicated a significant effect on the interaction of time × groups [$F_{(48,768)} = 7.367$; $P < 0.0001$].

The mean proportion (%) of day/night activity corresponding to the 12-h food access/12-h fasting phase indicated that the restricted feeding schedules induced a significant day/night change in general activity ($P < 0.01$; **Figure 1C** top and bottom). The two-way ANOVA for RM indicated a significant effect for the interaction of time × groups [**Figure 1C** top: $F_{(2,26)} = 129.2$; $P < 0.0001$; **Figure 1C** bottom:

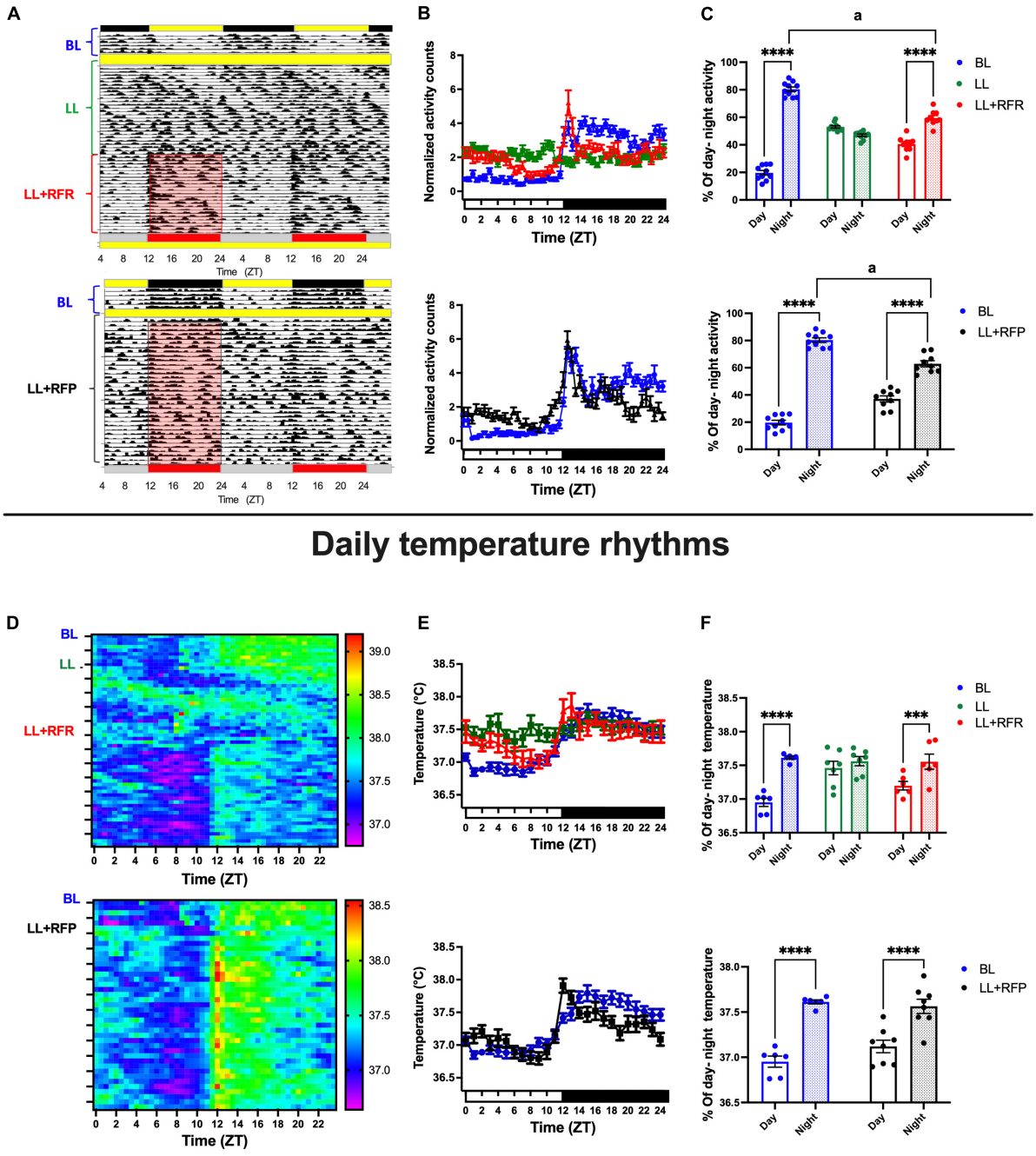

FIGURE 1
Daily activity and core temperature cycles in rats exposed to a light–dark cycle during the baseline (BL, blue lines and bars), to constant light (LL, green line and green bars), to LL followed by 3 weeks of the 12-h restricted feeding schedule as a rescue strategy (LL + RFR, red), or to LL paired simultaneously to the 12-h restricted feeding schedule as a preventive strategy for 6 weeks (LL + RFP, black). **(A** top) Representative actogram for the BL, LL, and the LL + RFR condition. **(A** bottom) Representative actogram for the BL, LL, and the LL + RFR condition. **(B** top and bottom) Daily activity patterns for the different experimental conditions. Data are expressed as the mean ± SEM; *n* = 9–10/group. **(C** top and bottom) Day–night percentage of activity for the experimental stages; light bars represent day, and dashed bars represent night. **(D** top) Mean heat map for BL, LL, and LL + RFR conditions. **(D** bottom) Mean heat map for the BL and LL + RFP conditions. **(E** top and bottom) Daily temperature patterns for the different experimental conditions; data are expressed as the mean ± SEM; *n* = 6–11/group. **(F** top and bottom) Day–night mean values for core temperature for the experimental stages; light bars represent day, and dashed bars represent night. Asterisk indicates a statistical difference between the day and the night value/group **** = ($P < 0.0001$). The letter a indicates a statistical difference between the nights of each group, a = ($P < 0001$).

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$F_{(1,17)} = 39.18$; $P < 0.0001$]. Importantly, in both conditions, LL + RFR and LL + RFP, the % of nocturnal activity driven by food access remained significantly lower than in the BL ($P < 0.001$).

Similar to that observed in general activity, the core temperature exhibited clear day/night cycles during the BL (Figure 1D). The exposure to LL induced a free-running rhythm for a long period that after 3 weeks resulted in a loss of rhythmicity (Figures 1D top,E top green line). The following 12-h feeding schedule (LL + RFR) induced the recovery of daily temperature cycles with increased levels during the 12 h of food access (Figure 1D top). The two-way ANOVA for RM indicated a significant interaction for time × groups [$F_{(48,360)} = 7.301$; $P > 0.0001$]. When the 12-h feeding schedule was imposed simultaneously with the LL protocol as a preventive strategy (LL + RFP), the loss of rhythmicity of core temperature was prevented (Figures 1D bottom,E bottom, black line). The two-way ANOVA for RM indicated a significant interaction for time × groups [$F_{(24,360)} = 10.39$; $P > 0.0001$].

The 12-h feeding/12-h fasting alternation induced in the mean core temperature a 12-h day/12-h night cycle ($P < 0.01$; Figure 1F top and bottom). The two-way ANOVA for RM indicated a significant effect for the interaction of time × groups [Figure 1C top: $F_{(2,16)} = 20.25$; $P < 0.0001$; Figure 1C bottom: $F_{(1,12)} = 12.59$; $P < 0.0001$].

Both the RFR and RFP conditions induced a nocturnal-like activation in rats kept in LL. However, as observed in Figures 1B,C, the intensity of the nocturnal activation was lower and statistically different from the BL condition. A comparison of the 1st half (ZT12–ZT18) and the 2nd half (ZT18–ZT24) of the night activity revealed that for the LL + RFR and LL + RFP groups, a constant nocturnal activation was not completely achieved with the 12-h feeding schedules (Supplementary Figure 2A). For both groups, general activity was significantly lower in the 2nd half of the night as compared to the 1st half of the night ($P < 0.01$). A similar effect was observed when comparing the mean core temperature for the 1st and 2nd half of the night (Supplementary Figure 2B; $P < 0.01$) and food ingestion (Supplementary Figure 2C; $P < 0.01$).

Daily-night c-Fos expression in the suprachiasmatic nucleus, arcuate nucleus, and anteroventral medial periventricular nucleus

To determine whether the RF can impose a day–night pattern of activity in the disrupted SCN as well as in nuclei involved in feeding/metabolism and in the estrous cycle, c-Fos expressing cells were counted. In the LD group, a clear day–night c-Fos activation was observed in the SCN with high cell counts at ZT2 and lower counts at ZT14 ($P < 0.01$). The constant light condition abolished this day–night variation, and this arrhythmic pattern remained in the LL + RFR and LL + RFP rats despite the cyclic feeding schedules (Figure 2A). The two-way ANOVA indicated a significant effect among groups

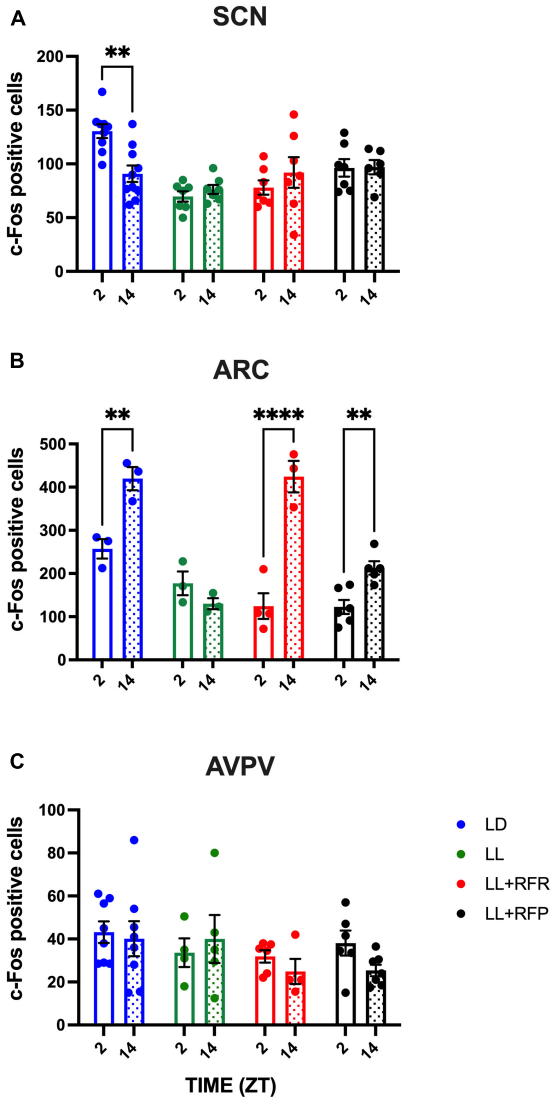

FIGURE 2
Day–night c-Fos positive cells in **(A)** the suprachiasmatic nucleus (SCN), **(B)** the arcuate nucleus (ARC), and **(C)** the posterior ventral preoptic area (AVPV), in rats exposed to a light–dark cycle during the baseline (BL, blue bars), to constant light (LL, green bars), to LL followed by 3 weeks of the 12-h restricted feeding schedule as a rescue strategy (LL + RFR, red bars), or to 6 weeks LL paired simultaneously to the 12-h restricted feeding schedule as a preventive strategy (LL + RFP, black bars). For each group, empty bars represent day (ZT2) and dashed bars represent night (ZT14). Data are expressed as the mean ± SEM; n = 4/time/group. Asterisks indicate a statistical difference between the day and the night values in the specified group; $^{**}P < 0.001$; $^{****}P < 0.0001$.

[$F_{(3,27)} = 8.726$; $P = 0.0003$], no effect in time [$F_{(1,25)} = 0.6557$; P = NS], and a significant interaction for groups × time [$F_{(3,25)} = 5.345$; $P = 0.005$].

Likewise, in the ARC, day–night c-Fos activation in the LD group was observed with high c-Fos activation at night as compared to the day ($P < 0.01$) coinciding with the activity

and feeding phase. This day–night rhythm was abolished by the LL condition but was reestablished (LL + RFR) and prevented (LL + RFP) by the 12-h feeding schedules (Figure 2B). The two-way ANOVA indicated significant differences among groups [$F_{(3,13)} = 20.67$; $P < 0.001$], in time [$F_{(1,9)} = 88.89$; $P < 0.001$], and a significant interaction for groups × time [$F_{(3,9)} = 27.29$; $P < 0.001$].

In contrast, in the AVPV, no day–night difference was observed in the LD group nor in the groups exposed to 12-h scheduled feeding (Figure 2C). The two-way ANOVA indicated no significant effects among groups, in time nor for the interaction of factors [$F_{(3,40)} = 0.6630$; P = NS]. Representative microphotographs are provided in Supplementary Figure 3.

Estrous cycle and ovary morphology

The vaginal smears indicated that 90% of LD rats maintained estrous cycles of 4–5 days (Figure 3A top) from which 30% of the time rats presented the estrous stage (Figures 3B top,C, blue bar). Constant light for 3 weeks resulted in the loss of estrous cycle for all the rats, inducing in 100% of the rats a predominant stage of estrus ($P < 0.0002$). The 12-h feeding schedule following 3 weeks of LL (LL + RFR) partially rescued the estrous cycle (Figure 3A). In the group LL + RFR, all rats remained with irregular cycles (Figure 3B). However, the days in estrus were significantly reduced (Figure 3D) reaching similar values as the LD group and significantly different from the LL group ($P < 0.04$). Imposing feeding schedules simultaneously to the LL as a preventive strategy (LL + RFP) had a similar effect in reducing the days of estrus. However, this was not different from LL (P = NS).

The one-way ANOVA indicated a significant difference among groups [$F_{(3,22)} = 10.23$; $P < 0.0001$].

Blood sample analysis indicated a low E_2/P ratio in LD rats and a significantly increased index in the LL group (Figure 3D). The 12-h restricted feeding reduced this ratio, especially in the LL + RFR females, which reached similar values as the LD group and statistically different values from the LL group ($P < 0.001$). In contrast, the LL + RFP rats remained statistically similar to the LL group and different from the LD ($P < 0.001$). The one-way ANOVA indicated a significant difference among groups [$F_{(3,15)} = 18.78$; $P < 0.0001$].

After the perfusion, both ovaries were extracted and weighed. Ovaries corresponding to LL rats were significantly smaller than those of the LD group ($P < 0.001$) and no improvement was observed associated with the feeding schedule (Figure 4A). The one-way ANOVA indicated a significant difference among groups [$F_{(3,19)} = 22.72$; $P < 0.0001$], and this was due to a significant difference between groups exposed to LL and their LD control.

The histology of the ovaries revealed fewer total follicles in rats exposed to LL as compared to LD rats ($P < 0.05$). The 12-h feeding schedules did not restore (LL + RFR) or prevent (LL + RFP) this effect (Figure 4B). The one-way ANOVA indicated a significant difference among groups [$F_{(3,19)} = 7.5$; $P < 0.0016$]. This difference was mainly due to a reduced number of primary and secondary follicles in all LL groups independent of the feeding schedule. The two-way ANOVA indicated a significant interaction of groups × follicle type [$F_{(6,57)} = 3.534$; $P = 0.004$; Figure 4C]. Remarkably, all groups exposed to LL displayed a significantly increased number of cysts within the ovaries, regardless of the feeding schedule (Figure 4D). Examples of ovarian histology are provided in Figure 5.

Experiment 2

Daily activity and temperature patterns

As observed in experiment 1, during the BL, all rats exhibited 24 h daily rhythms adjusted to the LD cycle with low activity counts during the day and high activity counts at night (Figures 6A top,B top; blue lines). Similar to experiment 1, rats developed first a free-running pattern that led to arhythmicity after 3 weeks in LL (Figure 6B, green line). Importantly, the activity profile of rats with the distributed food pulses (LL + PR) exhibited increased activation during the four bouts of food access resulting in a continuous activation along the 24 h (Figure 6B top, pink line). The two-way ANOVA for RM indicated a significant interaction time × condition [$F_{(96,768)} = 5.481$; $P < 0.0001$]. Likewise, the distributed feeding schedule induced a 12-h activity pattern when it was imposed simultaneously with the start of the LL protocol (LL + PP), preventing the initial free-running and the following arrhythmic activity pattern (Figures 6A,B bottom, gray line and Supplementary Figure 4A). The two-way ANOVA for RM indicated a significant interaction time × condition [$F_{(48,576)} = 3.774$; $P < 0.0001$].

The mean% of day/night activity corresponding to the 12-h day/12-h night or to the 12-h fasting/12-h food access showed a significant day/night alternation in general activity ($P < 0.0001$) for the BL and the LL + PR (Figure 6C, top). The two-way ANOVA for RM indicated a significant effect on the interaction of time × groups [$F_{(2,21)} = 50.26$; $P < 0.0001$]. Day–night alternation in general activity was also observed in the LL + PP group (Figure 6C bottom). The two-way ANOVA for RM indicated a significant effect on the interaction of time × groups [$F_{(1,13)} = 14.03$; $P = 0.0024$]. Despite the improved 12-h activation achieved with the distributed feeding, in both groups (LL + PR and LL + PP), the % of nocturnal activity remained significantly lower than the BL ($P < 0.01$).

Similar to those observed in general activity, the core temperature exhibited clear day/night cycles during the BL and during the distributed 12-h feeding schedules (Figure 6D). Food distributed in pulses caused a daily temperature cycle with increased levels during the 12 h of food access for both the LL + PR [two-way ANOVA interaction of time × groups

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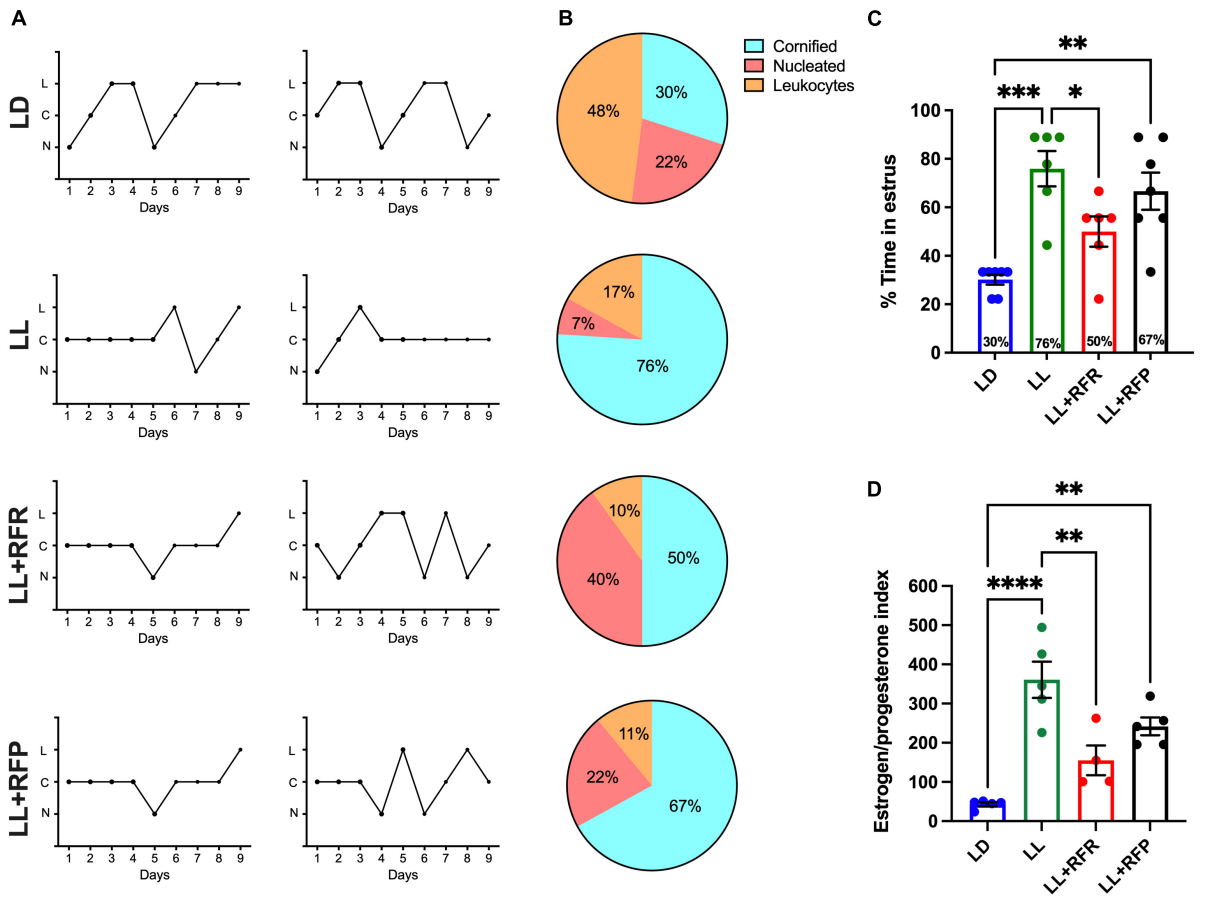

FIGURE 3

Estrous cycles obtained by 9-day vaginal smears. **(A)** Two representative cycles obtained with the cytology in the vaginal smears of rats exposed to a regular light–dark cycle (LD) from rats exposed to constant light (LL, second top) and rats exposed for 3 weeks to LL followed by 3 weeks of 12-h restricted feeding as a rescue strategy (LL + RFR) and rats exposed to LL simultaneously to RF for 6 weeks as a preventive strategy (LL + RFP). **(B)** Percentage of rats showing predominance for a given cellular type at each day of the estrous stage according to vaginal cytology. **(C)** Proportion of days in which rats displayed estrus. **(D)** Estrogen/progesterone (E_2/P) ratio. Data are expressed as the mean ± SEM; n = 4–5/group. Asterisks indicate *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; ****$P < 0.0001$. C, cornified cells; L, leukocytes; N, nucleated cells.

$F_{(48,456)} = 10.06$; $P < 0.0001$] and LL + PP groups [Figure 6E: $F_{(24,240)} = 5.353$; $P < 0.0001$].

The mean temperature for the 12-h day/12-h night or for the 12-h feeding/12-h fasting cycles (Figure 6F top and bottom) showed that both experimental groups, LL + PR and LL + PP, reached similar day/night values as the BL group ($P < 0.0001$). The two-way ANOVA indicated a significant interaction for time × groups [Figure 6F top: $F_{(2,19)} = 47.73$; $P < 0.0001$; Figure 6F bottom: $F_{(1,10)} = 10.75$; $P < 0.0001$].

In contrast to experiment 1, by imposing a distributed access to food, both the LL + PR and LL + PP groups displayed similar nocturnal activation between the 1st half (ZT12–ZT18) and the 2nd half (ZT18–ZT24) of the 12 h of food access (Supplementary Figure 4A) and similar food ingestion (Supplementary Figure 4C).

The two-way ANOVA for RM indicated a significant difference among groups [$F_{(3,22)} = 30.29$; $P < 0.0001$]; however, no significant interaction for time × groups [$F_{(3,22)} = 1.19$; P = NS]. A similar effect was observed when comparing the mean core temperature for the 1st and 2nd half of the night for the LL + PR group (Supplementary Figure 4B). However, the LL + PP still exhibited a significant reduction in temperature in the 2nd half of the night as compared with the 1st half ($P < 0.001$). The two-way ANOVA for RM indicated a significant interaction of time × groups [$F_{(3,25)} = 6.39$; $P = 0.002$].

Daily- night c-Fos expression in the suprachiasmatic nucleus, arcuate nucleus, and anteroventral medial periventricular nucleus

Under LD conditions, the SCN showed a clear day–night c-Fos activation, with high cell counts for ZT2 and lower counts for ZT14 ($P < 0.0001$). As described for experiment 1, LL abolished this day–night variation. Food distributed in

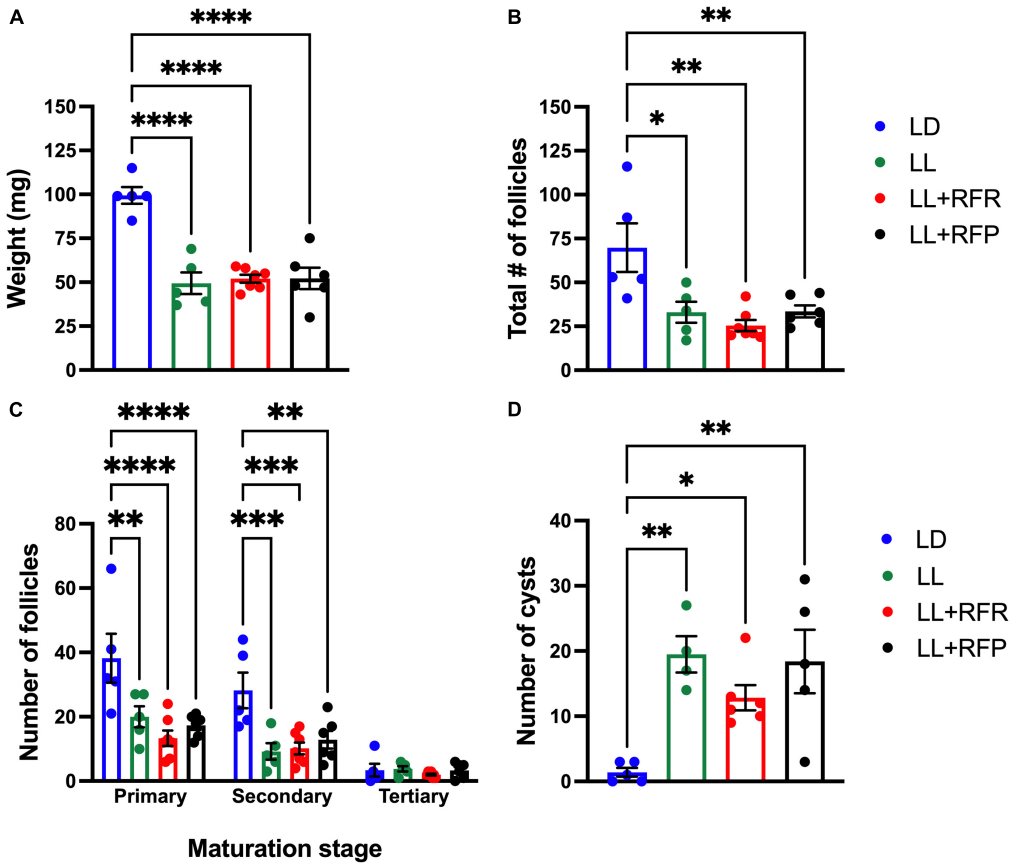

FIGURE 4
Ovary weight and morphology after LL exposure and the 12-h feeding schedules. **(A)** Ovary weight, **(B)** total number of follicles, **(C)** number of primary, secondary, and tertiary follicles, **(D)** number of cysts. Rats exposed to a regular light–dark cycle (LD, blue), exposed to constant light (LL, green), or exposed for 3 weeks to LL followed by 12-h restricted feeding as a rescue strategy (LL + RFR, red) and rats exposed to LL simultaneously to RF for 6 weeks as a preventive strategy (LL + RFP, black). Data are expressed as the mean ± SEM; n = 5–7/group. Asterisks indicate $^{*}P < 0.01$, $^{**}P < 0.001$, $^{***}P < 0.001$ and $^{****}P < 0.0001$.

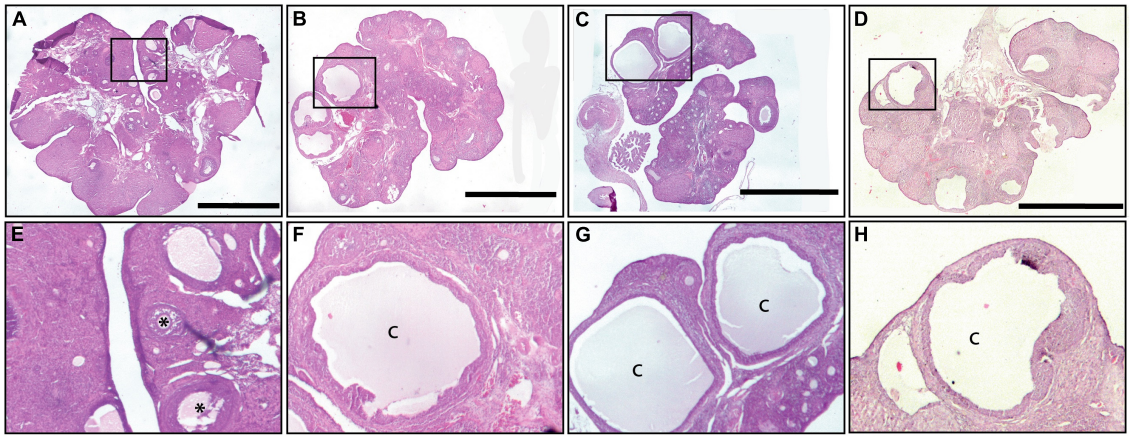

FIGURE 5
Ovary morphology. An example of a light–dark (LD) control ovary is shown in **(A,E)**, of a constant light (LL) ovary in **(B,F)**, of an ovary from a rat exposed for 3 weeks to LL followed by 12-h restricted feeding as a rescue strategy (LL + RFR) in **(C,G)**, and of an ovary from a rat exposed to LL simultaneously to RF for 6 weeks as a preventive strategy (LL + RFP) in **(D,H)**. Asterisks indicate growing follicles, and "c" indicates cysts. Scale bar = 2 mm.

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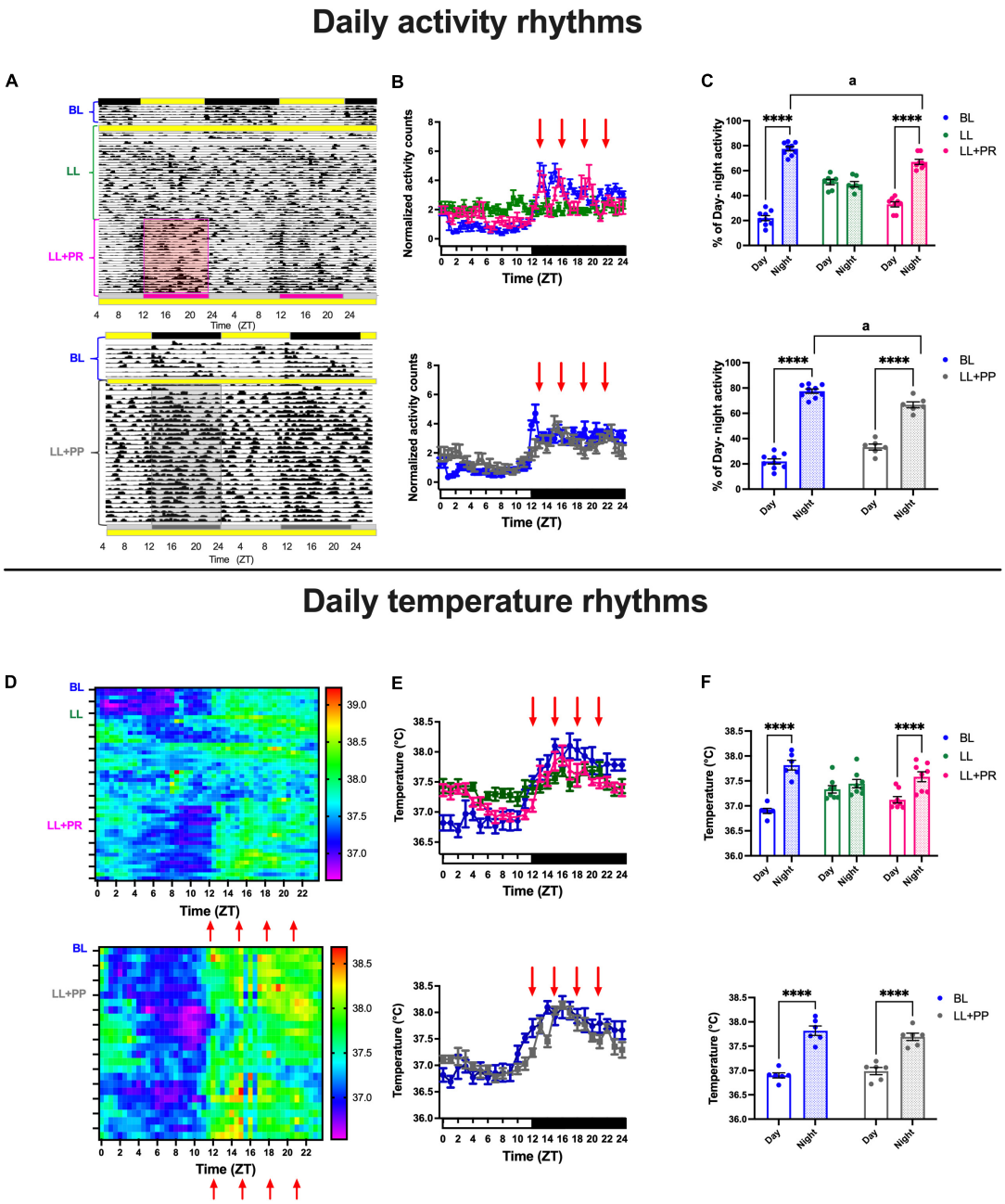

FIGURE 6
Daily activity and core temperature cycles in rats exposed to a light–dark cycle during the baseline (BL, blue lines, and bars), to constant light (LL, green lines and green bars), to LL followed by 3 weeks of 12-h feeding protocol distributed in 4 pulses feeding events every 3 h as a rescue strategy (LL + PR, pink), or to LL simultaneously to 4 food pulses as a preventive strategy for 6 weeks (LL + PP). **(A** top) Representative actogram from the BL, LL, and the LL + PR condition. **(A** bottom) Representative actogram from the BL and the LL + PP condition. **(B** top and bottom) Mean daily activity profiles for the different experimental conditions: the four feeding pulses indicated by red arrows. Data are expressed as the mean ± SEM; n = 7–9/group. **(C** top and bottom) Day–night percentage of activity for the experimental stages. **(D** top) Mean heat map for the BL, LL, and LL + PR conditions. **(D** bottom) Mean heat map for the BL and LL + PP conditions. **(E** top and bottom) Daily temperature patterns for the different experimental conditions. Data are expressed as the mean ± SEM; *n* = 6–8/group. **(F** top) Day–night mean values for core temperature for the three experimental stages. **(F** bottom) Day–night mean values for core temperature for the two experimental stages of the LL + PP group, light bars represent day, and dashed bars represent night. Red arrows indicate the four events when food was placed in the feeder. Asterisks indicate a statistical difference between the day and the night value/group (****$P < 0.0001$). For all graphs, light bars represent day (or subjective day) and dashed bars represent night (or subjective night).

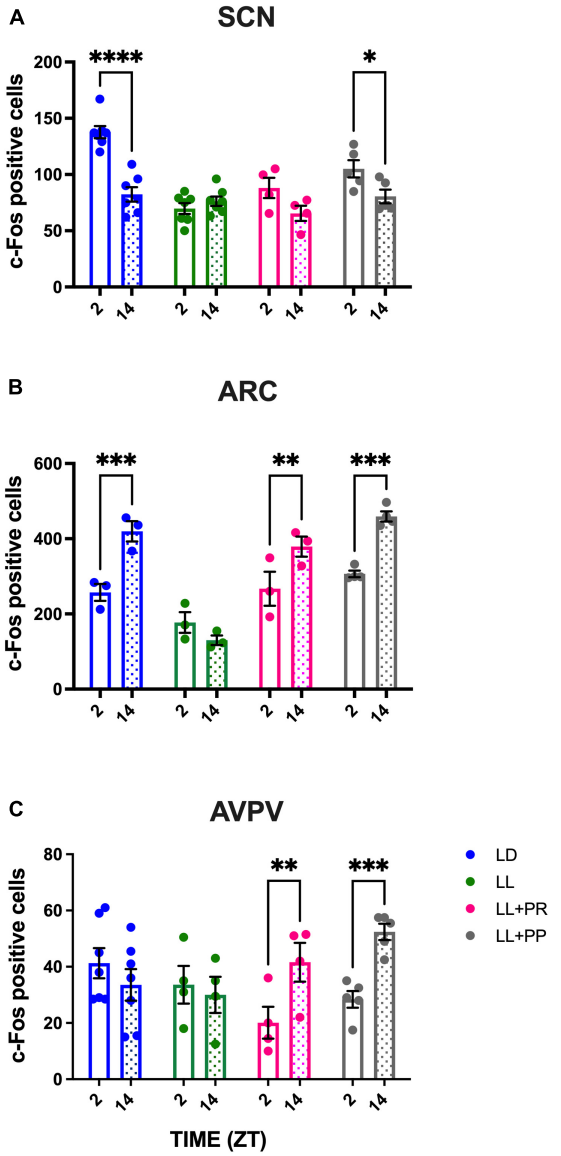

FIGURE 7
Day–night c-Fos positive cells in **(A)** the suprachiasmatic nucleus (SCN), **(B)** the arcuate nucleus (ARC), and **(C)** the posterior ventral preoptic area (AVPV), in rats exposed to a light–dark cycle during the baseline (BL, blue bars), to constant light (LL, green bars), to LL followed by 3 weeks of 12-h feeding protocol distributed in four pulses feeding events every 3 h as a rescue strategy (LL + PR, pink bars), or to LL simultaneously to four food pulses as a preventive strategy for 6 weeks (LL + PP, gray bars). For each group, empty unfilled bars represent day (ZT2) and dashed bars represent the night (ZT14). Data are expressed as the mean ± SEM; n = 3–4/time/group. Asterisks indicate a statistical difference between the day and the night values in the specified group; *$P < 0.01$, **$P < 0.001$; *** and **** = $P < 0.0001$.

four feeding pulses induced high c-Fos in the subjective day vs. the subjective night (Figure 7A); this was, however, only statistically significant for the LL + PP group ($P < 0.01$). The two-way ANOVA indicated a significant effect among groups [$F_{(3,19)} = 15.15$; $P < 0.0001$], in time [$F_{(1,\ 19)} = 034.10$; $P = 0.0001$], and a significant interaction for groups × time [$F_{(3,19)} = 12.23$; $P < 0.0001$].

As observed in experiment 1, in the ARC, a day–night c-Fos activation was observed in the LD group with high values during the night as compared to the day ($P < 0.001$), coinciding with the activity, and feeding phase. This day–night rhythm was abolished by the LL condition but was reestablished (LL + PR) and maintained (LL + PP) by the distributed 12-h feeding schedule (Figure 7B). The two-way ANOVA indicated significant difference among groups [$F_{(3,9)} = 23.16$; $P < 0.0001$], in time [$F_{(1,9)} = 62.29$; $P < 0.0001$], and a significant interaction for groups × time [$F_{(3,9)} = 15.90$; $P = 0.006$].

Importantly in the AVPV, the distributed feeding schedules imposed a day–night rhythm with low values during the fasting phase and high values during the feeding (active) phase (Figure 7C). The two-way ANOVA indicated a significant effect in time [$F_{(1,16)} = 15.24$; $P = 0.0013$] and for the interaction of group × time [$F_{(3,16)} = 15.60$; $P < 0.0001$]. Representative microphotographs are provided in Supplementary Figure 5.

Estrous cycle and ovarian morphology

Under LD conditions, vaginal smears of 90% of the females indicated estrous cycles of 4–5 days (Figure 8A top) from which 30% of the samples corresponded to the stage of estrus (Figure 8B top,C). As previously shown, LL for 3 weeks resulted in the loss of estrous cycles for all the rats inducing a predominant stage of estrus as compared with the LD group ($P < 0.0001$). The distributed feeding pulses significantly reduced the number of days spent in the estrous stage reaching similar values as the LD group and significantly different from LL (Figure 8C; $P < 0.01$). However, 100% of the rats exposed to LL despite the feeding schedule exhibited an irregular estrous cycle. When comparing the number of days displaying estrous cytology, the one-way ANOVA indicated a significant difference among groups [$F_{(3,24)} = 13.72$; $P < 0.0001$].

Blood sample analysis indicated a low E_2/P ratio in LD rats and a significantly increased ratio in the LL rats (Figure 8D; $P < 0.0001$). Both conditions of distributed feeding schedules reduced this proportion significantly as compared to LL (LL + PR, $P = 0.024$; LL + PP $P < 0.0001$). However, only the LL + PS group achieved similar values as the LD group. The one-way ANOVA indicated a significant difference among groups [$F_{(3,14)} = 24.75$; $P < 0.0001$].

Ovaries from LL rats were significantly smaller than those of the LD group ($P < 0.001$), and no change was observed associated with the distributed feeding pulses (Figure 9A). The one-way ANOVA indicated a significant difference among groups [$F_{(3,\ 20)} = 15.29$; $P < 0.0001$].

The histology of ovaries revealed a decreased number of total follicles in rats exposed to LL as compared to LD females. The number of follicles in rats exposed to 12-h distributed

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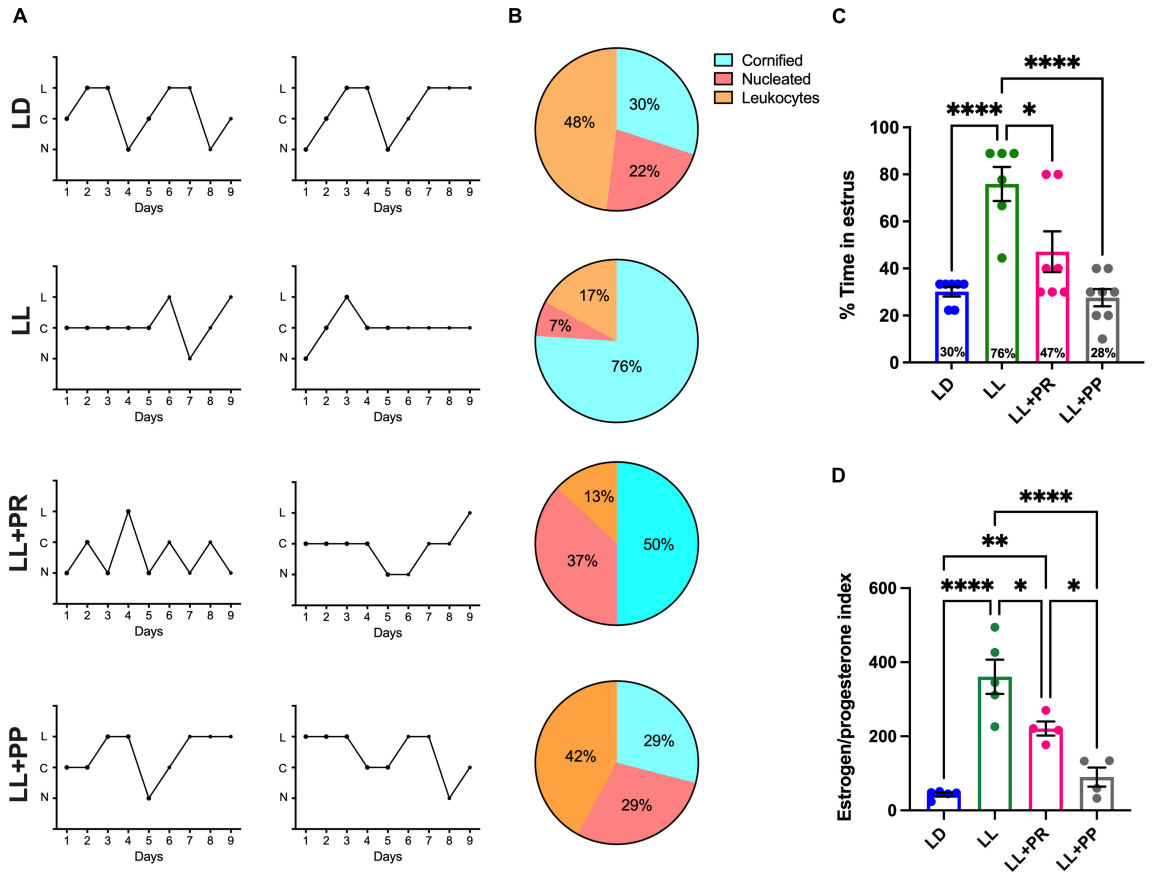

FIGURE 8

Estrous cycles obtained by 9-day vaginal smears. **(A)** Two representative cycles obtained with the cytology in the vaginal smears of rats exposed to a light–dark cycle during the baseline (BL, blue lines, and bars), to constant light (LL, green lines and green bars), to LL followed by 3 weeks of 12-h feeding protocol distributed in four pulses feeding events every 3 h as a rescue strategy (LL + PR, pink), or to LL simultaneously to four food pulses as a preventive strategy for 6 weeks (LL + PP). **(B)** Percentage of rats showing predominance for a given cellular type at each day of the estrous stage according to vaginal cytology. **(C)** Proportion of days in which rats displayed estrus. **(D)** Estrogen/progesterone (E_2/P) ratio. Data are expressed as the mean ± SEM; n = 4–5/group. Asterisks indicate *$P < 0.05$, **$P < 0.01$; ****$P < 0.0001$. C, cornified cells; L, leukocytes; N, nucleated cells.

feeding pulses exhibited similar values as the LD group (Figure 9B). The one-way ANOVA indicated no significant difference among groups for total follicles [$F_{(3,15)} = 3.7$; P = NS]. The effect of the 12-h distributed feeding pulses on the total number of follicles was mainly due to the increased number of primary and secondary follicles (Figure 9C). The two-way ANOVA indicated a significant effect of follicle type [$F_{(2,51)} = 44.73$; $P < 0.000$] and groups [$F_{(3,51)} = 4.10$; $P = 0.011$].

Notably, only the preventive strategy (LL + PP) reduced the number of cysts in the ovary as compared to LL rats inducing similar values as the LD group (Figure 9D). The one-way ANOVA indicated a significant difference among groups [$F_{(3,16)} = 22.39$; $P < 0.0001$]. In addition, several corpora lutea were observed in the ovaries of rats in both feeding regimes, suggesting the occurrence of ovulatory cycles. Examples of ovarian morphology can be observed in Figure 10.

Discussion

Timed restricted feeding is a powerful entraining stimulus for circadian function. Therefore, time-restricted feeding is suggested as a useful strategy to prevent or restore circadian disruption in conditions of experimental shift work or experimental jet lag (27, 29, 39). Also, scheduled feeding ameliorates circadian function under disrupting conditions due to a high-fat diet (40–42), and it improves the response to a glucose tolerance test (43) and is considered an efficient chrononutrition strategy for the treatment of obesity and diabetes (44, 45). Previous studies had evidenced that under constant darkness, feeding schedules can entrain the free-running activity rhythm (46, 47). The present study provides new evidence of the impact that timed restricted feeding can have on restoring disrupted daily rhythms due to ALAN. Here, we show that a cycle of 12-h feeding/12-h fasting

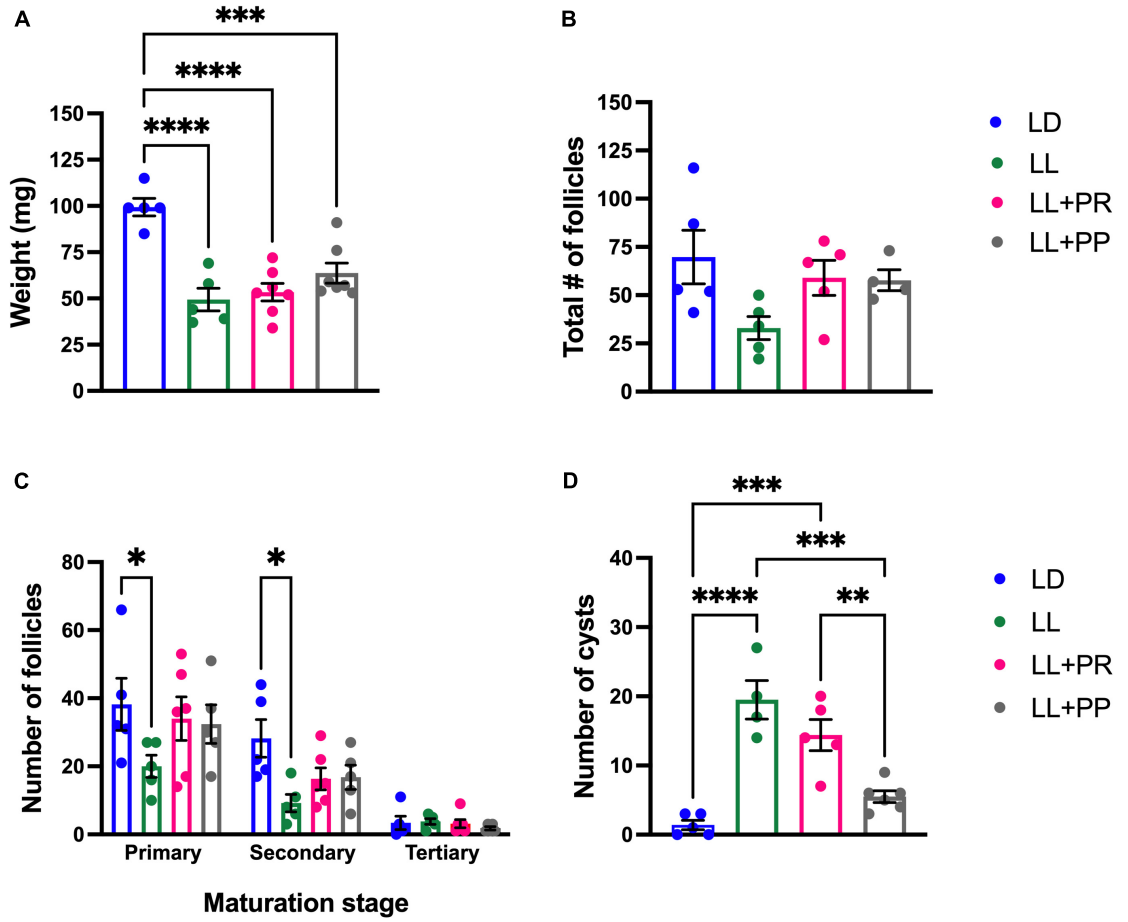

FIGURE 9
Ovarian weight and morphology after exposition to constant light (LL) and 12-h food distributed in four pulses events every 3 h. **(A)** Ovary weight, **(B)** total number of follicles, **(C)** number of primary, secondary, and tertiary follicles, and **(D)** number of cysts. Data are expressed as the mean ± SEM; n = 5–6/group. Asterisks indicate ****$P < 0.0001$.

can drive 12-h activity-12-h rest cycles of general activity and core temperature. This effect was improved when the access to food was distributed in pulses along the 12 h of scheduled feeding, as seen with the day–night amplitude of general activity and with core temperature. In both experiments, the ARC responded directly to the feeding/fasting cycles as observed with c-Fos activation. However, the AVPV only responded when food access was distributed along the 12 h of the subjective night. Interestingly, a day–night rhythm was observed for the SCN only when food was distributed in pulses along the 12 h of the subjective night as a preventive strategy (LL + PP). Likewise, the LL + PP schedule was more efficient for reducing the days spent in persistent estrus and improving the conditions of the ovary. Our findings confirm that feeding schedules can be an efficient entraining signal and that distributing food intake in pulses along the subjective night comprising a 12-h window that mimics a nocturnal feeding pattern for rodents may impose day–night rhythms on the behavioral, hypothalamic, and reproductive function.

Previous studies have evidenced that disruption of the circadian function in females results in the loss of synchrony for the LH surge and, thus, leads very soon to persistent estrus (23, 48). The estrous cycle requires the synchrony of multiple oscillators, and therefore, the loss of circadian rhythms due to LL would allow oscillators to free-run and drift apart from each other (49, 50). Following this line of thinking, regular 24 h cues might provide the regularity and fine-tuning for the oscillators involved in the reproductive function and therefore revert or prevent the state of continuous estrus under LL. Weber and Adler compared regular schedules with random schedules of vaginal smears in female rats exposed to different constant light intensities and observed that the onset of persistent estrus was delayed but not completely prevented by the daily regular manipulation (49).

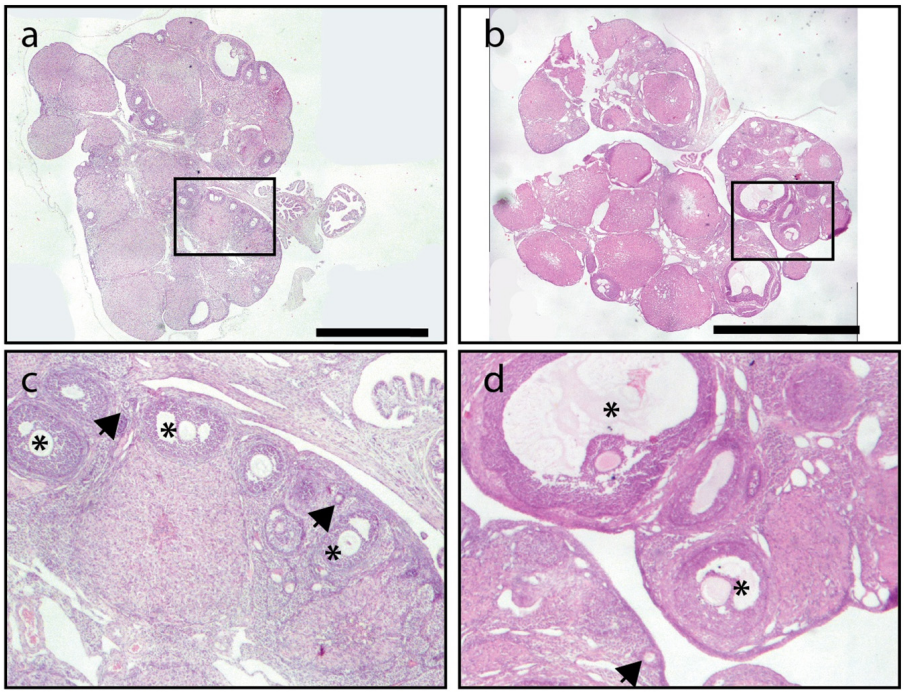

FIGURE 10
Example of an ovary from a rat exposed to LL followed by 3 weeks of 12-h feeding protocol distributed in four pulses feeding events every 3 h as a rescue strategy (LL + PR) **(a,c)** and an ovary from a rat exposed simultaneously to four food pulses as a preventive strategy for 6 weeks (LL + PP) **(b,d)**. Primary follicles are indicated with arrowheads and growing follicles with asterisks. Scale bar = 2 mm.

Scheduled exercise may be an alternative for restoring disrupted rhythms due to LL. Hughes et al. showed that scheduled voluntary exercise in mice with disrupted SCN activity drives a 24 h rhythm of wheel running and feeding activity (51). Using a model of polycystic ovary syndrome due to prenatal androgen exposure, voluntary wheel running improved the regularity of the estrous cycles, in spite that wheel running was performed *ad libitum* and did not impose a daily scheduled stimulus (52).

Since constant light inhibits melatonin production and melatonin is a relevant internal time signal for the circadian system (53), previous studies have tested the administration of exogenous melatonin as a strategy to restore or prevent the persistent estrus and the anovulatory state associated with LL. In female rats exposed to LL for 1 month, a chronic daily intravenous administration of 100 μl of melatonin induced metestrus and diestrus in 70% of rats, as compared with rats receiving only a single injection (54). Despite driving rats out of the persistent estrous stage, no regular estrous cycles were observed. In our present study, feeding cycles with food distributed in pulses along the subjective night also restored or prevented the persistent estrus in 90% of the female rats, leading rats to metestrus or diestrus, and likewise as observed with melatonin, females exhibited irregular estrous cycles.

Previous studies exploring mechanisms of food entrainment described that the SCN does not shift in response to feeding schedules and is mainly entrained by the light–dark cycle. However, the SCN has the capacity to respond to the metabolic state (fasting or feeding) by modifying its cellular activation as seen with c-Fos (55, 56) and in electrophysiological recordings (57). Also, studies using hypocaloric diets have shown that the SCN can respond to feeding events (58). Exposure to constant light disrupts cellular synchrony in the SCN driving individual cells out of phase. However, in LL, single neurons remain rhythmic (59). The loss of synchrony among VIP neurons may be a condition that facilitates their entrainment to non-photic time signals (60). Thus, the loss of intercellular synchrony in the SCN may have favored that the 12-h feeding/12-h fasting cycle synchronized c-Fos activation in the SCN, which allowed synchrony with other hypothalamic nuclei for the fine-tuning necessary for reproductive function. Importantly, when placing the total food as a single event every day, a constant nocturnal activation was not completely achieved in the LL + RFR and LL + RFP groups. High activation was observed during the first hours of the feeding schedule, and then, a significant decrease occurred. This had important consequences on the response of the SCN and the estrous cycle. In contrast, distributing food in pulses along the 12-h feeding window resulted in a stronger entraining signal for the SCN and the circadian

function, and this prevented deleterious effects on the estrous cycle. In this second experiment, the distributed schedule required continuous alertness from the rats, and this factor may have added up to the effect of the feeding schedule as a time signal.

The ovary is tightly driven by the circadian system, and it exhibits molecular clock mechanisms (61, 62); in this regard, the proposed feeding schedules might help to maintain an internal organization of the ovary clock under constant light conditions. In Siberian hamsters (*Phodopus sungorus*), changes in light cycles induced regression of ovarian and uterine size. In mice, 2 weeks of LL caused a reduction of ovarian follicle number accompanied by increased apoptosis of ovarian cells, increased levels of LH, FSH, and E2, and decreased progesterone levels (63). This is in accordance with our observations, and we report a persistent estrous stage, a significant reduction in ovarian size in addition to impaired follicular development, and an increased E_2/P ratio. Importantly, this condition was reverted with the 12-h feeding cycles, suggesting that in the disruptive circadian function, timed restricted feeding can prevent adverse effects on the ovary.

Interestingly, constant light induces the three major hallmarks of the polycystic ovarian syndrome (hyperandrogenism, polycystic ovarian morphology, and oligoovulation) (64). In our experiments, we observed the development of cysts in LL rats. Remarkably, the scheduled feeding regimes partially reverted these alterations in ovarian morphology. Polycystic ovarian syndrome (PCOS) is one of the most common endocrine disorders in women; despite that, its etiology is poorly understood. In addition, the treatment options for PCOS are not completely efficacious and often cause severe side effects. One emerging factor associated with the development of PCOS is the ALAN, which is affecting a growing proportion of the population. Further studies should explore better the beneficial effects of scheduled feeding regimes as an alternative intervention in the treatment of PCOS.

We provide evidence of the adverse effects of ALAN on reproductive function at the level of the hypothalamus, hormonal ratio, and ovary morphology. We demonstrated that 12-h feeding cycles can drive rest-activity cycles and that they can improve reproductive function. Moreover, forcing rats to eat along the subjective night proved to be more efficient than placing the food once/day in the feeder. Food distributed in pulses resembles better the feeding activity that rats perform during the night. Further studies are necessary to prove that this effect can persist if the 12-h feeding cycles are interrupted or whether the feeding cycle function as a daily hour-clock. Also, in LL conditions, the beneficial effects of the 12-h feeding cycles need to be tested for other systems, like metabolic and inflammatory perturbations described by previous studies (65).

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by the Committee for ethical evaluation at the Facultad de Medicina UNAM (FM/DI/140/2019).

Author contributions

CE: study conception and design and funding acquisition. NG-V, EE-B, RE, HL-M, MG-P, RN-E, MS, SB-W, ER-F, and BO: acquisition of data. NG-V and CE: statistical analysis and drafting of the manuscript. NG-V, EE-B, RE, and CE: analysis and interpretation of data. RB: critical revision. All authors contributed to the article and approved the final version of the article, including the authorship list.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.999156/full#supplementary-material

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Studying food entrainment: Models, methods, and musings

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The ability to tell time relative to predictable feeding opportunities has a long history of research, going back more than 100 years with behavioral observations of honeybees and rats. Animals that have access to food at a particular time of day exhibit "food anticipatory activity" (FAA), which is a preprandial increase in activity and arousal thought to be driven by food entrained circadian oscillator(s). However, the mechanisms behind adaptation of behavior to timed feeding continue to elude our grasp. Methods used to study circadian entrainment by food vary depending on the model system and the laboratory conducting the experiments. Most studies have relied on rodent model systems due to neuroanatomical tools and genetic tractability, but even among studies of laboratory mice, methods vary considerably. A lack of consistency within the field in experimental design, reporting, and definition of food entrainment, or even FAA, makes it difficult to compare results across studies or even within the same mutant mouse strain, hindering interpretation of replication studies. Here we examine the conditions used to study food as a time cue and make recommendations for study design and reporting.

KEYWORDS

circadian, food anticipatory activity (FAA), feeding, restricted feeding, time restricted eating, time-restricted feeding

Introduction

Given the over-abundance of food in modern society, it is easy to lose sight of the importance of feeding to animals in nature and in sculpting our own evolutionary history. For most animals, food is extremely scarce and being wise to opportunities to eat is essential for survival. As such, the circadian system has evolved to receive a number of different stimuli—i.e., light, temperature, and food—to keep biological processes coordinated and allow for adaptation to ever changing conditions (1, 2). Food is an often neglected zeitgeber, being outshined by light, which is more intensively studied and much better understood (3). The light entrainment pathway works *via* excitement of intrinsically photosensitive retinal ganglion cells that project to the suprachiasmatic nucleus (SCN), which is the major regulator of circadian rhythms (4). The SCN serves as a light entrained oscillator (LEO) and its near 24-h neuronal activity is so robust that it persists in explanted tissue for more than a year (5). Application of the same logic used to tease apart the neural circuitry and molecular clockwork of the LEO has not yielded great success when applied to studies of the food entrained oscillator(s) (FEO) (6). In this review, we describe attempts at delineating the circadian biology of food entrainment using a multitude of model systems, including extensive use of rodents, and given the

wide range of methodology we make suggestions for improvements and considerations in methods and interpretation of data.

Model systems used to study food entrainment

FAA research has a rich history with early published descriptions of the behavior dating back 100 years (7). In this time an array of organisms of varying complexities have been studied. Honey bees (*A. mellifera*) have been shown to entrain to restricted feeding schedules and exhibit phase differences in expression of cryptochrome2 (cry2) and period (per) with time-restricted foraging (8, 9). By contrast, studies in *D. melanogaster* show that restricted food availability is not able to entrain activity cycles (10, 11). Food anticipation has also been well-documented in fish. This behavior was first explored in 1964, when a pre-meal peak in locomotion was observed in both largemouth bass (*M. salmonides*) and bluegill (*L. macrochirus*) (12). Other studies have followed suit, utilizing goldfish (*C. auratus*) (13, 14) and zebrafish (*D. rerio*) (15, 16), among other species, to attempt to tease out an FEO separate from the LEO. Studies have demonstrated that fish align their activity periods with mealtimes; however, the presence of a FEO in fish that is separate and independent from the LEO is still an open question. Some unconventional mammalian systems have also been utilized; a recent study employed a pig model to show that ghrelin antagonist administration lowered FAA (17). While the bulk of research in the field has been conducted on rats and mice, alternative rodent models like Syrian hamsters (*M. auratus*), gerbils (*M. unguiculatus*), and the diurnal rodent *A. ansorgei* have been helpful in demonstrating the widespread nature of FAA and testing variables such as age and photoperiod (18, 19). For example, Syrian hamsters may express lower FAA under restricted feeding conditions due to a stronger sensitivity to the masking effect of daytime light conditions (20–23). Syrian hamsters have also been utilized for SCN ablation experiments to confirm that the SCN is not required for FAA in a rodent model aside from rats or mice (24).

Rabbits (*O. cuniculus*) naturally nurse their young once daily in the early morning hours, providing a natural system for studying FAA (25, 26). The eyes and ears of rabbit pups remain sealed until postnatal day (PD) 10; in addition, while retinal projections to the SCN are present at birth, its response to photic input is not fully developed until PD12 (27). Furthermore, circadian cycling of clock genes *Per1, Bmal1,* and *Cry1* in the SCN lacks a clear rhythm until PD45 (27). Even with the lack of rhythmic clock gene expression, young pups develop increased preprandial locomotor activity, altered expression of hormones such as ghrelin and corticosterone, and induction of c-Fos expression in several hypothalamic nuclei, including oxytocin neurons in the paraventricular nucleus (PVN) (28–31). These factors have made the rabbit pup an important model in which to explore the FEO with minimal interaction from the LEO.

Rats (*R. norvegicus*) have been a vital system for studying food entrainment and have been utilized for lesion experiments that provided many key negative results, ruling out structures of interest. Rats with lesions of the SCN lose their light:dark (LD) entrained rhythms when fed *ad libitum*, but maintain robust FAA when given restricted food access (32–34). These findings were repeated in mice (35), supporting the presence of a FEO separate from the LEO and that the SCN is not necessary for the development of food entrainment. Further ablation experiments have been conducted in rats in an attempt to find a region of control for FAA, showing that ablation of the olfactory bulb, dorsomedial hypothalamus, and thalamic paraventricular nucleus, as well as vagotomy, fail to prevent FAA (36–41). Historically these studies have lacked consistency, both in methodology and results, and as such the existence of a single structure regulating food entrainment remains a theoretical possibility (42).

Due to their increased size and complexity, many experiments have been completed in rats that would likely not be possible with mice. In a recent study, rats were taught to press a lever for food and then subjected to restricted food access, using lever pressing as a measure of FAA; the lever pressing showed a preprandial increase, even in animals with SCN lesions (43). Rats have also been shown to be more attuned to entrainment by palatable meals, developing FAA to the presence of a palatable treat provided in addition to standard chow *ad libitum* (44, 45). Another advantage of rats is their increased size in comparison to mice, making them more suitable for meal withdrawal experiments, which helped to rule out "hourglass" timers for food entrainment (46). Withdrawal of food is a key element to proof of entrainment; however doing so in mice, which are much smaller and extremely metabolically active, causes rapid weight loss and protocols with this withdrawal period are often not approved by local research ethics boards.

Even within the laboratory mouse (*M. musculus*), there is substantial variation in genetic makeup that has been shown to affect any number of phenotypes (47). Most studies of FAA in mice are done on the C57BL/6 background, with many utilizing the J substrain from Jackson labs; however, the BTBR strain, which is a popular model of autism (48), was recently shown to have superior FAA compared to C57BL/6J (49). Moreover, phase shifting experiments also demonstrated that the BTBR strain was better able to adjust to altered light or meal delivery times when compared to C57BL/6J mice (49). BALB/C mice are believed to have weakly-coupled circadian oscillators controlling their light-based rhythms (50). When these mice were used to study FAA, they quickly developed high levels of FAA when compared to the C57BL/6J strain (51). The inbred 129S1 strain has been tested under 60% calorie restriction (CR) conditions and showed that they redistributed almost all high activity

behaviors to the pre-meal window, displaying "exceptional" FAA (52). Historically, it has been shown that C57BL/6(J) mice have relatively weak FAA when compared to rats (35), which begs the question- why has this been the primary mouse strain utilized for studying FAA when other strains are available that are more attuned to scheduled feeding? We suggest that researchers continue to explore alternative strain backgrounds and consider using different strain backgrounds that demonstrate stronger FAA. Furthermore, prior research has found both major and minor variations in the genomes of commonly used strains of mice, such as C57BL/6J (B6/J) and C57BL/6N (B6/N). For example, a common mutation in *nicotinamide nucleotide transhydrogenase* (*Nnt*) gene, which is found solely in the B6/J strain, appears to negatively impact mice in metabolic studies (53). Mice with the *Nnt* mutation have an increased chance of diet-induced obesity due to glucose intolerance, as *Nnt* is necessary for the maintenance of ATP synthesis (53, 54). On the other hand, B6/N mice carry a frameshift mutation in *retinal degeneration 8* (*rd8*) and in the *cell polarity complex component* (*Crb1*), impairing vision (55). The effects of these particular mutations have not been studied for their effect on FAA.

Pendergast and Yamazaki (6) provided an authoritative review of food entrainment studies of mouse mutants, summarizing every knockout (KO) mouse tested (6). As a thought experiment, we describe the idealized mouse mutant that we have hoped to discover through genetic approaches to studying food entrainment. Firstly, this mouse would have normal light entrained rhythms and its total activity levels would be unaltered. Secondly, both its feeding, body weight, and body composition would be indistinguishable from controls. Third, its digestive and nutrient absorption system would be fully intact. Finally, it would have a complete deficit in predicting scheduled mealtime at a behavioral level–both when fed in the light cycle, dark cycle, and under constant conditions (DD or LL). Peripheral oscillations, such as liver gene expression, corticosterone secretion, and body temperature would all show normal entrainment. Whatever gene deletion is present in this mouse would be recapitulated in a conditional KO where this gene was removed from only a subset of neurons, indicating a brain region(s) that serves as the FEO. Alas, we are lacking such a mouse, but important strides have been made in this direction, including a mouse mutant lacking *Nr1d1* in neurons that cannot predict scheduled mealtime when fed during the day (56).

Food entrainment methodology in rodents

Since we study FAA in rodents, we are focusing on this system here but the methodological issues described herein can be applied similarly to other organisms. For studies of food entrainment, experimental conditions vary considerably. For example, temporal restriction (RF) of feeding is the most common method, but the duration of food availability has a large range. Similarly, some studies offer a larger temporal window of food availability that tapers down over time while others do not. Calorie restriction (CR), feeding anywhere between 60 and 80% of normal food intake, is another commonly employed method for inducing FAA. CR has the advantage of fewer handling steps (one feeding vs. delivering and removing food) but the disadvantage is that mice can potentially "ration" their food and may not consume it all during a tight window (we have not observed this to be the case after the first few days of CR). Despite these concerns, a side-by-side comparison of body temperature and FAA in mice on 60% CR vs. 3-h RF showed similar entrainment of activity and body temperature to feeding (57). Multiple feedings can also be employed: for example, Luby and colleagues demonstrated that mice can anticipate several meals per day but not with high precision while Petersen et al. demonstrated that rats are much better at anticipation of multiple mealtimes (43, 58).

Regarding RF, the temporal window of food access and when that occurs during the LD cycle varies a lot from study to study. At one extreme would be studies like that of del Rio-Martin et al. (59) that fed mice during the entire 12 h dark cycle, leading to minimal FAA in controls and the erroneous conclusion that a mutant mouse (*Pitx3*^{ak}) failed to show metabolic or behavior entrainment to scheduled feeding (59). When we studied the same mutant under a CR feeding regimen for many days (>40 of 60% CR), we observed robust FAA in the mutant and controls, highlighting the importance of feeding protocols and duration of experiments (60). Other studies like those of Li et al. (61) and Kaur et al. (62) give a 4-h access to food, but the overall duration of their experiments are quite short, lasting only 9–10 days of RF (61, 62). Such short duration studies can be problematic. For example, when we studied the same orexin KO mouse used by Kaur et al. (62), it had a slight delay in acquisition of FAA by about 1 week compared to littermate control mice (57). However, by 2 weeks of timed 60% CR, there were no differences between groups; moreover, the orexin KO showed resistance to weight loss on CR, making it difficult to interpret this delay in establishing FAA. Another important point discussed by Pendergast and Yamazaki (6) is the use of fasting days both pre- and post RF. For example, fasting a mouse for 24 h prior to beginning RF reduces both fat and lean mass and leads to much more rapid appearance of FAA, even within a few days (63). Some laboratories even combine temporal and calorie restriction (64). Finally, it will be important to examine the effect of repeated testing on the same animals for parameters of food entrainment. For example, LeSauter and colleagues concluded from their studies of D2 dopamine receptor overexpression transgenic mice that this manipulation lowered "motivation" for food, but in their studies they repeatedly tested the same mice in a short- (4 h), medium (6 h), and long-duration (8 h) RF schedule, finding that only under the longest duration RF that the D2 transgenic mice had less FAA (65). Between rounds of RF,

ad libitum access to food was given for variable amounts of time, ranging from 7 to 10 days but also including a fasting day in one of these respites (65). Expression of FAA should not necessarily be interpreted as lower motivation and it is important to apply instrumental assays to test this more explicitly.

Does restriction amount or the amount of weight loss matter? In a study using C57BL/6J male mice, we compared 60 vs. 80% CR fed in the middle of the light cycle (66). Both the 60% and 80% CR groups demonstrated FAA within 7 days of scheduled feeding, losing about 10% body weight during that first week and weight loss continued to decline for both groups until day 14. However, the 80% group's body weight rebounded slightly as the study progressed and their level of FAA declined after day 14, whereas weight loss in the 60% group continued and the magnitude of their FAA was almost twice that of mice on 80% CR at later time points when both groups had clearly developed FAA. Thus, the amount of restriction and accompanying weight loss appeared to dictate the amount and maintenance of FAA. However, and quite unexpectedly, examining individual mice within either the 60% or the 80% group showed that there was no correlation between the amount of weight loss experienced by each individual mouse and the magnitude of FAA that it expressed. Thus, weight loss or at least having mice in a negative energy balance appears to be an important aspect of obtaining maximal FAA (i.e., 60% has greater FAA than 80% after day 14), but at an individual level the weight loss is not predictive of the amplitude of FAA. In the future, a study that explicitly tests the effect of starting body weight on food entrainment would be of interest. For example, a cohort of same-aged mice could be either over-fed, under-fed (without a timing component), or normally fed prior to entering a timed feeding study. This would allow for comparison of overweight, normal weight, and under-weight status on induction and maintenance of FAA. It is important to note that some laboratories distinguish between hypo-and normocaloric food entrainment and obtain some measure of food entrainment by scheduled feeding that does not result in a net negative energy balance (67).

Diet plays a large role in metabolism and has effects on feeding behavior. The ability to achieve FAA on specific diets corresponds with a macronutrient restrictive food anticipatory study in which subsequent restriction of protein, carbohydrate, and fat nutrients in rats did not substantially affect locomotor behavior prior to reward stimulus (68). Binge-eating behaviors can lead to obesity and often stem from not only the addictive behavior of eating at a specific time during the day but even just from the sight or smell of palatable food (69). In the past, Mistlberger et al. attempted to observe the potential effects of food in relation to activity in mice by testing rats on two different types of diets. The main difference between these two diets was the lack of a simple sugar in unformulated or standard mouse chow, whereas formulated chow had sucrose. Rats in this study were deprived of either protein, carbohydrates, or high fat diet for about 2 weeks, then introduced to the missing macronutrient and tested for locomotor behavior using tilt cages (68). In other words, a palatable meal offered during caloric restriction is sufficient as an entrainment cue for food anticipation. These findings suggest an independence exists between a single macronutrient and FEO(s), thus diet in itself is not a sufficient cue to entrain any FEO(s).

High-fat palatable meals and "treats" have been shown to be a strong promoter of FAA. Palatable meals are loosely defined as being nutrient-rich with early studies utilizing a "nutrient-rich palatable mash" (45), but have included a variety of formulas such as high-fat rodent diets and treats like chocolate and cheese containing varying levels of sugar and fat content (52, 70). Overall, rats and mice have been shown to develop FAA and binge-type eating with the addition of high-fat meals, with correlating c-Fos activation in reward centers of the brain (44, 52, 71). A recent study also showed that, in rats, a high-fat diet stimulated higher c-Fos expression, stronger FAA, and more binge-type eating than a high-sugar diet (72). That being said, restricted access to a high-sucrose meal is known to activate c-Fos expression in the prefrontal cortex, lateral septum, nucleus accumbens and anterior lateral hypothalamus during the pre-meal window (73). It would be of interest to test whether the palatability, sweetness, and formulation of the diet would influence food entrainment, in particular the amount of FAA.

Factors other than food

Aside from diet and feeding times, there are a number of other variables that have been shown to impact FAA but are not always reported or considered. Overall, sex differences in FAA in rodents are modest, but should be studied and reported. Mice on RF schedules indicate clear differences between the sexes in regard to food intake, body weight, and food anticipation (61). This experiment, which separated male and female mice, depicts males to be generally more active and to have exhibited greater wheel-running activity during RF (61). Another study focused on sex differences in D1 receptor (D1R) KO mice also showed that female D1R KO mice had more severely attenuated FAA than the D1R males (74). In addition, ghrelin levels, the hormone responsible for triggering an appetite, were analyzed to be higher in female than male mice (61), which suggests gonadal hormonal differences can account for sex-related differences in regards to FAA. Supporting this, gonadectomized mice were observed to have similar food anticipation levels over a span of 10 days of RF (61). However, a series of follow-up experiments opposes this finding that differences in FAA are related to gonadal sex hormone differences. Instead, Aguayo et al. showed that singular manipulations to gonadal hormones, sex chromosomes, and developmental patterning in WT mice are not enough to explain the sex differences (75). This study used gonadectomized mice, sex chromosome copy mutants, and masculinized female mice that were treated with 17-β

estradiol during their neonatal period and still reported no differences between males and females while on a calorie-restricted feeding paradigm (75). Therefore, while there are reports that indicate male mice to be generally more active than females, gonadal hormone differences alone cannot account for these findings. We showed that sex difference in FAA was actually age dependent. Female mice that were 9–11 months old no longer had a decreased level of FAA compared to male mice (75).

In addition to the loss of sex differences in mice aged 9–11 months (75), age has also been shown to reduce amplitude and onset of FAA. A study comparing FAA in young and aged rats showed that rats aged 24–25 months, being of extreme old age, had a longer onset time and a lower amplitude of FAA when compared to rats aged 3–21 months (76). Another study examined FAA in dysrhythmic rats aged 13–18 months compared to young control rats and young SCN-lesioned rats; here, they found that the aged rats were able to develop FAA and successfully entrain to a restricted feeding schedule, although aged rats with intact LD cycling were not run in concurrence to compare onset and amplitude of activity (77). As it has been shown that aged mice display a decreased capacity for re-entrainment to LD phase shifts (78) it would stand to reason that the same would be true for entrainment to feeding schedules.

Manipulations in photoperiod have shown that the duration of the light cycle has a minimal effect on FAA and that the behavior is able to persist in both constant conditions (both LL and DD) (79, 80). Mice have also been tested in long- and short-day photoperiods, but neither consistently affected FAA (81). Interestingly, skeleton photoperiods enhanced FAA though an unknown mechanism (82).

Numerous studies of the gut microbiome have shown that time-restricted feeding or addition of specific microbiome derivatives (83) are capable of altering expression patterns of core clock genes both *in vitro* and *in vivo* [reviewed in Dass and de Ross (84)]. It has been shown that mice given standard chow *ad libitum* exhibit cyclical expression of gut microflora and that *ad libitum* access to high fat diet reduces these cyclical fluctuations; however, animals given time-restricted access to the same high fat diet retain the cyclical expression of the microflora (85). Another study demonstrated that disruption of LD rhythms by regular phase shifts resulted in changes in intestinal microflora only when mice were fed a high-fat diet, while mice fed normal chow *ad libitum* did not experience a similar change (86). In this study, it was discussed that a secondary stressor on top of phase shifting, such as alcohol consumption (87), high-fat & high-sugar diet, or general stress is often required to induce these microbiome changes and they may not occur in an "unchallenging" environment (88). While neither paper examined the gut microbiome of mice on a restricted feeding schedule of normal chow, given the stress likely induced by single housing and food restriction regimens, it would follow that such conditions may also induce changes in the gut microbiome of animals undergoing a "typical" FAA experiment, some of which may be cyclical as seen by Zarrinpar et al. Given the known interaction of the microbiome and expression of clock genes in peripheral tissues [reviewed in Dass and de Ross (84)], more investigation of how the microbiome affects behavior and FAA may be warranted. Initial studies could measure FAA in germ-free mice to establish the necessity of gut microbiota for food entrainment.

Other factors to consider are the activity measurement method and cage environment. For example, we observed that higher ambient temperature is suppressive of FAA (66) while others have obtained robust inductions of FAA and other readouts of entrainment at thermoneutral temperatures in mice (29°C) (89). There are a variety of methods utilized for quantification of food entrainment, including but not limited to wheel running, lever pressing, telemetry sensors, photobeams, motion sensors, and video-based homecage behavior analysis (40, 43, 52, 60, 90, 91). Direct comparison between telemetry sensors and motion sensors have shown that quantification through these methods are equivalent (92). Alternatively, the inclusion of a running wheel in a cage has been shown to increase FAA; even mice with previous wheel-running experience showed increased FAA when given a locked running wheel, although mice without prior wheel-running failed to show a similar increase (90). It was also shown that circadian organization, observed by Per2:Luc expression, is affected by wheel running. Phase-shifting of the lung, spleen, and liver was observed and shown to be wheel-running specific, as an enriched environment alone did not produce the same effect (93). A major setback of a number of behavioral analysis systems is the requirement of single housing the mice in a barren cage. One example of this is in Gallardo et al. (66), where video-based homecage behavior is analyzed using the HomeCageScan software (behavioral definitions described in 88). This method allows for a more detailed breakdown of behaviors than simple activity sensors or photobeam breaks, which has shown to be useful in certain mouse mutant lines. For example, in leptin KO mice we observed an increase in preprandial hanging and jumping behavior but walking was not increased (57). The sum of high activity behaviors was not significantly increased in leptin KO mice on CR, because this measurement is dominated by walking behavior. We concluded that the leptin KO mice had an intact FEO because of their timing of jumping and hanging, but other groups have reported that leptin negatively regulates FAA, making their measurements using locomotor activity *via* photobeam breaks (94). In this study leptin KO mice had enhanced FAA, consistent with studies in rats that harbor a homozygous null mutation in the *leptin receptor* gene (95). Administration of leptin blunted FAA in leptin KO mice but leptin administration in wild-type mice had no effect on FAA (94). That being said, there are downsides of video recording behavior that should be mentioned: most video-based methods require an empty cage

with minimal bedding, depriving the animals of enrichment (96). In Pendergast et al. (93), three separate measures of activity were taken by way of infrared motion sensors, wheel running, and infrared video analysis of eating behavior. This use of multiple techniques provides several measures of behavior while also allowing for an enriched environment for the animals (93). Given that FAA is likely an adaptation of animals to forage during periods of food availability, providing more forms of enrichment to allow for those behaviors may be ideal. Furthermore, studying food entrainment in socially housed animals has not been conducted to our knowledge, as most measures of activity require individual housing.

Recommendations and considerations for food entrainment studies

Based on the considerations and methodological issues described above, we advocate for the collection, presentation, and analysis of food entrainment data as follows:

- **Food intake values should be reported as raw and/or normalized to body weight**. These feeding measurements should be reported prior to restriction and during, if appropriate (i.e. during RF it is possible to measure food intake whereas during CR the value is fixed).
- **Although on its own, body weight loss is only loosely predictive of FAA, it is still important to report both as raw data (i.e. grams) and as a percentage of starting weight.** Ideally, measurements of lean and fat mass would be presented as well, especially in cases of mouse mutants that have adipose or metabolic phenotypes (57). For example, it was reported that orexin KO or orexin neuron ablated mice had less FAA (62, 97), but when we tested timed CR in orexin KO mice we observed that they were resistant to weight loss during CR and eventually developed comparable FAA to controls (57). Since starting body weight and/or fat stores can be affected by genetic manipulations and will likely affect the development of FAA, these data need to be considered and reported.
- **With respect to metabolism, measurements of indirect calorimetry (i.e. respiratory exchange ratios) are desirable especially when mutations cause metabolic phenotypes**. We are only aware of a couple of examples of studies of food entrainment that measure energy expenditure (60, 98) and recognize the difficulty in conducting them given the cost of obtaining the equipment. More studies of respiratory exchange ratios and energy usage under different feeding protocols and in different mouse mutants may reveal previously unappreciated principle components of food entrainment.
- **Raw and normalized behavioral activity data should be reported.** This will allow for i) visualizing total activity levels, which are often affected by gene deletions, and ii) for comparison to nocturnal activity peaks as a frame of reference. Thirdly, normalization to total activity corrects for differences within lines of mice and also allows for comparisons between studies (i.e. a 30% redistribution of activity to precede scheduled mealtime). In our study of sex differences in FAA using C57BL/6J mice, we observed no difference in premeal high activity behavior when comparing males vs. females, but when we normalized these data, the females clearly showed less FAA and higher nighttime activity (75).
- As recommended by Pendergast and Yamazaki (6), if permitted by the Institutional Animal Care and Use Committee, **include at least one fasting day or meal omission after FAA has been established** in order to discern if the mutation/manipulation in question is affecting the expression of FAA or the FEO itself.
- **Conduct initial studies of FAA in 12:12 LD conditions with feeding in the middle of the light cycle.** This can be followed by testing FAA for nighttime feeding as some mutant mice have specific deficits in FAA only for light cycle feeding [for example, see Podyma et al. (63)]. Finally, examining FAA in constant darkness or constant light and/or meal shifting experiments are an important third and fourth lines of inquiry that allow for studying FAA with less contribution from the LEO.
- **Conduct longer duration experiments or do a fasting day(s) prior to timed feeding for shorter duration experiments**. Studies of FAA range from less than a week (63) on the low end, to at least 2 months on the high end (60, 64). As starvation increases activity acutely (52), it is important to distinguish whether mice have a phenotype relating to hunger induced activity vs. food entrained activity, thus adding a week of RF/CR to a study can assist with interpretation of results in combination with body weight data (i.e. when weights have plateaued).
- **Report strain information** and track genetic purity of inbred rodent models to monitor genetic background and possible interacting alleles, especially in the context of KO/Cre transgenic mice made from ES cells on different genetic backgrounds where linkage disequilibrium makes it nearly impossible to obtain alleles on a pure background *via* backcrosses (47).
- **Report lab diet formulations.** As discussed above, foods with higher fat content tend to be suppressive of FAA while perplexingly, the palatable treats used to induce FAA in mice need to have high fat content (52, 70). It is also an outstanding question as to whether formulated diets will lead to more consistent or stronger food entrainment.
- **Report sexes and ages of animals.** Females are understudied in circadian biology and metabolism (99). The age of animals

affects their size and adiposity, and as discussed above can have an impact on FAA (75).

- **Conduct follow-up and replication studies, both within and between laboratories**. There are many interesting papers on FAA that lack followup; for example, a study indicating that the cerebellum harbors a FEO (100), interaction between SCN and the dorsomedial hypothalamus using dual lesions in rats (101), the complete lack of FAA in *Nr1d1* neuronal KO mice (56), and the dramatically decreased FAA in melanocortin 3 receptor KO (98), which was not replicated in a separate study by Ribeiro et al. (94). In our own experience, the deficit in FAA displayed by D1R KO mice diminished over time (66, 102). Follow-up studies using modern neurogenetic techniques and/or more refined deletion techniques, using viral vectors in combination with single or dual recombinase systems, could yield greater insights into the FEO using these initial observations as inroads should they prove reproducible. Furthermore, unclear or conflicting results from broad lesion experiments could be further refined with more detailed analyses of these brain regions. For example, c-Fos staining in the supraoptic nucleus and main body of the PVN in the rabbit and rat brain showed activation after feeding, while the posterior PVN showed activation prior to the scheduled feeding time (103, 104). These smaller subregions are more difficult to target and may be treated as a single, larger "region" under less precise techniques.
- **Come to a consensus on a more rigorous definition of "food anticipatory activity", and be more clear about whether FAA is reduced vs. eliminated.** Could the field agree on a mathematical definition or a heuristic rule for defining FAA? For example, conduct a one-way ANOVA comparing pre- and post-restriction with a significant effect of time? Or a simple rule such as the amplitude of the FAA peak must be at least half that nighttime activity peak to qualify as FAA? (52, 105). Other ideas to consider would be defining the start of FAA to be when activity surpasses the mean, or when an "acceleration" of a certain magnitude occurs (change in activity from previous bin). Given that most researchers collect food entrainment data in different ways, perhaps fitting a logistic curve to data could be useful since there is an upper limit to the amount of FAA that can be expressed. This objective will require a board consensus perhaps assisted by a metaanalysis of FAA or other food entrainment data. Finally, we need to appreciate the difference between a loss vs. a reduction in FAA or other metrics of entrainment. When FAA is reduced, it does not necessarily follow that the timing mechanism, or the FEO, is not working. As we collect more examples of mutations and manipulations that lower FAA, it will be important to measure other aspects of entrainment and potential masking effects (i.e., studies under DD, for example). In the future, it will be important to move beyond activity alone (as measured by running wheels, photobeam breaks, or computer vision) and try to assay motivation to feed using instrumental behavior assays (nose-poking, lever pressing, etc) (43). If we find that all mice with reduced FAA also have reduced instrumental behavior then we can simply use activity, but we cannot make this correlation until we have more examples of animals lacking FAA.

Author contributions

AS conceived of the manuscript. JT and AS wrote it together. Both authors contributed to the article and approved the submitted version.

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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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Black soybean seed coat polyphenol ameliorates the abnormal feeding pattern induced by high-fat diet consumption

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High-fat diet (HFD) consumption induces chronic inflammation and microglial accumulation in the mediobasal hypothalamus (MBH), the central regulator of feeding behavior and peripheral metabolism. As a result, the diurnal feeding rhythm is disrupted, leading to the development of obesity. Diet-induced obesity (DIO) can be prevented by restoring the normal feeding pattern. Therefore, functional foods and drugs that ameliorate hypothalamic inflammation and restore the normal feeding pattern may prevent or ameliorate DIO. Numerous functional foods and food-derived compounds with anti-obesity effects have been identified; however, few studies have been performed that assessed their potential to prevent the HFD-induced hypothalamic inflammation and disruption of feeding rhythm. In the present study, we found that polyphenols derived from black soybean seed coat (BE) significantly ameliorated the accumulation of activated microglia and pro-inflammatory cytokine expression in the arcuate nucleus of the hypothalamus of HFD-fed mice, and restored their feeding pattern to one comparable to that of standard diet-fed mice, thereby ameliorating DIO. Furthermore, cyanidin 3-*O*-glucoside—the principal anthocyanin in BE—was found to be a strong candidate mediator of these effects. This is the first study to show that BE has the potential to provide a variety of beneficial effects on health, which involve amelioration of the HFD-induced hypothalamic inflammation and abnormal feeding pattern. The results of this study provide new evidence for the anti-obesity effects of black soybean polyphenols.

KEYWORDS

obesity, feeding rhythm, cyanidin 3-*O*-glucoside, hypothalamus, microglia, inflammation, high-fat diet, polyphenol

Introduction

Diet-induced obesity (DIO) is the result of both the ingestion of inappropriate foods and eating at inappropriate times (1). Rodents normally feed during the dark/active period, rather than in the light/inactive period. Rodents fed an HFD *ad libitum* show an abnormal feeding pattern, involving hyperphagia during the light period, and develop obesity, diabetes, and metabolic syndrome (2). In contrast, HFD-feeding that is restricted to the dark period does not result in diet-induced obesity (DIO) (3), and the feeding of a standard diet during the light period does causes obesity (4, 5). In addition, repeated food consumption at the inactive period increases the risk of obesity and various other metabolic diseases in humans (6–8). These lines of evidence suggest that improperly timed meals, rather than the contents of the meals, are the principal cause of DIO. Therefore, DIO may be best prevented by maintaining appropriate meal timing. To further justify this approach, it is essential to understand how HFD consumption affects the *ad libitum* circadian feeding pattern.

Previous studies have shown that the abnormal feeding rhythm associated with HFD consumption is caused by hypothalamic inflammation (9, 10). Hypothalamic microglia, which play a macrophage-like role in the brain, are activated by long-chain saturated fatty acids, such as palmitic acid, a component of HFDs (9, 10). Microglial activation in the hypothalamic arcuate nucleus (ARC), the center for the control of feeding behavior, interferes with the function of neurons involved in appetite regulation, which disrupts the feeding rhythm (9, 11). This sequence of events occurs within just a few days of starting the consumption of an HFD (11), and HFD consumption for several months causes chronic inflammation, characterized by activation of the NF-κB pathway (12, 13). Chronic inflammation in the ARC reduces neuronal sensitivity to nutrients and hormones (9, 14, 15), leading to the dysregulation of feeding behavior and impairs peripheral metabolism (16, 17). The prevention of neuroinflammation mediated by activated microglia has been shown to improve the altered feeding pattern and suppress obesity (9, 11, 13, 15, 18). Thus, hypothalamic inflammation plays a key role in the pathogenesis of HFD-induced obesity, and its suppression might have anti-obesity effects.

Both anti-obesity drugs and functional food materials have been developed for the treatment of obesity, in addition to exercise and diet therapy. These functional foods include dietary antioxidants, such as polyphenols, alkaloids, isothiocyanates, vitamins, and carotenoids, which have anti-obesity effects (19, 20). Of the polyphenols, epigallocatechin gallate (21) and kaempferol (22) have been shown to reduce the hypothalamic microglial inflammation induced by HFD consumption in mice. Cyanidin 3-*O*-glucoside (C3G) and its metabolite, protocatechuic acid, suppress lipopolysaccharide-induced microglial inflammation in the VB-2 microglial cell line (23, 24). In addition, myricetin has been reported to inhibit microglial activation in hypoxic VB-2 cells (25). These results indicate that polyphenols and polyphenol-rich food materials might have both anti-obesity and anti-inflammatory effects. However, no studies have investigated the effects of functional foods on the combination of hypothalamic inflammation, abnormal feeding rhythm, and obesity.

We have obtained a polyphenol-rich extract from the seed coat of black soybean (*Glycine max L*) (BE), which is rich in (+)-catechin, (−)-epicatechin, and their polymeric procyanidins (PCAs), as well as anthocyanins, principally C3G. Previous studies have shown that BE has various beneficial effects, including anti-hyperglycemic (26), antioxidant (27), and anti-obesity (28) effects; an improvement in vascular function (29, 30); and the prevention of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis (31). We have previously shown that BE increases the expression of uncoupling proteins, which are responsible for heat production in brown/beige adipose tissue, and has an anti-obesity effect (28). However, the effects of this preparation on the abnormal feeding patterns and hypothalamic inflammation of models of obesity have not been evaluated. The hypothalamus is a central regulator not only of feeding behavior but also of the metabolism of peripheral adipose tissue and skeletal muscle (32). Therefore, food ingredients that reduce obesity and hyperglycemia might achieve these effects through a suppression of hypothalamic inflammation. In the present study, we aimed to determine whether BE would prevent the effects of HFD consumption on the feeding pattern and hypothalamic inflammation of rodents. In this way, we aimed to identify novel potential mechanisms for the anti-obesity effects of BE and provide new evidence for the effects of polyphenols on the central nervous system (CNS).

Abbreviations: 3V, third ventricle; ARC, arcuate nucleus of the hypothalamus; AgRP, agouti-related protein; BBB, blood brain barrier; BE, black soybean seed coat (polyphenol-rich) extract; C3G, cyanidin 3-*O*-glucoside; CART, cocaine- and amphetamine-regulated transcript; CNS, central nervous system; Cx3cr1, CX3C-motif chemokine receptor 1; DIO, diet induced obesity; EC, epicatechin and catechin; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Gfap, glial fibrillary acidic protein; HFD, high fat diet; Hsp70, 70kDa heat shock protein; Iba1, ionized calcium-binding adapter molecule 1; Il-1b, interleukin 1 beta; Il-6, interleukin 6; IκBα, inhibitor of nuclear factor-kappa B alpha; LPS, Lipopolysaccharides; MBH, mediobasal hypothalamus; Mcp1, monocyte chemotactic protein 1; ME, median eminence; NF-κB, nuclear factor-kappa B; NPY, neuropeptide Y; PCA, procyanidin; POMC, Pro-opiomelanocortin; SCN, suprachiasmatic nucleus; SD, standard diet; Tnf-α, Tumor necrosis factor alpha; VMH, ventromedial hypothalamic nucleus.

Materials and methods

Chemicals and reagents

BE was obtained from Fujicco Co., Ltd. (Kobe, Japan). The polyphenol composition of this BE was 9.2% cyanidin 3-*O*-glucoside (C3G), 6.2 EC, and 39.7% PCAs, including 6.1 dimers, 3.4 trimers, 0.5% tetramers, and higher degree of polymerized PCAs, which are more polymerized than tetramers; determined using high-performance liquid-chromatography (HPLC) and expressed as mass/mass ratio as previously described (28, 33). Silica gel (Chromatorex; #PSQ100B) was purchased from Fuji Silysia Chemical Ltd. (Aichi, Japan). Tissue-Tek® Paraffin WaxII60 was purchased from Sakura Finetek Japan Co., Ltd. (Tokyo, Japan). Glass slides (Crest Coat; SCRE-01) were purchased from Matsunami Glass Ind., Ltd. (Osaka, Japan). Methanol (HPLC grade) and fatty acid-free bovine serum albumin (BSA) (#013-15143) were purchased from Fujifilm Wako Pure Chemical Co., Ltd. (Osaka, Japan). Fetal bovine serum (FBS) was purchased from BioWest S.A.S. (Nuaillé, France). Domitor® (1.0 mg/ml medetomidine hydrochloride) was purchased from Nippon Zenyaku Kogyo Co., Ltd. (Fukushima, Japan). Pentobarbital sodium salt (#P0776) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Blocking One (#03953-95) and Blocking One-P (# 05999-84) were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Primary antibodies for western blotting; anti-heat-shock protein 70 (Hsp70) rabbit polyclonal antibody (#4872), anti-β-actin rabbit polyclonal antibody (#4967), anti-nuclear factor-kappa B (NF-κB) p65 rabbit polyclonal antibody (#3034), anti-phospho-NF-κB p65 (Ser536) (93H1) rabbit monoclonal antibody (#3033), anti-inhibitor of nuclear factor-kappa B alpha (IκBα) rabbit polyclonal antibody (#9242), anti-phospho-IκBα (Ser32/36) (5A5) mouse monoclonal antibody (#9246), and anti-glial fibrillary acidic protein (GFAP) (5GA) mouse monoclonal antibody (#3670), and secondary antibodies for western blotting; goat anti-rabbit IgG horseradish peroxidase-linked antibody (#7074), and horse anti-mouse IgG horseradish peroxidase-linked antibody (#7076) were purchased from Cell Signaling Technology Co., Ltd. (Danvers, MA, USA). All the other reagents used were of the highest grade available from commercial sources.

Separation of C3G, flavan 3-ols monomers, and the polymer fraction of BE

First, to extract the C3G and flavan 3-ols from BE, 10 g of BE powder was suspended in 100 ml of 0.1% (w/v) HCl and subjected to extraction using double volume of ethyl acetate. This process was repeated four times. The aqueous layer containing C3G (C3G-rich fraction) and the ethyl acetate fraction containing the flavan 3-ols were obtained and dried *in vacuo*. The C3G-rich fraction was subjected to HPLC separation under the conditions described in the Section Separation and purification of C3G using HPLC, to obtain 98% (w/w) pure C3G, while the ethyl acetate fraction was subjected to a silica gel column chromatography (5 × 75 cm, 100 μm mesh silica gel, charged with chloroform/methanol =4:1) to obtain a fraction containing monomeric flavan 3-ols, catechin, and epicatechin; and another containing the dimeric and higher-degree of polymerized PCAs. Elution was performed with mobile phase consisting of chloroform/methanol =4:1, and eluate was collected 20 ml each in test tubes. Each fraction was checked by a thin layer chromatography using a silica gel 60 F254 (#105549; Merck, Darmstadt, Germany) and chloroform/methanol =4:1, alongside flavan 3-ol standards. To detect the compounds in each fraction, the plate was exposed to UV light at a wavelength of 254 nm. The eluates containing catechin and epicatechin were combined, and the solvent was evaporated by a rotary evaporator. Obtained fraction was referred to as the Epicatechin (EC)-rich fraction. The eluates containing procyanidin B2, procyanidin C1, and cinnamtannin A2 were also evaporated separately, and this fraction was referred to as the PCA-rich fraction. The amount of each flavan 3-ol in the fraction was measured using a HPLC method (33): the EC fraction contained 64.98% (−)-epicatechin and 2.57% (+)-catechin; while the PCA fraction contained 39.13% procyanidin B2, 20.74% procyanidin C1, and 8.97% cinnamtannin A2. The HPLC chromatogram of EC-rich, PCA-rich and C3G-rich fractions were described in Supplementary Figures S6, S7.

Separation and purification of C3G using HPLC

Preparative HPLC was performed using a Shimadzu LabSolutions system (Shimadzu, Kyoto, Japan), an SPD-M20A photodiode array detector, a CTO-20A column oven, a CBM-20A communications bus module, and an LC-20AD binary pump. HPLC separation was performed with a gradient system using 0.1% (v/v) trifluoroacetic acid in water as mobile phase A and 0.1% (v/v), trifluoroacetic acid in methanol as mobile phase B, a Tsk-gel ODS-80Ts (20 mm × 250 mm, 5 μm; #0018409; Tosoh Co., Ltd., Tokyo, Japan), and a flow rate of 8.0 ml/min. The injection volume was 500 μL and the temperature of the column oven was maintained at 40°C. C3G was separated using a linear gradient, commencing with 33% B over 0–22.5 min; then followed by 90% B over 22.5–35 min, and 33% B over 35–50 min, with elution between 16 and 22 min. The eluate was collected and evaporated to obtain purified-C3G. The gradient from 35 min onwards was used to re-equilibrate the system between samples. The absorbance of C3G was monitored at 280 nm and 513 nm using a UV detector. The HPLC chromatogram of purified-C3G was described in Supplementary Figure S7.

Animal experiments

The animal experiments were approved by the Institutional Animal Care and Use Committee (approval number: 2020-10-13) and performed in accordance with the Guidelines for Animal Experiments of Kobe University. Male, 5-week-old C57BL/6J mice were purchased from Japan SLC, Inc. (Shizuoka, Japan), and were kept in a temperature- and humidity-controlled room (temperature: 23 ± 2°C, humidity: 50 ± 10%) under a 12:12–h light/dark cycle (lights on at 08:00). The mouse cages used were SEALSAFE® GM500 (Tecniplast Co. Ltd., West Chester, PA, USA) with 501 cm^2 floor area. Each mouse was housed in an individual cage. The mice were acclimatized to their environment for 1 week, with free access to standard diet [SD, containing 3.85 kcal/g, 10.0% kcal from fat (4.4 lard and 5.6% soybean oil), 20.0% kcal from protein, and 70.0% kcal from carbohydrates; #D12450J; Research Diets, Inc., New Brunswick, NJ, USA] and tap water. Their body weight and food intake were recorded periodically, at 08:00 (when the lights were turned on) or at 20:00 (when the lights were turned off), during the feeding period. The mice were used in the following two experiments and were fasted for 1 h before the sacrifice.

Experiment 1: Ninety-six mice were allocated to three groups of thirty-two and were fed an SD (#D12450J; Research Diets, Inc., New Brunswick, NJ, USA), a high-fat diet [HFD, containing 5.24 kcal/g, 60.0% kcal from fat (54.4% lard, 5.6% soybean oil), 20.0% kcal from protein, and 10.0% kcal from carbohydrates; #D12492; Research Diets, Inc.], or the HFD supplemented with 2.0% (w/w) BE. Pellet-type SD and HFD were pulverized and gave animals. BE was mixed with powdered HFD as outer percentage. Each group was further divided into four sub-groups of eight each for 3-day, 1-, 2-, and 4-week groups. All mice were housed individually, and mice in the 4-week group were used for measurement of food intake. Five mice of each group were euthanized by exsanguination *via* cardiac puncture, and remaining three mice by systemic perfusion with 4% (w/v) paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for immunohistochemical experiments, under anesthesia through an intraperitoneal injection of a mixture of sodium pentobarbital (65 mg/kg) as an anesthetic and medetomidine hydrochloride (0.3 mg/kg) as an analgesic. To obtain the hypothalamus, the collected brain was cut out with a range of bregma −1.00 to −2.50 using a rodent brain matrix (#RBM-2000C; Applied Scientific Instrumentation Inc., Eugene, OR, USA) on ice, under a binocular stereomicroscope. For immunohistochemistry, the cut-out coronal brain section was pre-fixed with 4% PFA-PBS; for total RNA and protein isolation, the hypothalamic block was obtained from the coronal section with the range of lateral −1.00 to 1.00 and interaural −0.50 to 2.00 using a scalpel. The hypothalamic block was stabilized in RNAlater® (#R0901; Sigma-Aldrich, St Louis, MO, USA) and stored at 4°C, and total RNA and proteins were isolated within 1 week. The liver and adipose tissues were collected, weighed, and then immediately placed at −80°C.

Experiment 2: Alternatively, thirty mice were allocated to five groups of six each, which were fed SD, HFD, and HFD containing 0.5% EC, 0.5% PCA, and 0.5% C3G for 4 weeks. Each component was mixed with the powdered diet as outer percentage and gave animals. At the end of the study, the mice were systemically perfused with 4% PFA in PBS under anesthesia as the same methods as ***Experiment 1***, and the brain was collected.

Measurement of food intake

As for the feeding of diets, powdered diets (5.0 g/day/mice) were placed into a powder feeder (#MF-3S; Shin Factory Co., Ltd., Fukuoka, Japan) and replaced to fresh ones every 2 or 3 days. The amount of remained diets was weighed and calculated the food intake at the timing of each replacement. On days 0, 1, 3, 7, 14, 21, and 28 of the feedings, the food intake was measured during the 12 h of the light (inactive) and dark (active) periods.

Isolation of RNA and RT-qPCR

RNA was isolated from hypothalamic blocks stored in RNA*later* using TRIzol™ Reagent (#15596018; Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. The quality and concentration of the RNA obtained were measured by spectrophotometry using a NanoDrop™ ND-1,000 spectrophotometer (Thermo Fisher Scientific Co., Ltd., Waltham, MA, USA.). The RNA samples were purified by digesting the residual DNA using DNase I recombinant (#4716728001; Roche, Basel, Switzerland) following the manufacturer's instructions. The DNase-treated RNA was reverse transcribed to cDNA using ReverTra Ace® (#TRT-101; Toyobo Co., Ltd.). cDNA was then subjected to RT-qPCR amplification using TB Green® Premix Ex Taq™ II (#RR820; Takara Bio, Kusatsu, Japan). The primer sequences used are listed in Supplementary Table S1. Real-time PCR reactions were performed using a TaKaRa PCR Thermal Cycler Dice® Real Time System II (#TP900; Takara Bio). Relative gene expression was calculated using the comparative CT method (34), using *Gapdh* as the reference gene. The results are expressed as fold-differences from the expression level of mice in the SD group.

Protein isolation from hypothalamic blocks

Hypothalamic protein was isolated from hypothalamic blocks stored in RNA*later*® using TRIzol™ Reagent, following the manufacturer's instructions. RNA and protein were isolated from the same mouse hypothalamic blocks. Briefly, 2-propanol was added to the phenol-containing layer to precipitate the

protein, and the protein pellet was thoroughly washed with 95% ethanol containing 0.3 M guanidine hydrochloride. The obtained protein pellet was homogenized with RIPA buffer [50 mM Tris, pH8.0, containing 150 mM sodium chloride, 1% (v/v) Non-idet P-40 (NP-40), 0.5% (w/v) deoxycholic acid, 0.1% (w/v) sodium dodecyl sulfate and 0.5 mM dithiothreitol] using a Misonix™ Microson™ XL-2,000 ultrasonic homogenizer (Qsonica Co., Newtown, CT, USA). The homogenates were incubated on ice for 60 min, with occasional mixing, and then centrifuged at 12,000 × g for 20 min at 4°C. The supernatants were used as the protein lysates.

Western blotting analysis

The protein concentrations of the obtained protein lysates were quantified using Lowry's method (35). Following this, the lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 10% gels for the detection of Hsp70, β-actin, NF-κB p65, p-NF-κB p65 and GFAP; and 15% gels for the detection of IκBα and p-IκBα. The separated proteins were transferred onto Immobilon® -P polyvinylidene difluoride membranes (#IPVH00010; Merck Millipore Ltd., Billerica, MA, USA), which were incubated with blocking solutions [Blocking One for unphosphorylated proteins and Blocking One-P for phosphoproteins), then treated with primary antibodies (1:5,000) in Can Get Signal® solution 1 (#NKB-101; Toyobo Co., Ltd., Osaka, Japan)] overnight at 4°C, and with the corresponding horseradish peroxidase-conjugated secondary antibody (1:50,000) in Can Get Signal® solution 2 for 1 h at room temperature. Protein bands were visualized using ImmunoStar® LD (#290-69904; Fujifilm Wako Pure Chemical, Osaka, Japan) and detected using LuminoGraphI (#WSE-6100; ATTO, Tokyo, Japan). Densitometric quantification of specific band was performed using ImageJ software (NIH, Bethesda, MD, USA).

Immunohistochemistry

The anesthetized mice were perfused with 4% PFA in PBS for 1 h, and then their brains were collected and post-fixed in 4% PFA in PBS for 48 h at room temperature, before being processed for embedding in paraffin. Hypothalamic coronal sections (10-μm-thick) were prepared using a microtome (#PR-50; Yamato Kohki Industrial Co., Ltd., Asaka, Japan), deparaffinized, and rehydrated using standard techniques. Antigen retrieval was performed using 10 mM sodium citrate buffer (pH 6.0) at 95°C for 30 min. The sections were blocked with 10% (v/v) heat-inactivated FBS, 5% (w/v) BSA, and 0.1% (w/v) sodium azide in PBS containing 0.1% (v/v) Triton X-100 for 1 h at room temperature, and then incubated with primary antibody in PBS containing 0.05% (v/v) Tween-20 and 3% (w/v) BSA overnight at 4°C. Subsequently, the sections were washed three times with PBS containing 0.05% (v/v) Tween-20 and incubated with secondary antibody in the same solution as the primary antibody for 3 h at 4°C. The nuclei were counterstained with 10 μg/ml 4',6-diamidino-2-phenylindole. For the detection of Iba1 in the hypothalamic arcuate nucleus, a rabbit monoclonal [HL22] primary antibody against Iba1 (1:200; #GTX100042; GeneTex, Irvine, CA, USA) and an Alexa Fluor™ 488-conjugated goat anti-rabbit IgG (1:1,000; #A-11008; Invitrogen) were used. For the detection of GFAP, a mouse monoclonal primary antibody (GA5) against GFAP (1:1000; #3670; Cell Signaling Technology) and an Alexa Fluor™ 594-conjugated goat anti-mouse IgG (1:1,000; #A-11005; Invitrogen) were used. Images were acquired using a fluorescence microscope (#FSX100; Olympus, Tokyo, Japan). The numbers of cells that were immunoreactive for Iba1 on matched sections within prespecified regions of interest in the ARC (bregma −1.40 to −1.70 mm) of each mouse were determined using ImageJ software. To count the number of immunoreactive cells, the threshold was defined as the intensity at which the cells were clearly immunoreactive to Iba1 on visual inspection, and then they were counted manually and the mean values for three sections per mouse were calculated. The microglial cell (the cell type that is immunoreactive to Iba1) size was measured using a thresholding protocol (ImageJ), which was followed by densitometric quantification, performed according to the previously published method (36).

Statistical analysis

Statistical analysis was performed using JMP statistical software, version 11.2.0. (SAS Institute, Cary, NC, USA). Data are presented as the mean and standard error (SE). Tukey-Kramer honestly significant difference test was performed, and $p < 0.05$ was considered to the represent statistical significance indicated by the different letters in each figure.

Results

BE reduces the accumulation of hypothalamic activated microglia and the gliosis induced by HFD consumption

The number of microglia was significantly higher at all of the time points in the HFD-fed group than in the SD-fed group, and the microglia cell size was also significantly larger 2-week after the feedings (Figures 1A–C; Supplementary Figure S1A). These findings are consistent with those of previous studies (9, 10). BE reduced the HFD-induced increases in the number

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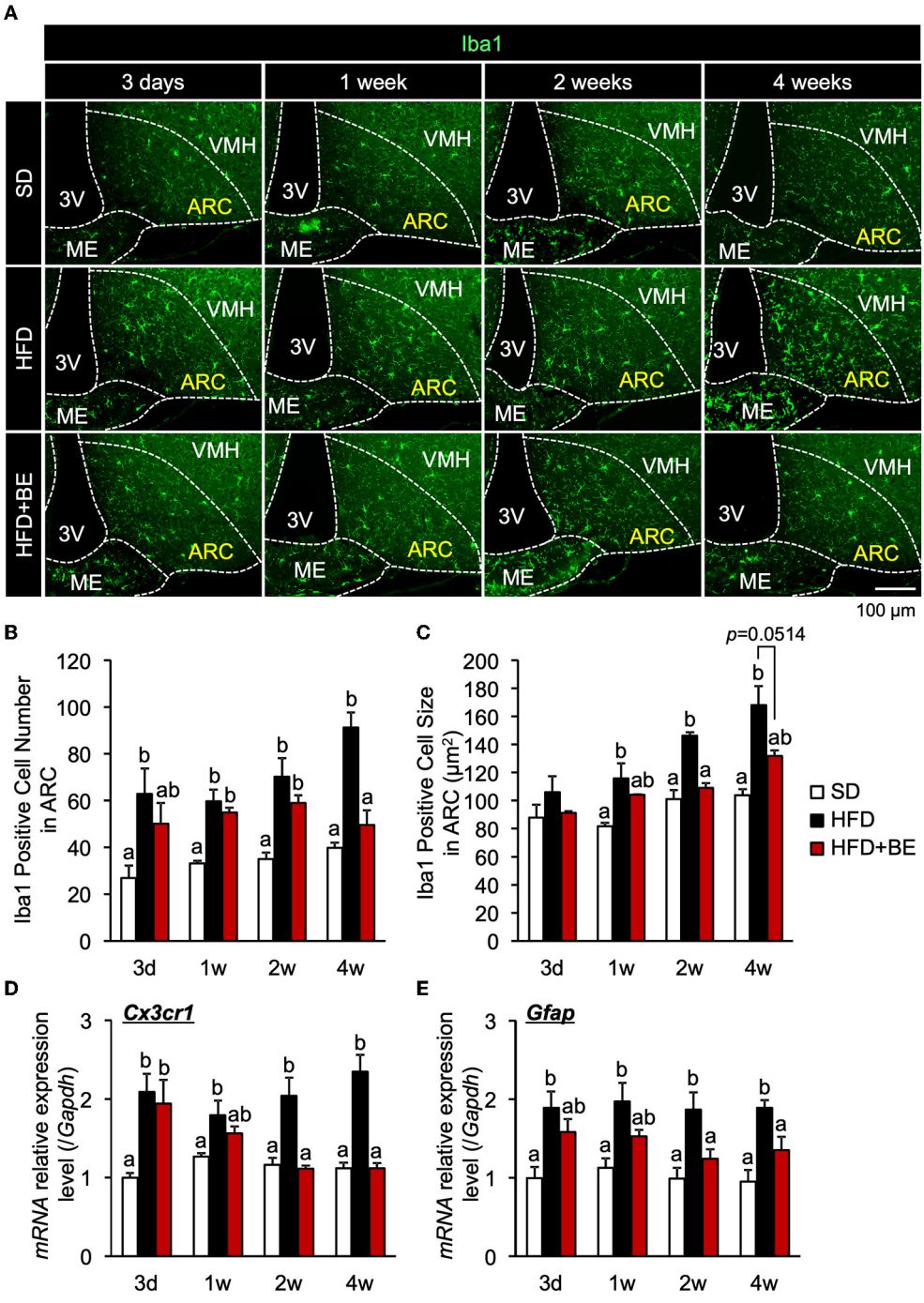

FIGURE 1
Effects of BE on HFD-induced hypothalamic gliosis in mice. **(A)** Sections of the mediobasal hypothalamus (MBH) of mice (10-μm-thick) were immunofluorescence-stained for Iba1 on Day 3 (3d), Week 1 (1w), Week 2 (2w), and Week 4 (4w) of the feedings. The third ventricle (3V), median eminence (ME), hypothalamic arcuate nucleus (ARC), and ventromedial hypothalamic nucleus (VMH) of the left side of the brain of mice in each group are shown. **(B,C)** Number and size of Iba1-positive cells, respectively, in the ARC. The cell number and size on sections through the ARC of the left and right hemisphere of the brain were measured and mean values calculated. $n = 3$. **(D,E)** Quantitative RT-PCR analysis showing high mRNA expression of gliosis marker, **(D)** *CX3C chemokine receptor 1* (*Cx3cr1*) and **(E)** *Glial fibrillary acidic protein* (*Gfap*), in the whole hypothalamus of mice on Day 3, Week 1, Week 2, and Week 4 of the study. Data shown are mean ± SE ($n = 5$). Different letters represent significant differences among the three groups at each experimental period by Tukey-Kramer honestly significant difference test ($p < 0.05$).

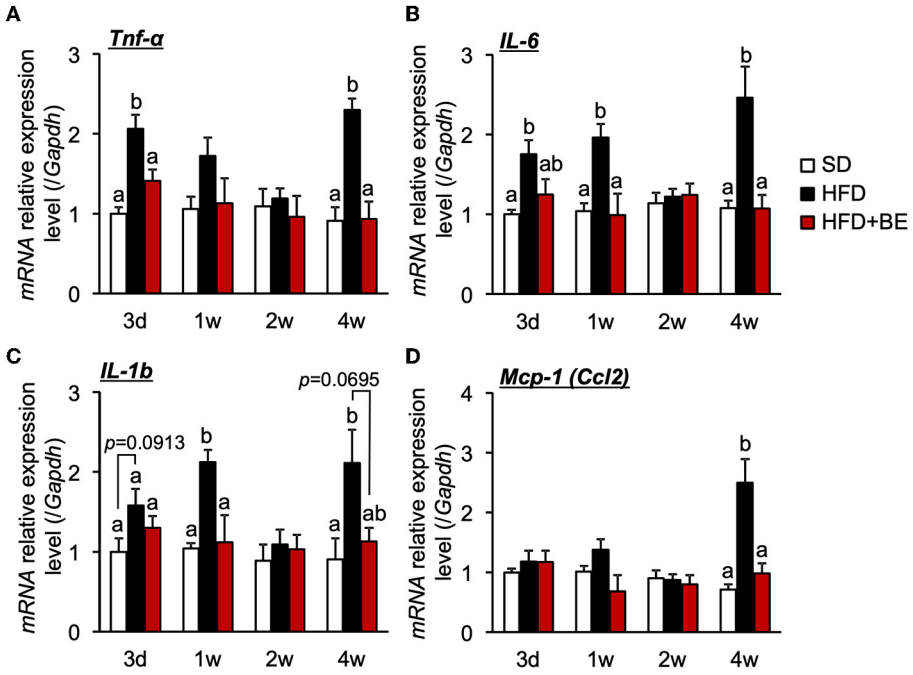

FIGURE 2
Effects of and HFD and BE on hypothalamic pro-inflammatory cytokine and chemokine gene expression. Quantitative RT-PCR analysis, showing high expression of genes encoding pro-inflammatory cytokines **(A)** *Tnf-α*, **(B)** *Il-6*, **(C)** *Il-1b* and a chemokine **(D)** *Mcp-1* in the whole hypothalamus of mice on Day 3 (3d), Week 1 (1w), Week 2 (2w), and Week 4 (4w) of the feedings. Data shown are mean ± SE ($n = 5$). Different letters represent significant differences among the three groups at each experimental period by Tukey-Kramer honestly significant difference test ($p < 0.05$).

and size of microglia at all the time points (Figures 1A–C; Supplementary Figure S1A). The expression of *CX3C chemokine receptor 1* (*Cx3cr1*; specific to activated microglia) and *Gfap* (specific to activated astrocytes), which is associated with the development of hypothalamic gliosis (9, 10, 15), was significantly higher in the HFD-fed mice than that in the SD-fed mice throughout the experiment (Figures 1D,E). In HFD+BE-fed mice, *Cx3cr1* expression was significantly higher than that in SD-fed mice until day 3, after which it returned to the level of SD-fed mice. In addition, HFD+BE-fed mice showed no significant difference in *Gfap* expression from that of SD-fed mice throughout the feeding period (Figures 1D,E). On the other hand, the visual morphology of GFAP-immunoreactive cells (astrocytes) in ARC and the expression levels of GFAP 4 weeks after the feedings were not different among the three groups (Supplementary Figures S2A,B). These results indicate that BE ameliorates the HFD-induced accumulation of activated microglia and the development of gliosis in the ARC.

BE reduces pro-inflammatory cytokine gene expression in the hypothalamus

Hypothalamic pro-inflammatory cytokine gene expression correlates with the degree of hypothalamic gliosis (9, 10, 15). Therefore, in the present study, we determined the effect of BE on the expression of genes encoding inflammatory cytokines. We found that HFD-feeding significantly increased the expression of hypothalamic *Tnf-α* on Day 3 (Figure 2A), and that of *Il-6* and *Il-1b* after 1 week (Figures 2B,C). The expression of these mRNAs decreased to the same level as that of SD-fed mice 2 weeks after the feedings, but was again high 4 weeks after the feedings. (Figures 2A–C). BE consumption prevented the HFD-induced increase in pro-inflammatory cytokines expression during the first week and caused a significant reduction in expression vs. the HFD-fed mice at the 4-week time point (Figures 2A–C). BE also prevented the HFD-induced increase in the expression of *Mcp-1* (encoding a chemokine) 4 weeks after the feedings (Figure 2D). Long-term HFD-intake induces inflammation in peripheral, especially visceral, adipose tissue (37). We also found that HFD significantly increased *Il-1b* expression in epididymal fat 4 weeks after the feedings, and supplementation of BE suppressed an increase in the expression of this inflammation marker (Supplementary Figure S3). Contrary, The *Il-1b* expression did not alter on Day 3. These results indicate that BE ameliorates the HFD-caused hypothalamic inflammation induced within 3 days, though it needs long-term to reduce inflammation in peripheral tissues. The inhibition of the acute hypothalamic inflammatory response might have prevented the chronic

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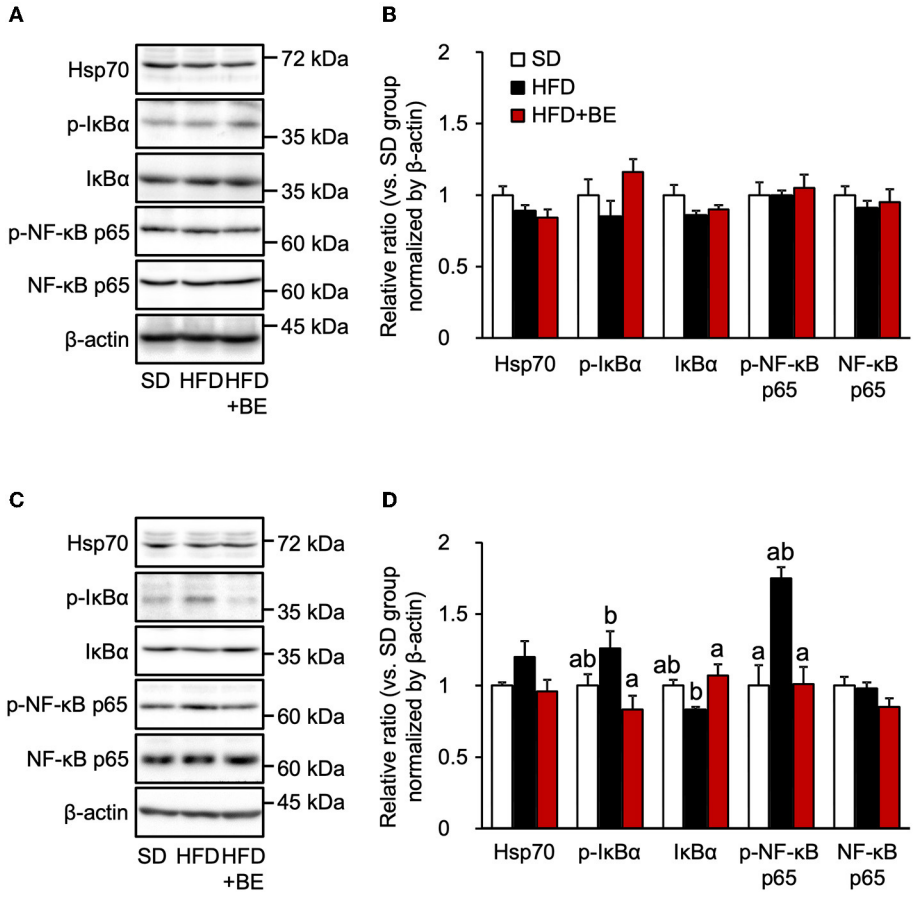

FIGURE 3
Western blotting analysis of the effects of BE on the expression of proteins involved in the NF-κB pathway. **(A,C)** Expression of 70 kDa heat shock protein (Hsp70), phosphorylated inhibitor of nuclear factor-kappa B alpha (IκBα), IκBα, phosphorylated nuclear factor kappa-B p65 subunit (NF-κB p65), NF-κB p65 and β-actin in the whole hypothalamus extracts of mice on **(A)** Day 3 (3d) and **(C)** Week 4 (4w) of the feedings. **(B,D)** Quantification of the expression levels of proteins in the whole hypothalamic fractions shown in **(A,C)**, respectively. Data shown are mean ± SE ($n = 5$). Different letters represent significant differences among the three groups at each experimental period by Tukey-Kramer honestly significant difference test ($p < 0.05$).

hypothalamic inflammation that is established 4 weeks after the feedings.

BE reduces the HFD-induced activation of the NF-κB pathway

The persistent production of proinflammatory cytokines in the hypothalamus leads to chronic inflammation, characterized by an upregulation of NF-κB transcriptional activity and neuronal stress (12, 13). The nuclear localization signal of NF-κB is usually masked by IκBα, to maintain it in an inactive state, but in response to pro-inflammatory cytokines or other stresses, IκBα is phosphorylated and proteasomally degraded, which permits the phosphorylation in cytoplasm and translocation of NF-κB to the nucleus, where it causes the transcription of genes encoding pro-inflammatory cytokines (38). In the present study, phosphorylation of NF-κB p65 and IκBα was observed 4 weeks after the feedings, though the expression level of IκBα was reduced 4 weeks after the feedings of HFD, as the same time point as the hypothalamic gliosis and inflammation were occurred (Figures 3C,D). Supplementation of BE completely canceled these alterations. The expression of NF-κB and Hsp70, which inhibits NF-κB in response to neuronal stress (39), did not significantly differ among the three groups at any of the time points (Figures 3A–D). These findings imply that the activation NF-κB pathway is involved in the increase in pro-inflammatory cytokine and chemokine gene expression after intake of HFD for long-term, whereas it did not associate with the increase in *Tnf-α* expression observed on Day 3. Thus, the intake of BE quickly reduced the expression of pro-inflammatory cytokine genes, thereby preventing chronic inflammation.

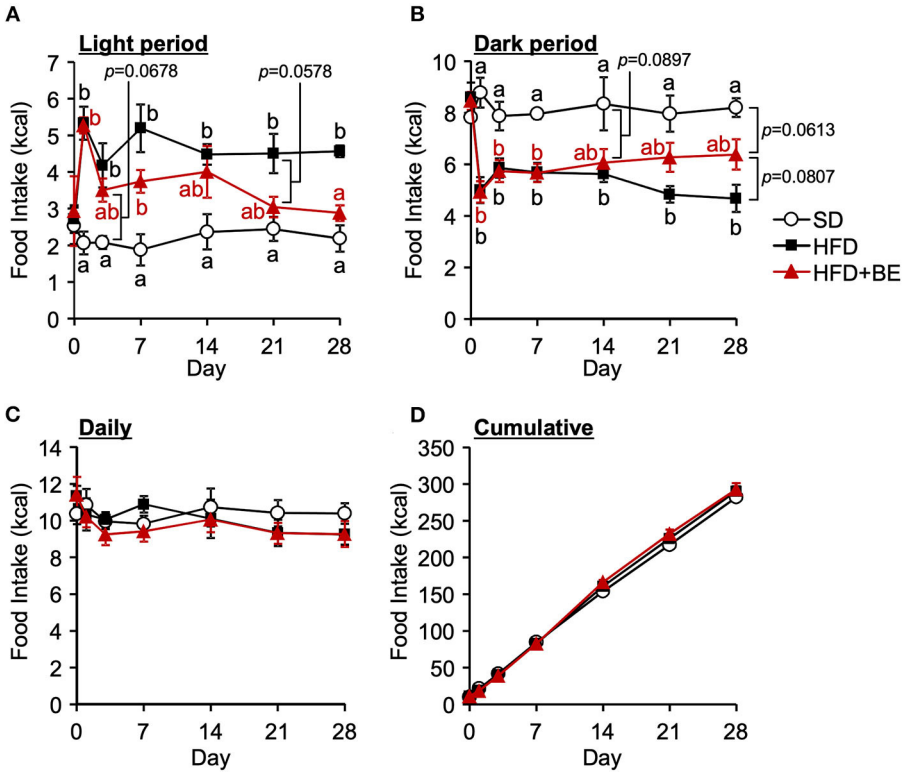

FIGURE 4
Effects of HFD and BE on the feeding pattern of mice. Food intake during **(A)** the light and **(B)** the dark periods; and **(C)** the daily and **(D)** cumulative food intake during the study period. Data shown are mean ± SE ($n = 5$). Different letters represent significant differences among the three groups by each experimental period by Tukey-Kramer honestly significant difference test ($p < 0.05$).

BE ameliorates the abnormal feeding pattern and obesity induced by HFD consumption

Next, we determined whether BE would improve the abnormal feeding pattern. HFD-fed mice significantly increased food intake during the light period and lowered the intake during the dark period from the start of the experiment compared with the SD-fed mice, in which exhibited a robust circadian feeding pattern, with 70–80% of their feeding occurring during the dark period (Figures 4A,B). The BE-fed mice exhibited the same abnormal feeding pattern as the HFD-fed mice until Day 14, but the rhythm was the same as that of the SD group by Day 28 (Figures 4A,B). However, no significant differences were observed in the daily or cumulative food intake among the groups (Figures 4C,D). These differences in feeding pattern were associated with microglial accumulation and the development of gliosis in the ARC. Furthermore, the body and white adipose tissue weights of HFD group were significantly higher between Day 14 and Day 28 of the feedings than those of the SD group. BE inhibited the above deterioration of body composition caused by HFD (Figures 5A–F). However, the brown adipose tissue and liver weight did not differ among the groups (Figures 5G,H). As for plasma lipids, HFD increased the level of non-esterified fatty acids, in particular 1 and 4 weeks after the feedings and BE tended to reduce it; both HFD and HFD+BE decreased the triacylglycerol level; and HFD increased the total cholesterol level 3 days and 4 weeks after the feedings and BE did not lower the HFD-increased total cholesterol level (Supplementary Figure S4). These results suggest that HFD-induced obesity is accompanied by a disruption of the normal circadian feeding pattern, but not necessarily any differences in daily or cumulative energy intake, and that BE ameliorates the effects of an HFD on feeding pattern and prevents obesity.

C3G reduces the obesity and hypothalamic inflammation induced by HFD consumption

We next attempted to identify the active compound in BE that is responsible for its beneficial effects on the HFD-induced hypothalamic inflammation, abnormality in feeding pattern, and obesity. The EC, PCA, and C3G fractions

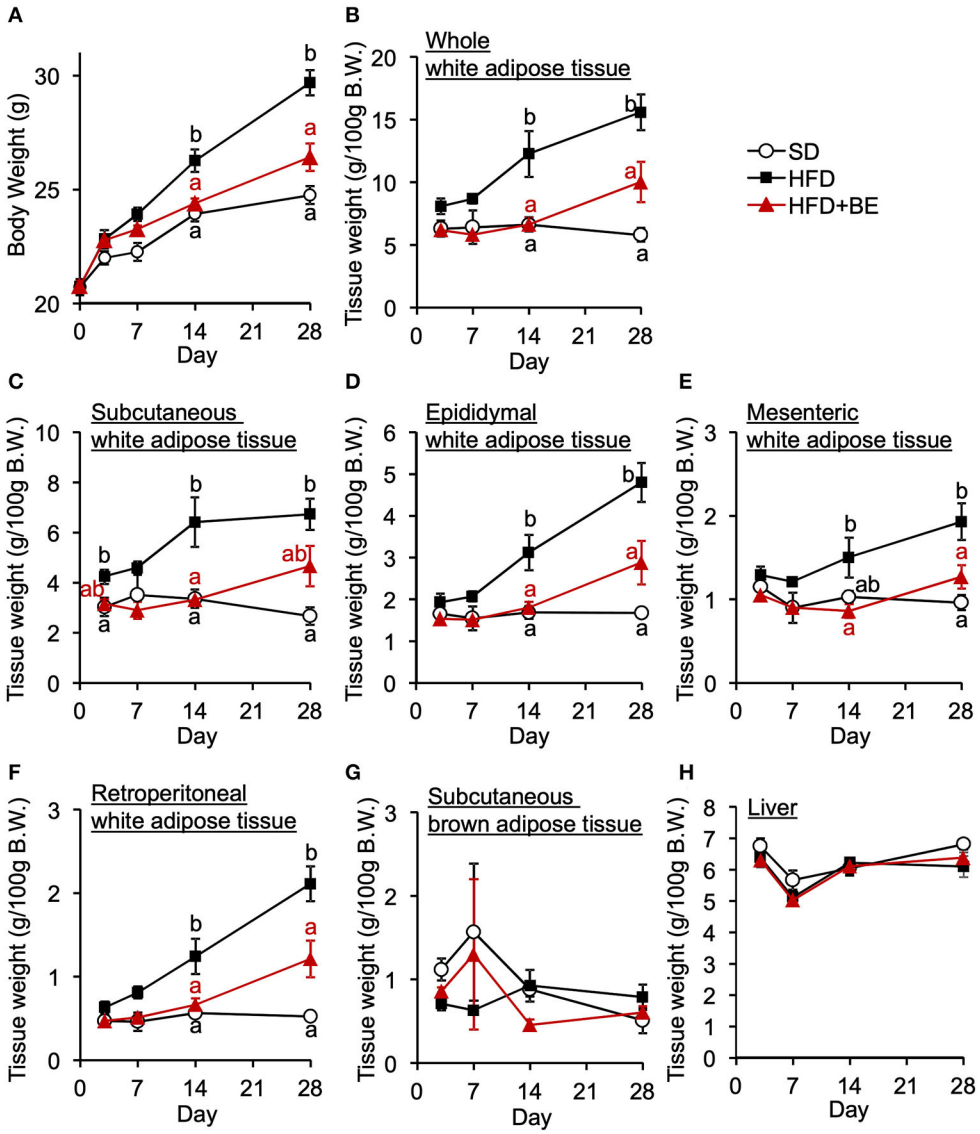

FIGURE 5
Effects of HFD and BE on the gains in body and fat weight. **(A)** Changes in body weight in each group during the 4-week experimental period. $n = 8$. Weight of **(B)** total white adipose tissue depots, **(C)** subcutaneous white adipose tissue, **(D)** epididymal white adipose tissue, **(E)** mesenteric white adipose tissue, **(F)** retroperitoneal white adipose tissue, and **(G)** subcutaneous brown adipose tissue at each experimental period. $n = 5$. **(H)** Liver weight at each experimental period. $n = 5$. Data shown are mean ± SE. Different letters represent significant differences among the three groups by each experimental period by Tukey-Kramer honestly significant difference test ($p < 0.05$).

were prepared from BE and separately given to mice for 4 weeks. Of these, only C3G significantly reduced the HFD-induced hypothalamic microglial accumulation (Figures 6A–C; Supplementary Figure S1B). C3G also tended to reduce the body weight gain induced by HFD consumption (Figure 7A) and reduced the light period hyperphagia (Figure 7B). The consumption of the EC or PCA fractions failed to prevent the weight gain or the abnormal feeding pattern induced by the HFD (Figures 7A–C). No differences were found in the daily or cumulative food intake among the groups (Figures 7D,E). These results suggest that C3G is the active compound in BE that is principally responsible for the prevention of the chronic inflammation in the ARC induced by HFD consumption and that it reduces obesity by preventing the effects of HFD on the circadian feeding pattern.

Discussion

The consumption of HFD causes obesity not only through high calorie intake but also by disturbing the normal feeding

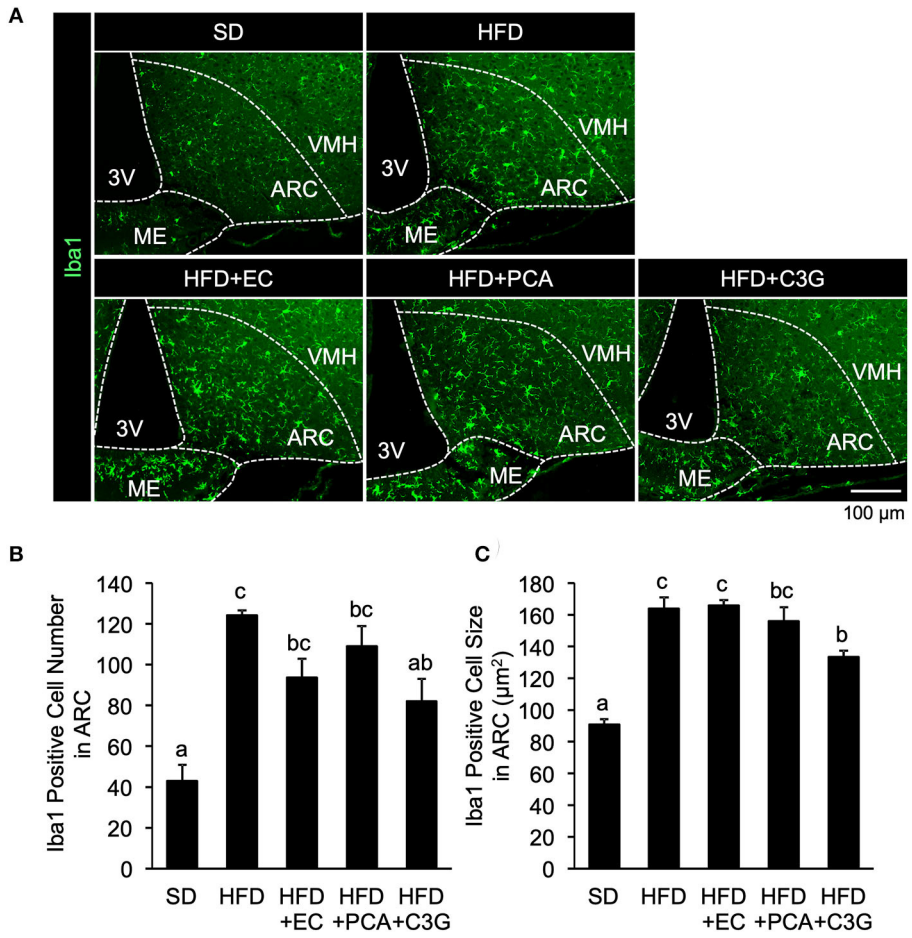

FIGURE 6
Effects of three fractions derived from BE on the HFD-induced hypothalamic gliosis of mice. **(A)** Sections of the mediobasal hypothalamus (10-μm-thick), immunofluorescence-stained for ionized calcium-binding adapter molecule 1 (Iba1) in mice fed standard diet (SD), high-fat diet (HFD), HFD+EC, HFD+PCA, or HFD+C3G for 4 weeks. The third ventricle (3V), median eminence (ME), hypothalamic arcuate nucleus (ARC), and ventromedial hypothalamic nucleus (VMH) of the left side of the brain of mice from each group are shown. Quantification of **(B)** the number and **(C)** size of Iba1-positive cells in the ARC. The cell number and size in the ARC of the left and right hemispheres of the brain were measured and mean values were calculated. Data shown are mean ± SE ($n = 3$). Different letters represent significant differences among the three groups by each experimental period by Tukey-Kramer honestly significant difference test ($p < 0.05$).

pattern. HFD consumption has been reported to rapidly induce a disruption in feeding rhythm, which is accompanied by hypothalamic inflammation, before significant increases in body or adipose tissue weight (11). Because these acute pathological defects, i.e., feeding rhythm disruption and hypothalamic inflammation, likely precede systemic metabolic disruption, suppression of pathological defects may represent an effective approach to the prevention of DIO. In the present study, we evaluated the effects of BE on the abnormal feeding pattern and hypothalamic inflammation induced by HFD-feeding, which precede HFD-induced obesity. We found that BE reduces HFD-caused pro-inflammatory cytokines expression (Figure 2) and prevents the development of chronic inflammation, characterized by activation of the NF-κB pathway 4 weeks after the feedings (Figure 3). BE ingestion reduces HFD-induced activated microglial accumulation in the ARC (Figure 1) and restores the normal feeding pattern (Figure 4), thereby preventing the marked weight gain induced by HFD (Figure 5). In addition, we have shown that C3G is an active compound in BE and principally mediates these effects (Figures 6, 7). This is the first study to show that a functional food material has an anti-obesity effect by ameliorating the hypothalamic inflammation and restoring the abnormal feeding pattern associated with DIO. Therefore, the results of the present study provide new insight into the anti-obesity effects of BE and its components.

HFD-induced hypothalamic inflammation impairs normal neurotransmission, resulting in a disruption to feeding rhythm (11). Within a week of HFD intake, the activation of microglia disrupts neurotransmission, in the form of excessive glutamate release from gap-junction hemichannels on the cell surface

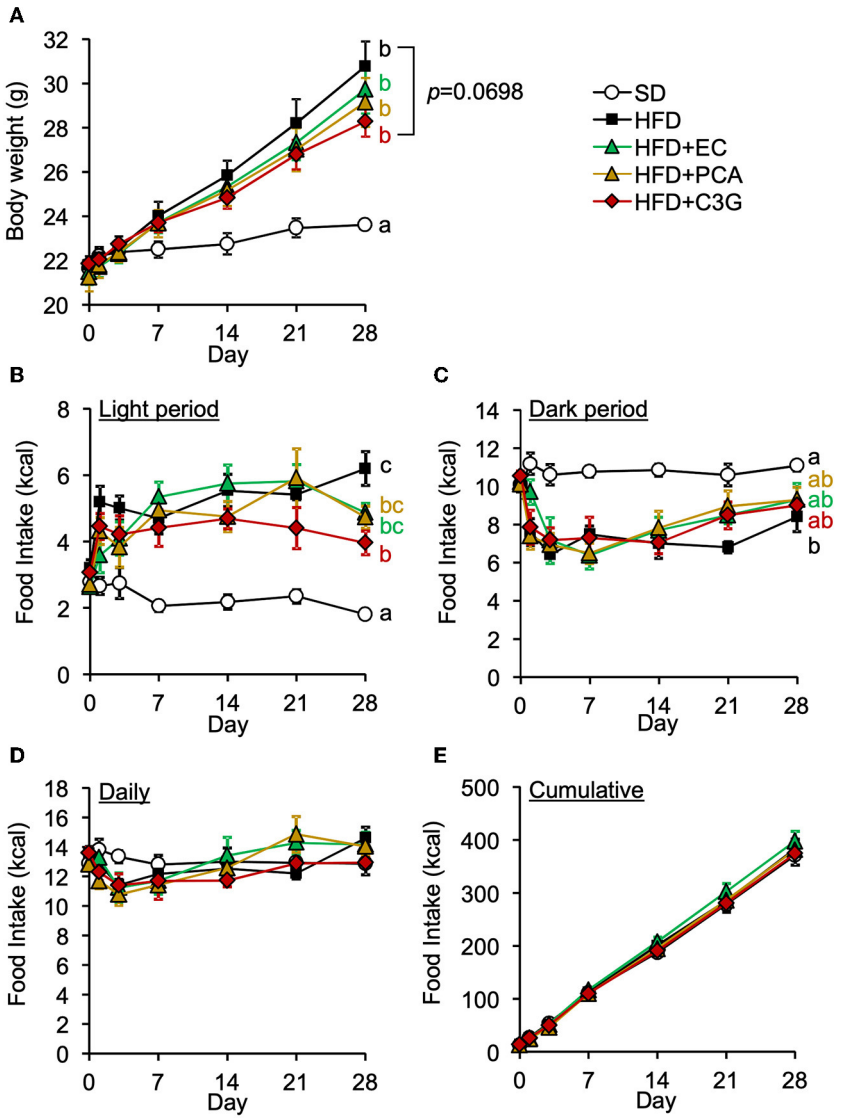

FIGURE 7
Effects of three fractions derived from BE on the HFD-induced body weight gain and abnormal feeding pattern of mice. **(A)** Changes in body weight in each group during the 4-week study period. Food intake during **(B)** the light and **(C)** dark periods; and **(D)** daily and **(E)** cumulative food intake during the experimental period. Data shown are mean ± SE ($n = 6$). Different letters represent significant differences among the five groups by each experimental period by Tukey-Kramer honestly significant difference test ($p < 0.05$). The results of statistical analysis are shown only at the end points and the statistical data for the other time points are listed in Supplementary Table S2.

(40), and this activation lead to the activation of microglia and astrocytes accompanied by the release pro-inflammatory cytokines (9, 10), which recruit peripheral myeloid cells to the ARC, leading to chronic inflammation, characterized by the activation of the NF-κB pathway (10, 13) and persistent neurological dysfunction, and subsequent disruption of the feeding rhythm and induction of obesity. In the present study, BE and C3G had beneficial effects on the abnormal feeding pattern induced by HFD from 2 weeks (Figures 4, 7). The inhibition of neurotransmission *via* the microglial gap-junction hemichannel pathway is transient (11, 40), occurring within the first few days of HFD-consumption. This suggests that BE does not reduce the activation of the microglial gap junction hemichannel pathway. However, BE attenuated the HFD-induced increase in hypothalamic pro-inflammatory cytokine gene expression at all of the time points during the feeding periods (Figure 2). This implies that the inhibition of inflammatory cytokine signaling is involved in the amelioration of the HFD-induced abnormality in feeding pattern by BE and C3G.

The decline in the neurological function of the hypothalamus that is caused by HFD-induced inflammation

(9, 13) induces a loss of feeding rhythm and obesity (41, 42). In the present study, BE consumption attenuated the reduction in hypothalamic *Pro-opiomelanocortin* (*Pomc)* expression caused 4 weeks after HFD-intake (Supplementary Figure S5). In the ARC, anorexigenic agouti-related peptide/neuro peptide Y (AgRP/NPY)-expressing neurons and orexigenic POMC/cocaine- and amphetamine-regulated transcript (CART)-expressing neurons sense systemic metabolic status by monitoring the concentrations of hormones and nutrients, delivered *via* the circulation or visceral sensory nerves, and precisely regulate feeding behavior (43). Recent studies using the designer receptor exclusively activated by designer drugs (also known as DREADD) system have shown that HFD consumption reduces the responsiveness of POMC- and AgRP-expressing neurons (44–46). In addition, chronic HFD consumption has been shown to induce the specific apoptosis of POMC-expressing neurons in the ARC (10). These neurons regulate feeding behavior and peripheral metabolism in response to leptin (47, 48), a satiety hormone that is released by adipose tissue. We found that BE ameliorates the loss of leptin sensitivity in the ARC that is induced by an HFD (data not shown). These findings may imply that BE ameliorates the neuronal hypersensitivity, abnormal feeding pattern, and systemic metabolic dysfunction through the suppression of hypothalamic inflammation.

Sustained abnormalities in feeding pattern ultimately disrupt the circadian rhythms of peripheral organs, causing metabolic dysfunction. The circadian rhythm of the suprachiasmatic nucleus (SCN) of the hypothalamus, the central clock that controls the systemic circadian rhythm, is reset by light arriving at the retina and is not affected by feeding. However, the feeding rhythm is disrupted within a day of the start of HFD consumption, which is earlier than disruptions occur in peripheral organs. This implies that the feeding rhythm is more strongly influenced by meals than the SCN and that the disruption of the feeding rhythm underpins HFD-induced obesity. In the present study, the total energy intake of each group was similar (Figures 4D, 7E), but only the HFD-fed mice, which exhibited an abnormal feeding pattern, showed significant weight gain (Figures 4A,B, 7B,C). Previous studies have shown that oral administration of D-allulose or pectin-containing carbonated water ameliorates obesity in mice through suppressing HFD-caused light period hyperphagia (49, 50). In fact, previous studies have shown that the restriction of HFD intake during the light phase alone prevents obesity and disruption to the circadian rhythm in peripheral tissues (3, 51, 52). These findings suggest that the keeping of an appropriate feeding rhythm is essential for the maintenance of metabolic homeostasis and the circadian rhythm.

In the present study, we found that C3G was the most effective compound for preventing HFD-induced hypothalamic inflammation and feeding rhythm disruption. The principal mechanism underpinning the anti-inflammatory effect of C3G has previously been shown to be a reduction in the oxidative stress induced by lipopolysaccharide (LPS) or saturated long-chain fatty acids, upstream of the NF-κB pathway (53, 54). C3G and its metabolites have been reported to inhibit the LPS- or saturated long-chain fatty acids-induced activation of NF-κB pathway and expression of pro-inflammatory cytokines in the cultured microglial cells (24, 55–60) and the cells in peripheral tissues. Furthermore, the intake of C3G-rich meals decreases the expression level of pro-inflammatory cytokines in the peripheral tissues of mice with DIO (59, 61). One possible mechanism for the inhibitory effect of C3G on hypothalamic inflammation is that C3G or its metabolites enter the hypothalamus and possess direct antioxidant effects. However, orally administered C3G is quickly metabolized to cyanidin, protocatechuic acid, phenolic acids, and phloroglucinol aldehyde, and only ∼12.4% is absorbed in its intact form (23, 62, 63). Nevertheless, C3G and its metabolites have been detected in various organs, including the hypothalamus (64). In addition, when rats feed C3G-enriched diets for ∼2 weeks, the concentrations of C3G and its metabolites in the brain are higher than those in the plasma (64, 65), suggesting that C3G and its metabolites may accumulate in the brain. Because C3G and its metabolites have been shown to be able to cross the blood-brain barrier (BBB) (62) and part of the ARC lacking area of BBB (66), these compounds may be able to access the ARC *via* the circulation. However, these previous findings are not enough to explain the anti-inflammatory effects of C3G, because C3G consumption also affects hormone secretion and the gut microbiota (67). Further characterization of the pharmacokinetics and other aspects of these substances should help clarify the mechanism by which C3G ameliorates inflammation *in vivo*. In the present study, we did not address to the detailed anti-inflammatory mechanism and pharmacokinetics of C3G and further study is needed in future.

In the present study, we found that C3G alone could not suppress the weight gain or correct the feeding rhythm disturbance as same as BE did. One possible explanation for this is that the duration of C3G consumption was too short to have an effect. In other studies, HFD-induced weight gain was significantly reduced by 8–16 weeks of ingestion of C3G through supplementation to the diet (68, 69), or the drinking water (70, 71), and orally administration (72). Thus, we should investigate absorption and accumulation of C3G and its metabolites and estimate whether concentration is enough to prevent DIO in future. It is also possible that the C3G and flavan 3-ols in BE have additive or synergistic effects to prevent DIO. Previous studies have shown that the oral administration of B-type PCAs promotes heat production *via* brain-peripheral organ axes (73, 74). Therefore, the suppression of hypothalamic inflammation by C3G may be coordinate with falvan 3-ols to improve metabolic function *via* the neural axis, resulting in a significant suppression of DIO by BE. However, further study is needed to evaluate this issue.

In conclusion, we have demonstrated that BE ingestion reduces HFD-induced hypothalamic inflammation, resulting in amelioration of the abnormal feeding pattern and DIO. Many researchers gradually pay an attention that the CNS is a therapeutic target for DIO, and the effect of functional foods on the CNS is a growing area of interest. The results from the present study indicate that BE is an attractive functional food material for regulating the CNS function and provide new insights into the mechanism by which it prevents obesity, in particular DIO.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary files, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee in Kobe University (permission number: 2020-10-13). Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

K-yH, YY, and HA designed and conceived the experiments and wrote the manuscript. K-yH performed the experiments. All authors contributed to the data interpretation, drafting of the manuscript, manuscript writing, and approved the submitted version.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.1006132/full#supplementary-material

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Dietary ketosis improves circadian dysfunction as well as motor symptoms in the BACHD mouse model of Huntington's disease

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Disturbances in sleep/wake cycles are common among patients with neurodegenerative diseases including Huntington's disease (HD) and represent an appealing target for chrono-nutrition-based interventions. In the present work, we sought to determine whether a low-carbohydrate, high-fat diet would ameliorate the symptoms and delay disease progression in the BACHD mouse model of HD. Adult WT and BACHD male mice were fed a normal or a ketogenic diet (KD) for 3 months. The KD evoked a robust rhythm in serum levels of β-hydroxybutyrate and dramatic changes in the microbiome of male WT and BACHD mice. NanoString analysis revealed transcriptional changes driven by the KD in the striatum of both WT and BACHD mice. Disturbances in sleep/wake cycles have been reported in mouse models of HD and are common among HD patients. Having established that the KD had effects on both the WT and mutant mice, we examined its impact on sleep/wake cycles. KD increased daytime sleep and improved the timing of sleep onset, while other sleep parameters were not altered. In addition, KD improved activity rhythms, including rhythmic power, and reduced inappropriate daytime activity and onset variability. Importantly, KD improved motor performance on the rotarod and challenging beam tests. It is worth emphasizing that HD is a genetically caused disease with no known cure. Life-style changes that not only improve the quality of life but also delay disease progression for HD patients are greatly needed. Our study demonstrates the therapeutic potential of diet-based treatment strategies in a pre-clinical model of HD.

KEYWORDS

BACHD mice, circadian rhythms, ketogenic diet (KD), sleep, motor performance, activity rhythm

Introduction

Huntington's disease (HD) is a progressive degenerative disorder that results in cognitive, psychiatric and motor dysfunction (1, 2). HD is caused by a CAG repeat expansion within the huntingtin (*HTT*) gene, which encodes an enlarged polyglutamine tract in the N-terminal fragment of the protein, causing mutant huntingtin to fold abnormally (3). The resulting protein misfolding and aggregations lead to pathophysiology and cell death in the brain (4) but also impacts function throughout the body (5). Among the diverse set of HD symptoms, altered central and peripheral metabolism of glucose, lipids and carbohydrates has been well described in HD patients (6, 7). In addition, sleep disorders are common among patients and animal models recapitulate the progressive breakdown of the circadian rest/activity cycle seen in the patients including delays in sleep onset, sleep fragmentation, and an increase in cycle-to-cycle variability (8–10). Given the many links between sleep/wake cycles and metabolism (11, 12), these symptoms could be co-dependent. Hence, HD represents a promising target for chrono-nutrition-based interventions.

Among the most developed of the chronotherapies is time-restricted feeding [TRF; (13)] for which there is a detailed biochemical understanding in both liver (14, 15) and pancreas (16). In addition, there is an emerging literature on clinical trials using TRF protocols (17–20). Over 90 clinical trials labeled with the key word "time-restricted feeding" are presently listed on the ClinicalTrials.gov online database (as of September, 2022). In preclinical mouse models of HD, a TRF protocol (6 h feeding aligned to the middle of the active phase and 18 h fasting) has been shown to effectively improve circadian parameters, autonomic functions as well as motor performance (21, 22). An important consequence of TRF is an increase in ketone bodies (23) and there has been speculation that at least some of the benefits of TRF are due to the switch from glucose to lipids as fuel source and the subsequent reduction in reactive oxygen species. This raises the question of whether a ketogenic diet (KD), similar to TRF, could also be beneficial for HD and other neurodegenerative disorders (24–26).

Therefore, in the current study, we first sought to demonstrate that KD is biologically impactful, both peripherally and centrally, in the BACHD mouse model of HD. The possible effects of the diet were first evaluated on body weight, serum levels of β-hydroxybutyrate (βHB), one of the most abundant ketone bodies, species composition of fecal microbiome and gene expression in the striatum in wild-type (WT) and mutant mice. Then, we examined the impact of the KD on sleep behavior, activity rhythms and motor performance (rotarod, grip strength, challenging beam).

Materials and methods

The work presented in this study followed all the guidelines and regulations of the UCLA Division of Animal Medicine that are consistent with the Animal Welfare Policy Statements and the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

Animals

The BACHD mouse model used in this study expresses the full-length of the human mutant HTT gene encoding 97 glutamine repeats under the control of the endogenous regulatory machinery (27). BACHD dams, backcrossed on the C57BL/6J background for a minimum of 12 generations, were bred with C57BL/6J (WT) males from The Jackson Laboratory (Bar Harbor, Maine) in our facility at UCLA. Data were collected from male WT and heterozygous for the BACHD transgene littermates. Genotyping was performed at 15 days of age by tail snips, and after weaning at postnatal day 21, littermates were group housed, unless otherwise noted. All animals were housed in soundproof chambers with controlled temperature, humidity and lighting conditions, 12 h light, 12 h dark cycle (12:12 LD, intensity 350 lux) for at least 2-weeks prior to any experimentation or change in diet. For all experiments, a light meter (BK precision, Yorba Linda, CA) was used to measure light-intensity (lux). Each chamber holds 8 cages of mice, grouped together by feeding treatment. The animals received cotton nestlets and had water available at all times.

Experimental groups and diet

Male WT and BACHD mice (3 months old) were randomly assigned to either a Normal Diet (ND) or KD group. The mice had *ad libitum* access to either a custom KD (Teklad diet TD.10911.PWD, Envigo, Madison, WI) or ND (Teklad diet 7013, NIH-31 Modified Open Formula diet, Envigo) for 3 months. The KD used (77.1% fat, 22.4% protein, 0.5% carbohydrate) has moderately high protein, no sugars and predominantly healthy fats (with a 2:1 ratio of n-3 to n-6 fatty acids from medium chain triglycerides with a little flax and canola oil). The level of proteins in the KD diet is consistent with recommendations for optimal health. The food was refreshed every 4 days, new and unconsumed food were weighed. At the end of the treatment, the animals (6–7 months old) were used to assess motor functions and then euthanized for tissue collection.

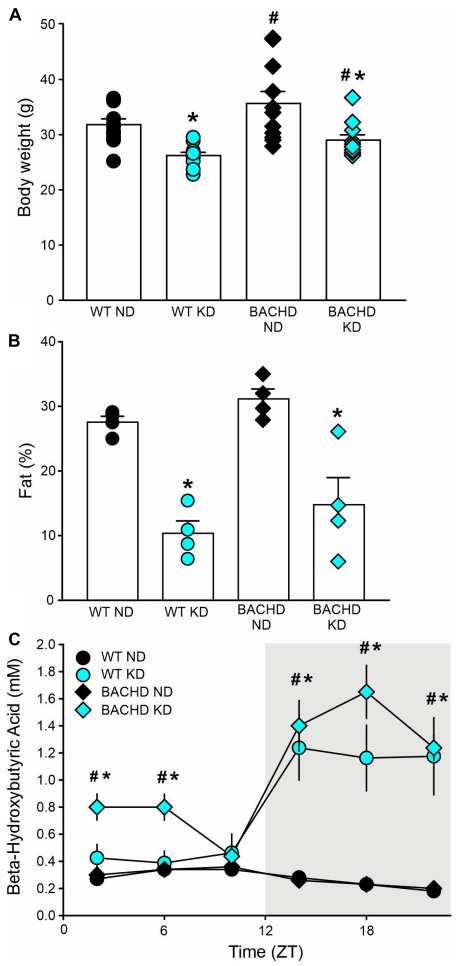

FIGURE 1
The ketogenic diet (KD) is effective in the BACHD mouse model of HD. Male WT and BACHD mice were placed on a KD at 3 months of age and held on this diet for 3 months. Controls of each genotype were kept on their normal diet (ND). **(A)** Body weight measurements at the end of the study indicated that the BACHD on ND were heavier than the WT, while the KD reduced the body weight in both genotypes (n = 8 mice per group). Two-way ANOVA indicated significant effects of both genotype [$F_{(1,\ 43)}$ = 6.241, P = 0.017] and diet [$F_{(1,\ 43)}$ = 21.128, $P < 0.001$]. **(B)** The adiposity of separate cohorts of mice (n = 4 per group) were evaluated using NMR spectroscopy. Both groups of mice on the KD were leaner as indicated by the lower% of fat in the body composition. Two-way ANOVA indicated no effect of genotype [$F_{(1,\ 15)}$ = 2.631, P = 0.131] but an unquestionable effect of the diet regimen [$F_{(1,\ 15)}$ = 46.0538, $P < 0.001$]. **(C)** To ensure that the BACHD mice fed the KD would undergo ketosis, tail blood was sampled at 6 time-points throughout the 24-h cycle (ZT 2, 6, 10, 14, 18, 22) and βHB measured (n = 10 per group). Both genotypes exhibited robust daily rhythms in ketones that peaked in the night. The BACHD mice also presented with elevated levels of βHB at some phases during the day. Two-way ANOVA found significant effects of time [WT: $F_{(5,\ 107)}$ = 8.833, $P < 0.001$; BAHCD: $F_{(5,\ 107)}$ = 20.443, $P < 0.001$] and diet [WT: $F_{(1,\ 107)}$ = 16.267, $P < 0.001$; BACHD: $F_{(1,\ 107)}$ = 76.599, $P < 0.001$]. Data are presented as means ± S.E.M. values. Significant differences were calculated using a two-way ANOVA followed by Holm–Sidak's multiple comparisons tests: $^{*}P < 0.05$ vs. mice on ND (effect of diet); $^{\#}P < 0.05$ between genotypes (same diet).

Body weight and body composition

For the first 3 weeks, to ensure that the KD did not hinder the animals' normal growth, WT and BACHD mice (n = 8 animals/genotype) on either ND or KD were weighed on the same day the food was refreshed (every 4 days), then only once every 4 weeks, and again at the end of the treatment period. The animals were weighed in the middle of the LD cycle between Zeitgeber time (ZT) 10 and ZT12. The body weights recorded at the end of the treatment period are shown.

Body composition (n = 4 animals/genotype/diet regimen) was determined at about 6+ months of age with a Mouse Minispec apparatus (Bruker Woodlands, TX) with Echo Medical Systems (Houston, TX) software. This apparatus uses NMR spectroscopy for fat and lean mass measurements with coefficients of variation of < 3%. Correlation between NMR and gravimetric measurements is better than 0.99.

β-hydroxybutyrate measurements

Tail vein blood samples were obtained from 6 months old WT and BACHD mice (n = 10 animals/genotype) kept for 3 months on a ND or KD at 6 time points during the sleep/wake cycle (ZT 2, 6, 10, 14, 18, 22). A small incision was made to access the tail vein to permit repeated withdrawals (under 3 μL per collection) with minimal pain and distress to the mice. At each specific ZT, mice were retrieved from the cages, placed on a stable surface and minimally restrained by the tail. Blood sampling was performed under normal room lighting (350 lux) for the testing times in the day, between ZT 0 and 12, and under dim red-light (3 lux) for those during the night (between ZT12 and 24). Blood flow was stopped by applying pressure with a sterile gauze to achieve hemostasis. Metabolite measurements were made in 1.5 μL blood samples using a commercially available ketone meter (Precision Xtra Ketone Monitoring System, Abbott Laboratories, Chicago, IL).

Microbiome measurements

Fecal samples were collected at ZT6 from WT and BACHD mice (n = 5 animals/genotype) that had been on ND or KD for at least 3 months. The fecal samples were then sent to TransnetYX (TransnetYXyx, Inc., Cordova, TN) for sequencing of the gut microbiome. The composition of the gut microbiome and the species relative abundances were analyzed using the One Codex (One Codex, San Francisco, CA) platform.

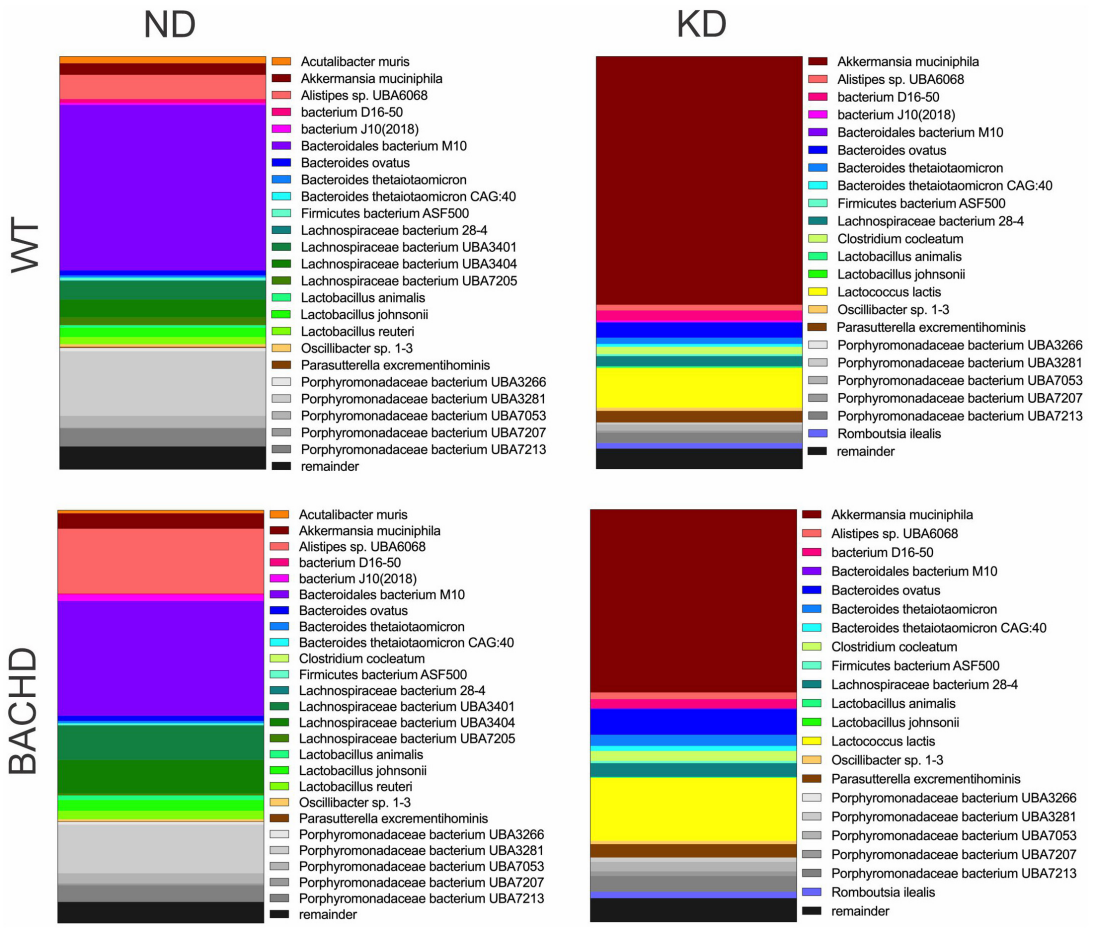

FIGURE 2
Ketogenic diet drove large changes in the microbiome of the BACHD model of HD. Male WT and BACHD mice (3 months old) were fed a normal diet (ND) or a KD for 3 months. For all four groups, fecal samples were collected at ZT6 when the mice were 6 months of age (n = 5 mice per group). The species abundance determined from each animal was averaged to produce a picture of the species composition for each of the four groups. The top 25 bacterial species across all 4 groups were identified and each is indicated by a different color. The probiotic *Akkermansia muciniphila* (shown in brick red) dramatically increased in abundance under KD. See **Table 1** for values analyzed by two-way ANOVA with genotype and diet as factors, followed by Holm-Sidak's multiple comparisons test.

RNA extraction and nanostring analysis

WT and BACHD (6–7 months old) mice (n = 5–6 animals/genotype) on either ND or KD were euthanized with isoflurane at ZT 14. The left and right striati were rapidly dissected out, separately frozen and stored at -80°C. Samples were lysed using the Invitrogen™ TRIzol™ reagent (Thermo Fisher Scientific; Carlsbad, CA). Total RNA was extracted using the RNeasy® Mini kit (Qiagen). Concentration and purity of the samples were assessed using a ThermoScientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Canoga Park, CA). Gene analysis was performed in 150 ng of total RNA (at a concentration of 20 ng/μl) at the UCLA Center for Systems Biomedicine (genetic engineering platform) using the Nanostring nCounter® Neuropathology Panel designed to interrogate 770 transcripts specific for neurodegenerative processes/disease. Data quality and normalization of the sample signals were performed using the nSolver analyses software. The Rosalind® software[1] was used to identify differentially expressed genes, as well as to obtain fold changes and p-values within two groups comparison (4 combinations) with genotype and diet as attributes as described in the ROSALIND® Nanostring Gene Expression Methods. The Reactome database was used to identify the top enriched biological pathways (Rosalind interactive analysis).[2] The identified genes along with selected genes known to be circadian regulated were further analyzed by two-way ANOVA followed by the Holm-Sidak's multiple comparisons test using the relative expression values obtained with the nSolver software.

1 https://rosalind.bio/
2 https://app.rosalind.bio/

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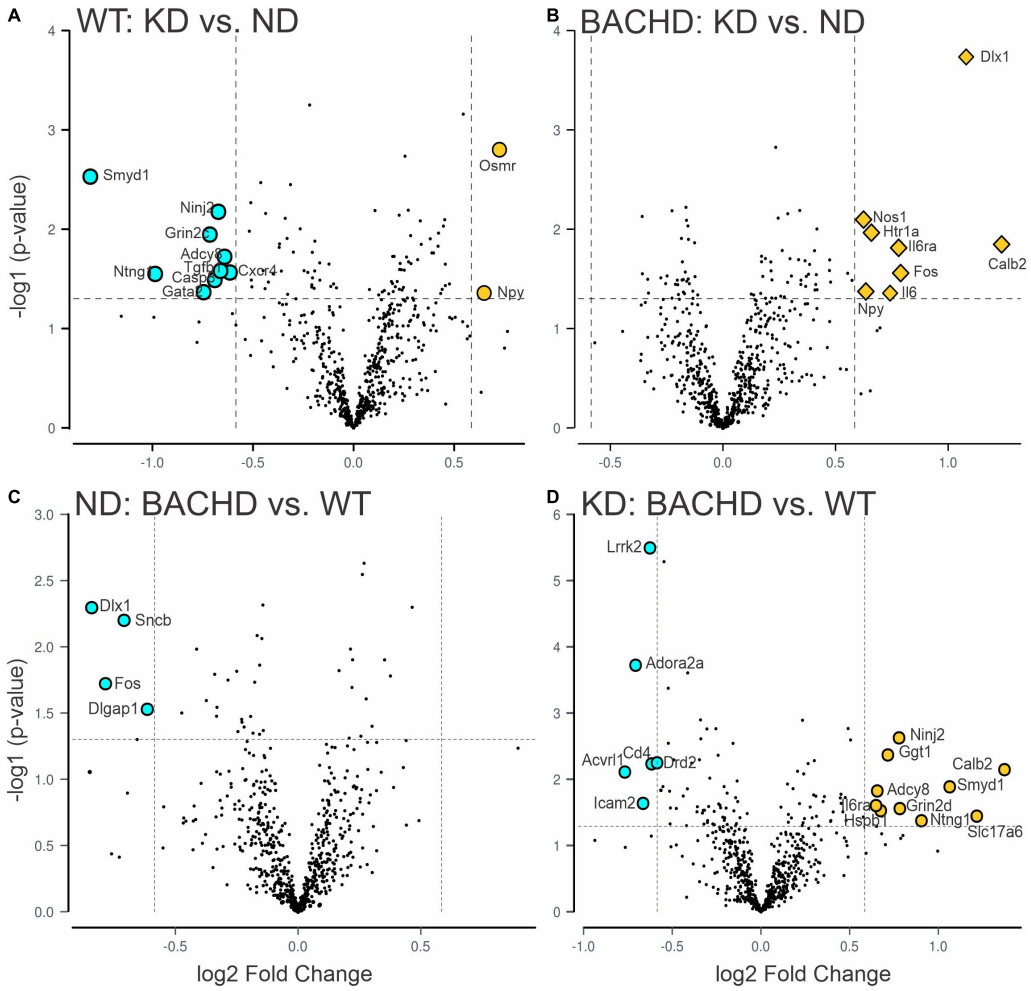

FIGURE 3

Changes in the transcriptional landscape of the BACHD striatum driven by the Ketogenic diet (KD). Striatal tissue samples were collected at ZT 14 from 6 to 7 months old WT and BACHD male mice fed a normal diet (ND) or a KD for 3+ months until euthanized (n = 5–6 animals per group). NanoString transcriptomic analysis was performed using the Nanostring nCounter® Neuropathology Panel and analyzed using the software rosalind. The volcano plots illustrate the differentially expressed transcripts and similarities between WT and BACHD on ND or KD (two-groups comparisons). Volcano plot in **(A,B)** were obtained by comparing the effects of the KD within each genotype, while the plots in **(C,D)** illustrate the differences in gene expression in animals on the same diet. The gray points indicate genes with no significant changes, while significantly increased genes are in dark yellow and those decreased are shown in cyan. See **Table 2** and **Supplementary Figure 2** for expression levels analyzed by two-way ANOVA with genotype and diet as factors followed by Holm-Sidak's multiple comparisons test.

Locomotor activity rhythms

WT and BACHD mice on ND or KD (n = 8 animals/genotype) were singly housed in cages with IR motion sensors to record their locomotor activity with a 12:12 h LD cycle and entrained for 2 weeks before beginning data collection. Activity data were collected in the 2–3 weeks prior to the motor performance tests when the mice were between 5 and 6 months of age and analyzed using the El Temps (A. Diez-Nogura, Barcelona, Spain)[3] and ClockLab programs. Locomotor activity was recorded as previously described (28) using Mini Mitter (Bend, OR) data loggers in 3-min bins, and 7–10 days of data were averaged for analysis. The data were analyzed to determine the period and rhythmic strength as previously described (28). The periodogram analysis uses a χ^2-test with a threshold of 0.001 significance, from which the amplitude of the periodicities is determined at the circadian harmonic to obtain the rhythm power. The amount of cage activity over a 24 h period was averaged and reported here as the arbitrary units (a.u.)/h. The number of activity bouts and the average length of bouts were determined using Clocklab, where each bout was counted when activity bouts were separated

3 http://www.el-temps.com/principal.html

TABLE 1 Species abundance of microbiome measured in fecal samples from WT and BACHD mice fed a normal (ND) or a ketogenic diet (KD).

	WT		BACHD			
Species	**ND**	**KD**	**ND**	**KD**	**Diet**	**Genotype**
Akkermansia muciniphila	2.8 ± 3.6	**60.2 ± 22.9***	3.8 ± 3.9	**44.4 ± 30.2***	**$F_{(1,19)}$ = 32.756; P < 0.001**	$F_{(1,19)}$ = 0.757; P = 0.397
Bacteroidales bacterium M10	40.1 ± 13.9	**0.2 ± 0.2***	27.8 ± 6.8	**0.2 ± 0.3***	**$F_{(1,19)}$ = 95.124; P < 0.001**	$F_{(1,19)}$ = 3.145; P = 0.095
Alistipes sp. *UBA6068*	5.8 ± 1.9	1.4 ± 0.6	**15.7 ± 8.5#**	**1.6 ± 0.3***	**$F_{(1,19)}$ = 22.080; P < 0.001**	**$F_{(1,19)}$ = 6.450; P = 0.022**
Bacteroides ovatus	1.2 ± 0.3	3.6 ± 3.5	1.2 ± 1.1	**6.2 ± 4.3***	**$F_{(1,19)}$ = 8.472; P = 0.010**	$F_{(1,19)}$ = 1.091; P = 0.312
Parasutterella excrementihominis	0.3 ± 0.3	2.8 ± 3.8	0.2 ± 0.1	3.3 ± 3.5	**$F_{(1,19)}$ = 5.977; P = 0.026**	$F_{(1,19)}$ = 0.016; P = 0.901
Lachnospiraceae bacterium 28-4	0.2 ± 0.2	2.5 ± 2.5	0.1 ± 0.1	**3.3 ± 3.1***	**$F_{(1,19)}$ = 9.744; P = 0.007**	$F_{(1,19)}$ = 0.190; P = 0.669
bacterium D16-50	0.9 ± 1.6	2.4 ± 2.5	0.1 ± 0.1	3.3 ± 31	$F_{(1,19)}$ = 3.904; P = 0.066	$F_{(1,19)}$ = 0.237; P = 0.633
Bacteroides thetaiotaomicron	0.5 ± 0.1	1.5 ± 1.5	0.5 ± 0.5	**2.7 ± 1.9***	**$F_{(1,19)}$ = 8.368; P = 0.011**	$F_{(1,19)}$ = 0.290; P = 0.290
Porphyromonadaceae bacterium UBA7213	4.4 ± 1.0	2.5 ± 1.6	4.1 ± 1.0	3.8 ± 3.4	$F_{(1,19)}$ = 1.336; P = 0.265	$F_{(1,19)}$ = 0.293; P = 0.596
Porphyromonadaceae bacterium UBA7053	2.8 ± 0.7	1.5 ± 1.0	2.5 ± 0.6	2.3 ± 1.9	$F_{(1,19)}$ = 1.336; P = 0.265	$F_{(1,19)}$ = 0.293; P = 0.596
Lactobacillus reuteri	1.7 ± 2.7	0.0 ± 0.0	2.5 ± 0.6	2.3 ± 1.9	$F_{(1,19)}$ = 4.407; P = 0.052	$F_{(1,19)}$ = 0.037; P = 0.849
Lactobacillus johnsonii	2.3 ± 3.0	0.1 ± 0.1	2.6 ± 3.3	0.1 ± 0.1	**$F_{(1,19)}$ = 5.674; P = 0.030**	$F_{(1,19)}$ = 0.035; P = 0.853
Porphyromonadaceae bacterium UBA3281	15.6 ± 6.2	**0.4 ± 0.6***	11.8 ± 3.8	**0.9 ± 2.0***	**$F_{(1,19)}$ = 59.532; P < 0.006**	$F_{(1,19)}$ = 1.003; P = 0.332
Lachnospiraceae bacterium UBA3401	4.4 ± 9.8	0.0 ± 0.0	8.3 ± 6.3	**0.0 ± 0.0***	**$F_{(1,19)}$ = 5.953; P = 0.027**	$F_{(1,19)}$ = 0.552; P = 0.468
Lachnospiraceae bacterium UBA3404	4.3 ± 9.0	0.0 ± 0.0	8.1 ± 6.3	**0.0 ± 0.0***	**$F_{(1,19)}$ = 5.812; P = 0.028**	$F_{(1,19)}$ = 0.532; P = 0.476
Lachnospiraceae bacterium UBA7205	1.9 ± 9.6	0.0 ± 0.0	0.5 ± 6.3	0.0 ± 0.0	**$F_{(1,19)}$ = 7.375; P = 0.015**	$F_{(1,19)}$ = *2.494*; P = 0.134
Acutalibacter muris	1.6 ± 1.9	**0.0 ± 0.0***	0.7 ± 0.5	0.0 ± 0.0	**$F_{(1,19)}$ = 8.266; P = 0.011**	$F_{(1,19)}$ = *1.213*; P = 0.287
Porphyromonadaceae bacterium UBA3266	0.8 ± 1.8	**0.1 ± 0.1***	0.7 ± 0.5	**0.1 ± 0.0***	**$F_{(1,19)}$ = 58.352; P < 0.001**	$F_{(1,19)}$ = *0.145*; P = 0.709
Lactococcus lactis	0.0 ± 0.1	**9.7 ± 0.1***	0.0 ± 0.3	**15.3 ± 0.2***	**$F_{(1,19)}$ = 26.198; P < 0.001**	$F_{(1,19)}$ = 1.325; P = 0.267
Clostridium cocleatum	0.0 ± 0.0	**1.7 ± 6.5***	0.0 ± 0.2	**2.4 ± 8.7***	**$F_{(1,19)}$ = 41.479; P < 0.001**	$F_{(1,19)}$ = 1.066; P = 0.317
Romboutsia ilealis	0.0 ± 0.0	1.4 ± 1.3	0.0 ± 0.0	**1.5 ± 0.5***	**$F_{(1,19)}$ = 9.192; P = 0.008**	$F_{(1,19)}$ = 0.036; P = 0.852
Oscillibacter sp. *1-3*	0.6 ± 0.4	0.8 ± 0.4	0.5 ± 02	0.8 ± 0.3	$F_{(1,19)}$ = 2.610; P = 0.126	$F_{(1,19)}$ = 0.217; P = 0.647
Bacteroides thetaiotaomicron CAG:40	0.2 ± 0.1	0.7 ± 0.7	0.2 ± 0.2	**1.2 ± 0.9***	**$F_{(1,19)}$ = 8.137; P = 0.012**	$F_{(1,19)}$ = 1.173; P = 0.295
Firmicutes bacterium ASF500	0.5 ± 0.5	0.5 ± 0.3	0.2 ± 0.0	0.5 ± 0.2	$F_{(1,19)}$ = 1.1280 P = 0.275	$F_{(1,19)}$ = 0.692; P = 0.418
bacterium J10(2018)	0.5 ± 1.1	0.3 ± 0.8	1.5 ± 2.2	0.0 ± 0.0	$F_{(1,19)}$ = 2.159; P = 0.161	$F_{(1,19)}$ = 0.399; P = 0.537

Two-way ANOVA followed by Holm-Sidak's multiple comparisons test of the abundance of the 25 most common species (Figure 2). Data are expressed as the percentage of the total number of species in each sample and are the mean ± SD of 5 animals/genotype/diet regimen. Degrees of freedom are reported within parentheses, alpha = 0.05. Asterisks indicate significant difference within genotype (i.e., diet effect); crosshatches indicate differences between genotypes (i.e., same diet). There were no significant interactions except for *Alistipes* (see text for values). Bold type indicates statistical significance.

by a gap of 21 min (maximum gap: 21 min; threshold: 3 counts/min). The onset variability was determined using Clocklab by drawing the best-fit line over the recorded days and averaging the differences between activity onset and best-fit regression of each day.

A separate cohort of WT and BACHD mice on ND or KD was held in LD for about 2 months followed by 4 weeks in constant darkness (DD). Cage activity was recorded to obtain free-running activity.

Sleep behavior

Immobility-defined sleep was determined as described previously (28) in 6–7 months old WT and BACHD mice (n = 8 animals/genotype) held for 3+ months on ND or KD. Animals were housed in see-through plastic cages containing bedding (without the addition of nesting material). A side-on view of each cage was obtained, with minimal occlusion by the food bin or water bottle, both of which were top-mounted. Cages were top-lit using IR LED lights. Video capture was accomplished using surveillance cameras with visible light filters (Gadspot Inc., City of Industry, CA) connected to a video-capture card (Adlink Technology Inc., Irvine, CA) on a custom-built computer system. ANY-maze software (Stoelting Co., Wood Dale, IL) was used for automated tracking of mouse immobility.

Immobility was registered when 95% of the area of the animal stayed immobile for more than 40 s, as was previously determined to have 99% correlation with simultaneous EEG/EMG defined sleep (29). Continuous tracking of the mice was performed for a minimum of 5 sleep-wake cycles, with randomized visits (1–2 times/day) by the experimenter to confirm mouse health and video recording. The 3rd and 4th sleep-wake cycles were averaged for further analysis. Immobility-defined sleep data were exported in 1 min bins, and total sleep time was determined by summing the immobility durations in

TABLE 2 Ketogenic diet impacts gene expression in the striatum of WT and BACHD male mice.

	WT		BACHD				
GOI	**ND**	**KD**	**ND**	**KD**	**Diet**	**Genotype**	**Interaction**
Dlx1	65 ± 15	65 ± 12	43 ± 9	**81 ± 33***	$\mathbf{F_{(1,20)} = 5.325; P = 0.032}$	$F_{(1,20)} = 0.144; P = 0.709$	$\mathbf{F_{(1,20)} = 5.552; P = 0.029}$
Smyd1	30 ± 9	**15 ± 2****	28 ± 5	**27 ± 6**$^{\#}$	$\mathbf{F_{(1,18)} = 11.00; P = 0.004}$	$F_{(1,18)} = 3.441; P = 0.080$	$\mathbf{F_{(1,18)} = 7.907; P = 0.012}$
Bdnf	80 ± 34	105 ± 35	50 ± 24	83 ± 27	$\mathbf{F_{(1,17)} = 4.695; P = 0.045}$	$F_{(1,17)} = 3.825; P = 0.067$	$F_{(1,17)} = 0.110; P = 0.744$
Fos	190 ± 65	250 ± 72	116 ± 20	**258 ± 107***	$\mathbf{F_{(1,18)} = 10.77; P = 0.004}$	$F_{(1,18)} = 1.138; P = 0.300$	$F_{(1,18)} = 1.728; P = 0.205$
Il6	55 ± 24	79 ± 30	45 ± 15	69 ± 7	$\mathbf{F_{(1,20)} = 7.991; P = 0.010}$	$F_{(1,20)} = 1.465; P = 0.240$	$F_{(1,20)} = 8.6e\text{-}008; P = 0.999$
Kcnb1	349 ± 99	412 ± 52	332 ± 53	386 ± 31	$\mathbf{F_{(1,20)} = 5.049; P = 0.036}$	$F_{(1,20)} = 0.686; P = 0.417$	$F_{(1,20)} = 0.030; P = 0.864$
Osmr	31 ± 7	**47 ± 8***	40 ± 7	47 ± 10	$\mathbf{F_{(1,20)} = 11.64; P = 0.003}$	$F_{(1,20)} = 2.040; P = 0.169$	$F_{(1,20)} = 1.764; P = 0.199$
Cxcr4	34 ± 10	26 ± 6	39 ± 5	34 ± 3	$\mathbf{F_{(1,20)} = 6.252; P = 0.021}$	$\mathbf{F_{(1,20)} = 6.321; P = 0.021}$	$F_{(1,20)} = 0.125; P = 0.726$
Dlgap1	131 ± 40	146 ± 35	83 ± 12	134 ± 25	$\mathbf{F_{(1,16)} = 5.895; P = 0.027}$	$\mathbf{F_{(1,16)} = 4.857; P = 0.042}$	$F_{(1,16)} = 1.732; P = 0.207$
Il6ra	18 ± 2	21 ± 3	21 ± 5	**29 ± 7**$^{*,\#}$	$\mathbf{F_{(1,20)} = 9.145; P = 0.007}$	$\mathbf{F_{(1,20)} = 9.334; P = 0.006}$	$F_{(1,20)} = 1.757; P = 0.199$
Nos1	140 ± 36	168 ± 36	99 ± 22	146 ± 18	$\mathbf{F_{(1,19)} = 9.747; P = 0.006}$	$\mathbf{F_{(1,19)} = 6.899; P = 0.017}$	$F_{(1,19)} = 0.623; P = 0.439$
Npy	233 ± 118	**398 ± 132***	176 ± 45	272 ± 36	$\mathbf{F_{(1,19)} = 11.86; P = 0.003}$	$\mathbf{F_{(1,19)} = 5.799; P = 0.026}$	$F_{(1,19)} = 0.827; P = 0.375$
Adora2a	1134 ± 266	1422 ± 358	1115 ± 207	**885 ± 134**$^{\#\#}$	$F_{(1,20)} = 0.079; P = 0.781$	$\mathbf{F_{(1,20)} = 7.135; P = 0.015}$	$\mathbf{F_{(1,20)} = 6.223; P = 0.021}$
Lrrk2	904 ± 120	**1098 ± 167***	857 ± 110	**723 ± 67**$^{\#\#\#}$	$F_{(1,20)} = 0.364; P = 0.553$	$\mathbf{F_{(1,20)} = 18.03; P = 0.001}$	$\mathbf{F_{(1,20)} = 10.92; P = 0.004}$
Ntng1	702 ± 309	**314 ± 146***	668 ± 81	**767 ± 125**$^{\#\#}$	$F_{(1,16)} = 3.007; P = 0.102$	$\mathbf{F_{(1,16)} = 6.276; P = 0.023}$	$\mathbf{F_{(1,16)} = 8.541; P = 0.010}$
Drd2	789 ± 296	882 ± 185	705 ± 124	598 ± 75	$F_{(1,20)} = 0.007; P = 0.935$	$\mathbf{F_{(1,20)} = 5.694; P = 0.027}$	$F_{(1,20)} = 1.685; P = 0.209$
Ggt1	28 ± 6	23 ± 4	31 ± 6	**33 ± 3**$^{\#}$	$F_{(1,20)} = 0.485; P = 0.494$	$\mathbf{F_{(1,20)} = 9.976; P = 0.005}$	$F_{(1,20)} = 2.923; P = 0.103$
Htr1a	37 ± 8	35 ± 9	32 ± 7	27 ± 7	$F_{(1,20)} = 1.394; P = 0.252$	$\mathbf{F_{(1,20)} = 4.408; P = 0.049}$	$F_{(1,20)} = 0.159; P = 0.695$
Nfkbia	180 ± 25	187 ± 29	201 ± 16	218 ± 18	$F_{(1,19)} = 1.756; P = 0.201$	$\mathbf{F_{(1,19)} = 7.324; P = 0.014}$	$F_{(1,19)} = 0.302; P = 0.589$
Ninj2	42 ± 11	30 ± 6	49 ± 11	**47 ± 7**$^{\#}$	$F_{(1,20)} = 3.674\ P = 0.069$	$\mathbf{F_{(1,20)} = 10.84; P = 0.004}$	$F_{(1,20)} = 1.522; P = 0.232$
Sncb	235 ± 75	259 ± 87	154 ± 21	212 ± 33	$F_{(1,20)} = 2.725; P = 0.114$	$\mathbf{F_{(1,20)} = 6.598; P = 0.018}$	$F_{(1,20)} = 0.459; P = 0.505$
Acvrl1	58 ± 9	92 ± 33	70 ± 18	59 ± 13	$F_{(1,19)} = 1.716; P = 0.206$	$F_{(1,19)} = 1.258; P = 0.276$	$\mathbf{F_{(1,19)} = 6.656; P = 0.018}$
Adcy8	319 ± 126	208 ± 74	248 ± 20	327 ± 37	$F_{(1,19)} = 0.548; P = 0.468$	$F_{(1,19)} = 0.237; P = 0.632$	$\mathbf{F_{(1,19)} = 8.467; P = 0.009}$
Calb2	240 ± 139	144 ± 79	158 ± 60	**363 ± 158**$^{*,\#}$	$F_{(1,20)} = 1.322; P = 0.264$	$F_{(1,20)} = 2.092; P = 0.163$	$\mathbf{F_{(1,20)} = 10.05; P = 0.005}$
Gata2	28 ± 7	21 ± 7	24 ± 5	29 ± 3	$F_{(1,20)} = 0.229; P = 0.637$	$F_{(1,20)} = 0.678; P = 0.419$	$\mathbf{F_{(1,20)} = 5.545; P = 0.029}$
Homer1	4964 ± 462	**6080 ± 795***	5376 ± 635	**4614 ± 563**$^{\#\#}$	$F_{(1,20)} = 0.480; P = 0.496$	$F_{(1,20)} = 4.252; P = 0.052$	$\mathbf{F_{(1,20)} = 13.51; P = 0.001}$

Two-way ANOVA followed by Holm-Sidak's multiple comparisons test of selected genes identified by Nanostring transcriptomic analysis. A total of 30 differentially expressed genes was identified in 2 groups-comparisons with the highest number in the BACHD-KD vs. WT-KD comparison (Figure 3 and Supplementary Figure 2). Data are shown as the mean ± SD of 5–6 animals/genotype/diet regimen. Degrees of freedom are reported within parentheses, alpha = 0.05. Asterisks indicate significant difference within genotype (i.e., diet effect), whilst crosshatches those between genotypes (i.e., same diet). Bold type indicates statistical significance. ND, normal diet; KD, Ketogenic diet.

the rest phase (ZT 0–12) or active phase (ZT 12–24). An average waveform of hourly immobile-sleep over the two sleep-wake cycles was produced per genotype and treatment for graphical display. Variability of sleep onset, sleep offset, and sleep fragmentation were determined using Clocklab (Actimetrics, Wilmette, IL).

Motor behavior

All the motor behavioral tests were performed in WT and BACHD mice fed with either ND or KD at about 6.5–7 months of age (n = 8–10 mice/genotype) after monitoring sleep and activity rhythms.

Grip strength test

Grip strength was used to measure neuromuscular function as maximal muscle strength of forelimbs. The grip strength ergometer (Santa Cruz Biotechnology, Santa Cruz, CA) was set up on a flat surface with a mouse grid firmly secured in place. The grid was cleaned with 70% ethanol and allowed to dry before testing each cohort. Peak mode was selected to enable measurement of maximal strength exerted. The sensor is reset to zero before each trial. Well-handled mice were tested in their active phase under dim red light (3 Lux) and acclimated to the testing room for 10 min prior to testing. Mice underwent five trials with an inter-trial interval of at least 2 min. For each trial, each mouse was removed from its home cage by gripping the tail between the thumb and the forefinger. The mice were lowered slowly over the grid, and only their forepaws were allowed to grip the grid. Mice were pulled by the tail ensuring the torso remains horizontal until they were no longer able to grip the grid. Mice were then returned to their cages. The maximal grip strength value of each mouse was utilized.

TABLE 3 Top enriched biological pathways extrapolated using the Reactome database (https://app.rosalind.bio/).

				WT KD vs. ND	BACHD KD vs. ND	ND BACHD vs. WT	KD BACHD vs. WT
Term ID	**Term name**	**# Genes in term**	**# Genes in cluster**	**FDR-adjusted *P*-value**	**FDR-adjusted *P*-value**	**FDR-adjusted *P*-value**	**FDR-adjusted *P*-value**
R-MMU-450341	Activation of the AP-1 family of transcription factors	10	1	–	0.0992	**0.0341**	–
R-MMU-9018519	Estrogen-dependent gene expression	118	1	–	0.1179	**0.0408**	–
R-MMU-2871796	FCERI mediated MAPK activation	29	1	–	0.0992	**0.0341**	–
R-MMU-1059683	Interleukin-6 signaling	9	2	–	**0.0026**	–	0.0866
R-MMU-112411	MAPK1 (ERK2) activation	8	2	–	**0.0026**	–	0.0866
R-MMU-110056	MAPK3 (ERK1) activation	9	2	–	**0.0026**	–	0.0866
R-MMU-6794361	Neurexins and neuroligins	31	1	–	–	**0.0341**	–
R-MMU-2559580	oxidative stress induced senescence	109	1	–	0.0992	**0.0341**	–

Only the terms with a FDR adjusted *p*-value of less than 0.05 in at least one group comparison are reported. *P*-value adjustments were performed using the Benjamini-Hochberg method of estimating false discovery rates (FDR) as reported in the ROSALIND® Nanostring Gene Expression Methods statement. Bold type indicates statistical significance. ND, normal diet; KD, ketogenic diet.

Rotarod test—Accelerating version

The rotarod apparatus (Ugo Basile, Varese, Italy) is commonly used to measure motor coordination and balance. This apparatus is, in essence, a small circular treadmill. It consists of an axle or rod thick enough for a mouse to stand over the top of when it is not in motion and a flat platform a short distance below the rod. The rod was covered with smooth rubber to provide traction while preventing the mice from clinging to the rod. In this study, mice were placed on top of the rubber-covered rod. When the mice moved at the pace set by the rotation rate of the rod, they would stay on top of it. When mice no longer moved at the selected pace, they dropped a short distance to the platform below. The time a mouse remains on the rod, before dropping to the platform is the latency to fall. Following a 15-min habituation to the testing room, mice were placed on the slowly rotating rod. The rod gradually accelerates from 5 to 38 rpm over the course of the trial. The length of time the mouse stayed on the rod was recorded. A 2-day protocol for the accelerating rotarod tests was used. On the first day, the mice were trained on the rotarod over 5 trials. The maximum length of each trial was 600 s, and mice were allowed to rest for a minimum of 60 s between trials. On the second day, mice were tested on the rotarod and the latency to fall from the rotarod was recorded from 5 trials. Mice were again allowed to rest for a minimum of 60 s between trials. Data from each mouse were analyzed after averaging the times from all five trials. The apparatus was cleaned with 70% alcohol and allowed to dry completely between trials. A dim red-light (3 lux) was used for illumination during active (dark) phase testing.

Challenging beam test

In our version of the test, a beam was placed between two cages. The beam narrows in 4 intervals from 33 mm > 24 mm > 18 mm > 6 mm, with each segment spanning 253 mm in length. The home cage of each mouse was put on the end of the beam as the motivating factor. In this study, animals were trained on the beam for 5 consecutive trials on two consecutive days. During each trial, each mouse was placed on the widest end of the beam and allowed to cross with minimal handling by the experimenter. On the testing day, a metal grid (10 × 10 mm spacing, formed using 19-gauge wire) was overlaid on the beam. This overlaid grid increased the difficulty of the beam traversal task and provided a visual reference for foot slips made while crossing the grid. Each mouse was evaluated on five consecutive trials conducted during their active (dark) phase. Trials were recorded by a camcorder under dim red-light conditions (2 lux), supplemented with infrared lighting for video recording. The videos were scored *post hoc* by two independent observers

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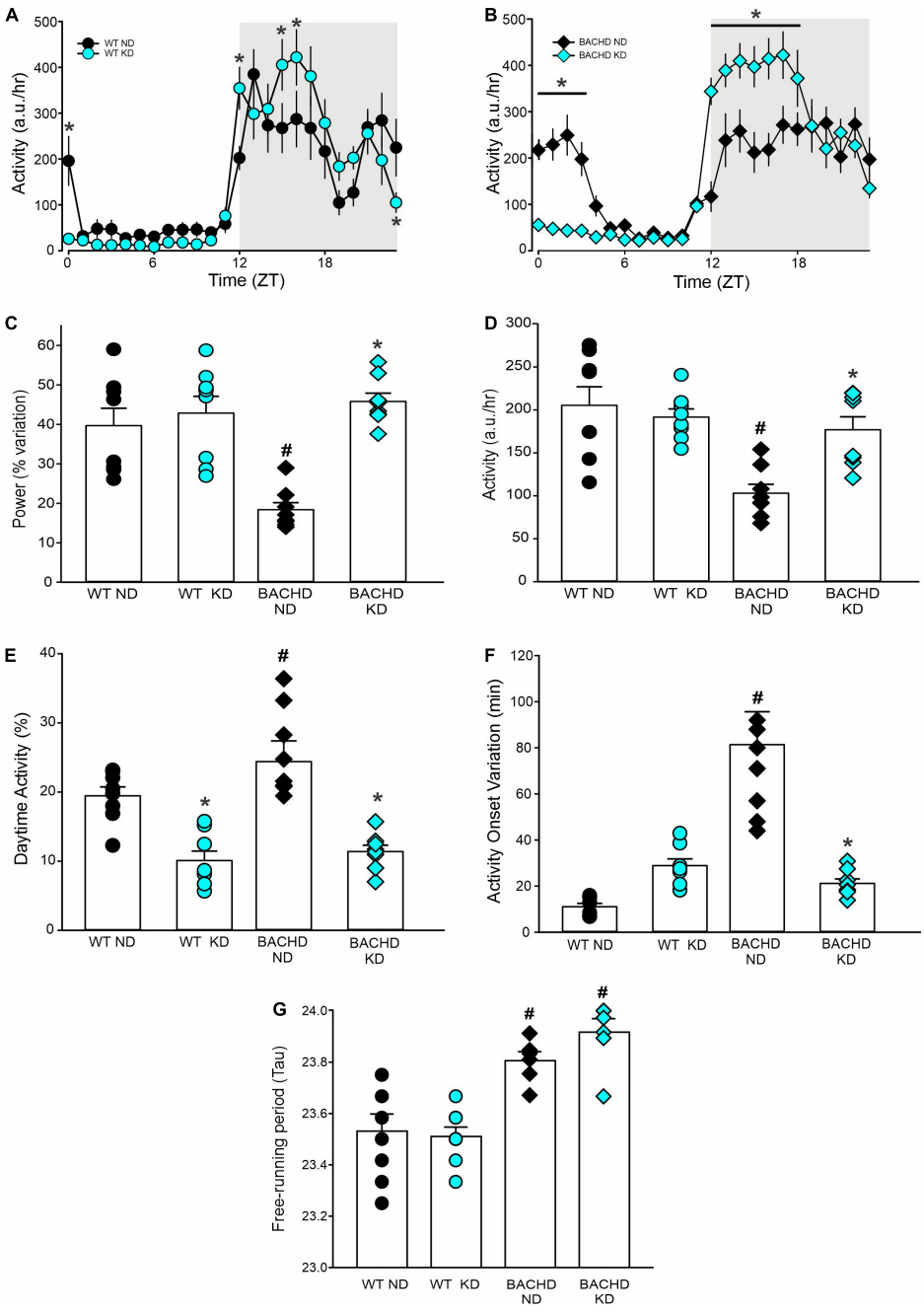

FIGURE 4

Ketogenic diet (KD) strongly improved the daily activity rhythms in male BACHD mice. The activity rhythms were monitored throughout the 3 months of exposure to the KD. The recordings of the last 3 weeks were analyzed and are shown (n = 8 mice per group). **(A,B)** Waveforms of daily rhythms in cage activity in 5–6 months old WT (circles) and BACHD (diamonds) mice under normal diet (ND; black) or KD (cyan). The gray shading indicates nighttime when mice were in the dark. The activity waveform (1 h bins) of each group was analyzed using a two-way ANOVA for repeated measures with treatment and time as factors. For WT mice, there were significant effects of time [$F_{(23,\ 359)}$ = 21.406, $P < 0.001$] but not of diet [$F_{(1,\ 359)}$ = 0.731, P = 0.393] along with a significant interaction of time and diet [$F_{(23,\ 359)}$ = 10.165, $P < 0.001$]. Similarly, for the BACHD mice, there were significant effects of time [$F_{(23,\ 383)}$ = 26.533, $P < 0.001$] but not diet [$F_{(1,\ 383)}$ = 3.768, P = 0.053]. Again, there was a significant interaction between the two variables [$F_{(23,\ 383)}$ = 17.329, $P < 0.001$]. $^{*}P < 0.05$ differences between the 1 h bins, Holm-Sidak's multiple comparisons test. **(C)** The KD restored the strength of the rhythms in the BACHD mice, while **(D)** increasing their activity per hour. **(E)** KD significantly decreased the improper activity during the light phase and **(F)** improved the activity onset. **(G)** The free-running circadian period (Tau) of the mice in constant darkness was measured. The BACHD mice exhibited a longer Tau that was not altered by the diet. Histograms show the means ± SEM with the values from the individual animals overlaid. Properties of the daily activity rhythms were analyzed using a two-way ANOVA with genotype and treatment as factors followed by the Holm-Sidak's test for multiple comparisons (n = 8 per group) (see also Table 4). $^{*}P < 0.05$ vs. mice on ND (effect of diet); $^{\#}P < 0.05$ between genotypes (same diet).

TABLE 4 Impact of KD on activity rhythms and sleep.

	WT		BACHD				
	ND	KD	ND	KD	Diet	Genotype	Interaction
Activity rhythms							
Total activity (a.u./24 h)	2659 ± 1585	3648 ± 1180	4115 ± 812	4324 ± 982	$F_{(1,30)}$ = 0.05; P = 0.820	**$F_{(1,30)}$ = 1.72; P = 0.02**	$F_{(1,30)}$ = 0.06; P = 0.800
Day activity (a.u./12 h)	651 ± 476	**256 ± 58***	**1323 ± 204#**	**472 ± 112***	**$F_{(1,30)}$ = 45.95; P < 0.001**	**$F_{(1,30)}$ = 23.35; P = 0.03**	**$F_{(1,30)}$ = 6.17; P = 0.019**
Day activity (%)	19.5 ± 3.7	10.1 ± 3.9	25.6 ± 6.4	11.4 ± 2.6	**$F_{(1,30)}$ = 58.72; P < 0.001**	**$F_{(1,30)}$ = 5.86; P = 0.022**	$F_{(1,30)}$ = 2.53; P = 0.123
Night activity (a.u./12 h)	3009 ± 1255	3393 ± 1168	2792 ± 811	3853 ± 893	$F_{(1,30)}$ = 3.72; P = 0.064	$F_{(1,30)}$ = 0.11; P = 0.748	$F_{(1,30)}$ = 0.82; P = 0.374
Power (% variance)	39.7 ± 12.5	42.8 ± 12.0	**18.4 ± 5.0#**	**45.8 ± 5.9***	**$F_{(1,30)}$ = 23.50; P < 0.001**	**$F_{(1,30)}$ = 5.39; P = 0.03**	**$F_{(1,30)}$ = 10.15; P = 0.004**
Onset variability (min)	11.0 ± 4.1	**28.8 ± 8.3***	**81.4 ± 40.4#**	**21.6 ± 5.6***	**$F_{(1,30)}$ = 8.23; P = 0.008**	**$F_{(1,30)}$ = 17.96; P < 0.001**	**$F_{(1,30)}$ = 27.90; P < 0.001**
Sleep							
Total sleep (min/24 h)	648 ± 74	667 ± 50	616 ± 20	**606 ± 51#**	$F_{(1,33)}$ = 0.07; P = 0.791	**$F_{(1,33)}$ = 6.14; P = 0.019**	$F_{(1,33)}$ = 0.62; P = 0.437
Day sleep (min)	454 ± 48	473 ± 13	444 ± 38	438 ± 37	$F_{(1,33)}$ = 0.40; P = 0.529	$F_{(1,33)}$ = 0.40; P = 0.529	$F_{(1,33)}$ = 2.61; P = 0.117
Night sleep (min)	194 ± 47	194 ± 43	**172 ± 35#**	167 ± 29	$F_{(1,33)}$ = 0.08; P = 0.768	**$F_{(1,33)}$ = 4.38; P = 0.045**	$F_{(1,33)}$ = 0.41; P = 0.527
Fragmentation (# bouts)	10.7 ± 2.1	10.5 ± 1.7	12.8 ± 2.0	**9.4 ± 1.6***	**$F_{(1,33)}$ = 5.93; P = 0.021**	$F_{(1,33)}$ = 0.04; P = 0.84	**$F_{(1,33)}$ = 4.33; P = 0.046**
Sleep onset (ZT)	23.8 ± 0.5	0.4 ± 0.3	1.8 ± 1.4	0.2 ± 0.7	$F_{(1,33)}$ = 2.60; P = 0.118	**$F_{(1,33)}$ = 8.47; P = 0.007**	**$F_{(1,33)}$ = 12.81; P = 0.001**

Comparisons of WT and BACHD mice fed a normal (ND) or a ketogenic diet (KD). Data were analyzed by two-way ANOVA using genotype and diet as factors followed by the Holm-Sidak's multiple comparisons test and are expressed as the mean ± SD of 8 mice/genotype/diet regimen. Degrees of freedom are reported within parentheses, alpha = 0.05. Asterisks indicate significant difference within genotype (i.e., diet effect), and crosshatches those between genotypes (animals on same diet). Bold type indicates statistical significance. au, arbitrary units.

for the number of missteps (errors) made by each mouse. The observers were masked as to the treatment group of the mice that they were scoring. An error was scored when any foot dipped below the grid. The number of errors was averaged across the 5 trials per mouse to give the final reported values. The apparatus was cleaned with 70% alcohol and allowed to dry completely between trials.

Statistical analysis

The sample size per group was determined by both our empirical experience with the variability in the prior measures in the BACHD mice and a power analysis (SigmaPlot, SYSTAT Software, San Jose, CA) that assumed a power of 0.8 and an alpha of 0.05. Data sets were examined for normality (Shapiro-Wilk test) and equal variance (Brown-Forsythe test). To determine the impact of the diet on temporal activity, sleep, and ketone waveforms, we used a two-way analysis of variance (two-way ANOVA) with treatment and time as factors. All the other data were analyzed using two-way ANOVA with diet and genotype as factors. Pairwise Multiple Comparison Procedures were made using the Holm-Sidak's method. Between-group differences were determined significant if $p < 0.05$. Values are reported in the tables as mean ± standard deviation (SD) or in the figures as mean ± standard error of the mean (SEM).

Results

In these experiments, we examined the effect(s) of a KD on BACHD and WT mice in comparison with mice on ND. Both KD and ND groups had *ad libitum* access to food from 3 to 6–7 months of age. By the end of the study, the body weights of both WT and BACHD mice held on KD were reduced as compared to their counterparts fed a ND (Figure 1A), with significant effects of both genotype and diet. Body composition analysis carried out on a separate cohort of mice showed that adiposity was higher in the BACHD and WT mice under the ND compared to those on the KD (Figure 1B), with no effect of genotype but an unquestionable effect of the diet regimen. Thus, mice on KD were leaner independent of their genotype.

Since HD patients present with metabolic deficits, to ensure that the BACHD mice would undergo ketosis in response to the KD, we sampled tail blood at 6 time-points throughout the 24-h cycle and measured βHB. Both WT and mutant mice exhibited pronounced rhythms in βHB under KD but not under control feeding conditions (Figure 1C). The βHB levels in the WT and BACHD on KD exhibited significant effects of time and diet. Interestingly, the βHB levels were significantly elevated in the mutants at most phases (ZT 2, 6, 14, 18, and 22) of the daily cycle, whilst in WT mice, the increase was only observed during the dark phase (ZT 14, 18, 22). Therefore, both genotypes responded to the KD with the rhythmic production of ketone bodies, albeit more pronounced in the BACHD mice.

The gut microbiota is a very dynamic entity influenced by environmental and nutritional factors, which, in prior work has reported to be altered by KD (30, 31). The possible effect of KD on species composition of the gut microbiota in both genotypes was examined by sequencing fecal samples collected from WT and BACHD on both diets for 3 months. KD elicited dramatic changes in the relative abundances of the bacteria species present in the fecal samples in both genotypes (Figure 2). The top 15 species in each of the four groups (as determined by

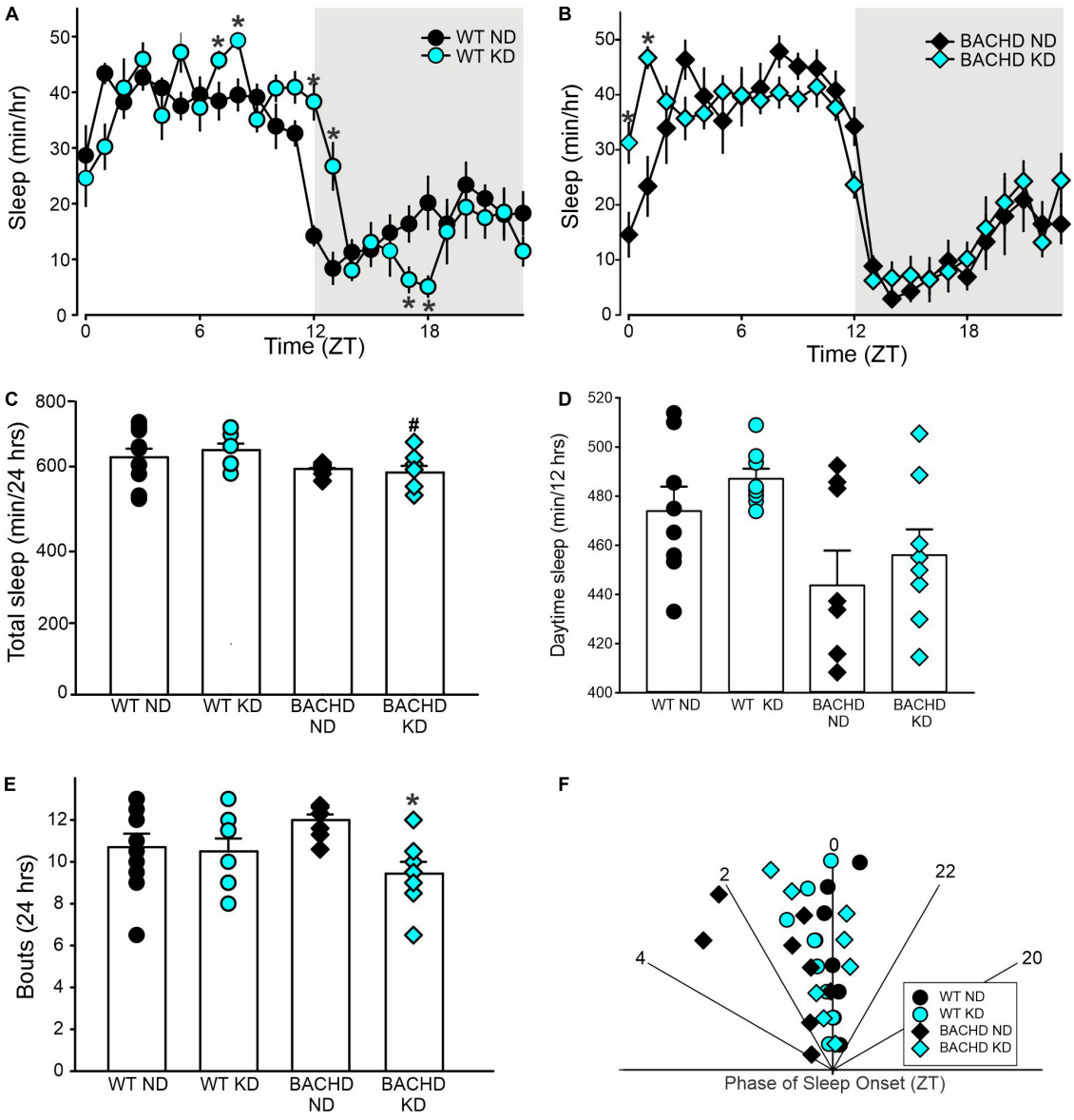

FIGURE 5
Modest improvement of the daily sleep behavior rhythms driven by the Ketogenic diet (KD) in BACHD mice. Sleep was measured in male WT and BACHD mice on normal diet (ND) or KD for 3+ months at 6–7 months of age (n = 8 mice per group). **(A,B)** Waveforms of daily rhythms in sleep behavior in WT (circles) and BACHD (diamonds) mice under ND (black) or KD (cyan). The gray shading indicates nighttime. BACHD mice on KD displayed an increase in sleep at ZT 0 and 1. The activity waveform (1 h bins) of each group was analyzed using a two-way ANOVA for repeated measures with treatment and time as factors. **(A)** For WT mice, there were significant effects of time [$F_{(23,\ 431)}$ = 30.483, $P < 0.001$] but not diet [$F_{(1,\ 431)}$ = 0.525, P = 0.469], as well as a significant interaction between the two variables [$F_{(23,\ 431)}$ = 4.149, $P < 0.001$]. **(B)** For the BACHD mice, there were significant effects of time [$F_{(23,\ 383)}$ = 32.808, $P < 0.001$] but not diet [$F_{(1,\ 383)}$ = 0.843, P = 0.359]. Again, there was a significant interaction of the two factors [$F_{(23,\ 383)}$ = 2.3619, $P < 0.001$]. **(C,D)** The KD did not affect total sleep or sleep in the day-time in the BACHD mice; still, **(E)** a significant decrease in the number of sleep bouts in the 24 h was observed. **(F)** KD reduced the phase delay in sleep onset shown by the BACHD on ND. Hence, the BACHD mice on KD were almost indistinguishable from WT. Histograms in **(C–E)** show the means ± SEM with the values from the individual animals overlaid. Properties of the daily sleep rhythms were analyzed using two-way ANOVA with genotype and treatment as factors followed by the Holm-Sidak's test for multiple comparisons (n = 8 per group) (see also **Table 4**). *$P < 0.05$ vs. mice on ND (effect of diet); $^{\#}P < 0.05$ between genotypes (same diet).

relative abundances) were compiled, and the overlapping species identified (**Table 1**). Most of the species displayed statistically significant differences in relative abundances due to the diet, with no effects of genotype. It is noteworthy that the probiotic *Akkermansia municiphila* dramatically increased in abundance in both KD groups. Under ND, this bacterium represented 2.8 and 3.8% of the total species found in WT and BACHD, respectively, while, under KD, this species increased to 60.2% in

WT and 44% in the mutants. Both diet and the genotype had a significant effect on the abundance of one species (*Alistipes* sp. UBA6068) (Table 1) along with a significant interaction of the two factors [$F_{(1,19)} = 5.928$; $P = 0.027$]. The evidence that effective changes were driven in the microbiome by the KD suggests that the diet is actively influencing both biological and metabolical pathways in these mutants.

One of the key pathological features of HD are transcriptional changes in the striatum (32, 33). Hence, we sought to determine if KD altered gene expression patterns in the striatum using NanoString transcriptomic analysis. A total of 30 differentially expressed genes was identified in 2 groups-comparisons (effect of diet within genotype or between genotypes), with the highest number in the BACHD-KD vs. WT-KD comparison (Figure 3 and Supplementary Figure 1). Eight genes were found to be upregulated in the BACHD-KD compared to BACHD on ND (Figure 3 and Supplementary Figure 1), while KD elicited changes in 11 genes in WT, with 2 genes upregulated and 9 downregulated. Among these, only one gene was found to be altered in both genotypes, *Npy*. When comparing the effect of the KD in BACHD and WT, there were 16 genes differentially expressed (10 upregulated and 6 downregulated), with two of the upregulated genes, *Calb2* and *Il6ra*, showing a similar change to that observed in the BACHD-KD vs. BACHD ND comparison (Figure 3 and Supplementary Figure 1). There were only 4 genes differentially expressed between the BACHD and WT mice on ND, all downregulated in the BACHD. It is notable that 2 of them, *Dlx1* and *Fos*, were upregulated in the BACHD by the KD, suggesting that this diet may influence cell function in the striatum.

Further analyses of the 30 identified genes using two-way ANOVA with diet and genotype as factors (Table 2 and Supplementary Figure 2) revealed that among those significantly affected by the diet were the neurotrophic factor *Bdnf*, the immediate early gene *Fos*, the proinflammatory cytokine *Interleukin 6* (*Il6*), and the oncostatin M receptor *Osmr* (type I cytokine receptor family). Both diet and genotype had a significant effect on genes involved in various physiological response, including inflammation and stress (*Il6ra*, *Nos1,* and *Npy*). A larger number of transcripts (neurotransmitter receptors, transcription factors, factors involved in neurodegenerative disorders, Table 2) exhibited a significant effect of genotype and, perhaps, more importantly, a significant interaction between the two variables. For example, transcripts exhibiting interactions between genotype and diet (Table 2 and Supplementary Figure 2) include *Adora2a*, an adenosine receptor involved in inflammation, and regulation of sleep, along with genes involved in metabolic and neurodegenerative diseases (*Adcy8, Lrrk2*), as well as in neuronal excitability and synaptic activity (*Calb2*, *Dlx1*). Strikingly, the expression of the sleep regulated gene *Homer1* was significantly influenced by the interaction between the two variables, and its levels were significantly increase in the WT-KD in comparison to the WT-ND and BACHD-KD. To reveal the enrichment of certain biological pathways, we used the Reactome Database in Rosalind.bio and reported in Table 3, only those terms reaching statistical significance at a FDR < 0.05 in at least one comparison as these could reflect a biological response to the KD. This analysis showed changes in a diverse set of gene categories/biological functions, such as oxidative stress, transcription factors, cell adhesion molecules and inflammation. No overlaps were observed, and strikingly, the comparison with the most enriched pathways was the BACHD ND vs. WT ND (Table 3). Hence, the KD altered the expression of a number of transcripts in the striatum, some of which (e.g., *Bdnf, Homer1, Il6ra, Lrrk2, Scnb*) implicated in neurodegenerative disorders, inflammation or sleep and could be potential targets for future work.

BACHD mice exhibit altered sleep-wake cycles (34, 35). As shown in Figure 4, a number of parameters of the temporal pattern of cage activity (Figures 4A,B) were improved by the KD, in WT as well as mutants. Analysis of the activity waveform by two-way ANOVA with time and diet as factors indicated significant effects of time in both WT and BACHD mice, along with a significant interaction of the two variables. Most importantly, in the BACHD, the KD significantly reduced the non-characteristic activity during the day. Further analysis of the activity rhythms for all 4 groups (Table 4) found that the power of the rhythms (Figure 4C) was significantly altered by diet as was the average amount of activity per hour in the BACHD was increased by the KD (Figure 4D). The non-characteristic daytime activity as well as the variation in the onset of activity rhythms seen in the mutants were improved by the KD (Figures 4E,F). Finally, the lengthening of the free-running circadian period observed in the BACHD mice (Figure 4G) was not altered by the diet suggesting that the KD might not influence the molecular clock. Overall, the BACHD mutants exhibited weaker activity rhythms than WT, and KD improved many of the activity parameters.

The amount of sleep is controlled by homeostatic mechanisms and was largely unaltered by the KD. For WT and BACHD mice, analysis of the sleep waveform (Figures 5A,B) indicated significant effects of time, but not diet, as well as a significant interaction between the factors. The total amount of sleep in a 24-h cycle was modestly reduced in both BACHD groups and unaltered by the diet (Figure 5C and Table 4). Overall, the KD did not significantly increase sleep behavior during the day (Figure 5D) despite the increase seen at ZT 0 and 1. The BACHD on KD displayed a reduced total number of sleep bouts in a 24-h period (Figure 5E and Table 4) as well as an advance in sleep onset (Figure 5F and Table 4). The mutant mice on ND started sleeping at ZT 1.8 ± 1.4 (108 min after lights on), while those on the KD at ZT 0.2 ± 0.7 or 12 min after lights-on. In summary, the KD produced a modest reduction in sleep fragmentation and corrected the phase delay in sleep onset seen in the BACHD mice.

The defining symptoms of HD are centered on motor dysfunction hence, we hypothesized that KD should also improve motor performance in the BACHD model. Motor performance was assessed using well-defined tests: the accelerating rotarod, grip strength, and challenging beam tests (**Figure 6** and **Table 5**). As previously described, the BACHD mice exhibited worse performance in all three measures compared to WT at 6 months of age. The KD improved the performance of the mutant mice in the rotarod and challenging beam, but did not impact grip strength (**Figure 6** and **Table 5**). The KD did not alter the performance of the WT mice in the rotarod or grip strength assays while the challenging beam was not evaluated. Overall, the improvement in motor performance exhibited by the BACHD on KD is a key finding of this study.

Discussion

We have previously shown that scheduled feeding improves the sleep/wake cycle and motor performance in mouse models of HD (21, 22). These types of time-restricted feeding schedules produce a range of changes including an increase in ketosis (13). Hence, we became interested in the possibility that ketogenesis could underlie the improvements seen in these earlier works (36). However, HD patients and mouse models are known to exhibit a range of metabolic dysfunctions (6, 7) and so it was important to confirm that KD produced the intended effects in the BACHD model. We utilized a KD with moderately high protein, no sugars and predominantly healthy fats, quite distinct from that in some prior work: a "high-fat diet" with high sugar (averaging a stunning 20% of total Kcals from sucrose). Measuring body weight and composition, we found that the BACHD mice held on KD weighed less and exhibited lower body fat compared to the mutant mice held on ND (**Figure 1**). The mutant mice exhibited higher levels of βHB than WT on a normal diet and, importantly, the *ad libitum* KD generated a rhythm in ketone bodies peaking in the night (**Figure 1**). This is consistent with prior work by the Sassone-Corsi laboratory, who also found that KD-fed mice exhibit a robust oscillation in βHB serum levels (37). These rhythms are presumably driven by daily rhythm in feeding along with the well-characterized rhythm in the production of ketones in the liver (23, 38). These experimental observations provide a nice example of how a change in diet can generate a rhythm in a key metabolic parameter and thus fall into the category of chrono-nutrition.

Microbiome

Prior work has provided evidence that the KD drives changes in the microbiome that can be measured in fecal samples (30, 39). Therefore, we sought to determine if the KD produced similar changes in the BACHD model. As measured

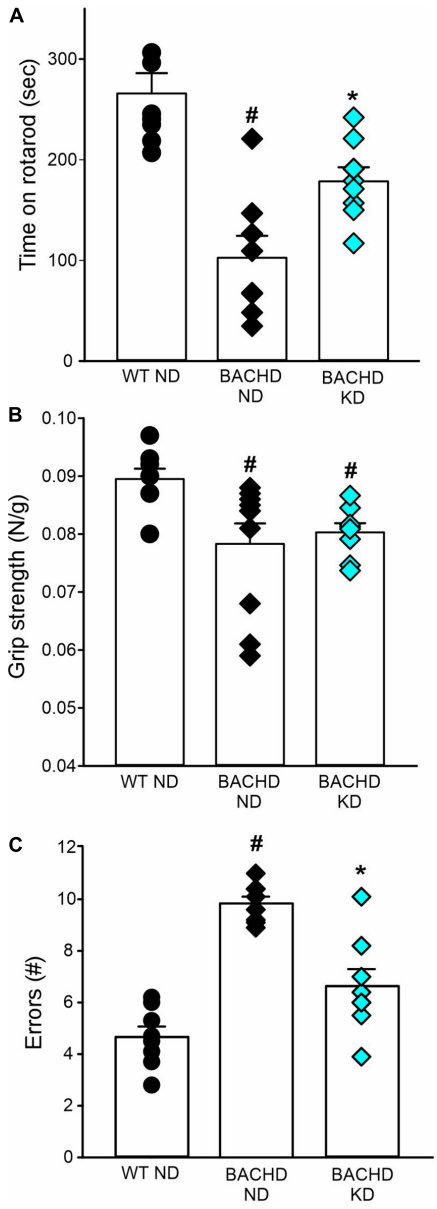

FIGURE 6
Ketogenic diet (KD) resulted in improved motor functions in the BACHD mice. Behavioral measures including time on rotarod, grip strength, and errors on the challenging beam were measured in WT and BACHD mice fed with either ND or KD at about 6.5–7 months of age (n = 8–10 mice per group) after monitoring sleep and activity rhythms. We have previously found that the BACHD exhibit deficits in all these tests and WT data are shown for comparison. **(A)** The time and performance on the rotarod were significantly improved in the BACHD on the KD. **(B)** Grip strength was not affected by the diet in the BACHD, that are not different by their counterpart on ND. **(C)** The total number of errors on the challenging beam test was significantly reduced in the BACHD mice on the KD. Histograms show the means ± SEM with the values from the individual animals overlaid. Motor data were analyzed using two-way ANOVA with genotype and treatment as factors followed by the Holm-Sidak's test for multiple comparisons (see also **Table 5**). $^{*}P < 0.05$ vs. mice on ND (effect of diet); $^{\#}P < 0.05$ between genotypes (same diet).

TABLE 5 Impact of Ketogenic diet on motor performance.

	WT		BACHD				
	ND	KD	ND	KD	Diet	Genotype	Interaction
Rotarod (s)	265.8 ± 57	276.8 ± 54	102.6 ± 62$^{\#}$	178 ± 40*	$\boldsymbol{F_{(1,31)} = 8.34; P = 0.007}$	$\boldsymbol{F_{(1,31)} = 63.33; P < 0.001}$	$F_{(1,31)} = 2.30; P = 0.140$
Grip strength (N/g)	0.089 ± 0.005	0.087 ± 0.006	0.078 ± 0.004$^{\#}$	0.080 ± 0.002$^{\#}$	$F_{(1,33)} = 0.25; P = 0.622$	$\boldsymbol{F_{(1,33)} = 6.62; P = 0.015}$	$F_{(1,33)} = 0.03; P = 0.871$
Errors on beam (#)	19.5 ± 3.7	NA	25.6 ± 6.4$^{\#}$	11.4 ± 2.6*	$\boldsymbol{F_{(1,23)} = 58.72; P < 0.001}$	$\boldsymbol{F_{(1,23)} = 5.86; P = 0.022}$	$F_{(1,23)} = 2.53; P = 0.123$

Grip strength measures for each animal were normalized to its body weight. Data were analyzed by two-way ANOVA with genotype and diet as factors followed by Holm-Sidak's multiple comparisons test and are expressed as the mean ± SD of 8–10 mice/genotype/diet regimen. Degrees of freedom are reported within parentheses, alpha = 0.05. Asterisks indicate significant difference within genotype (i.e., diet effect), whilst crosshatches those between genotypes (i.e., same diet). Bold type indicates statistical significance. ND, normal diet; KD, ketogenic diet; NA, not available.

by changes in species abundance, we confirmed that KD drove dramatic changes in the microbiome of both BACHD and WT mice (**Figure 2** and **Table 1**). Changes in microbiota composition under KD could be due to either a maladaptive or an adaptive condition of the gut. Broadly, the patterns in the changes in species abundance looked similar between the two genotypes. It is noteworthy that the probiotic *Akkermansia muciniphila* dramatically increased in abundance under KD. For example, under ND, this species represented 4% of the total species found in the BACHD while, under KD, this species increased to 44%. These observations are very similar to prior work showing that the KD can dramatically increase *A. muciniphila* (40, 41). Although not the focus of the present study, *A. muciniphila* is associated with improved metabolic health in both animal and human studies (42–44). This species could be an interesting focus of future experiments designed to determine if the changes in the microbiome impacted the behavioral outcomes. Interestingly, work in a *Drosophila* model of HD, suggest that the gut bacteria can regulate the pathology of HD (45), indicating that KD-driven changes in the microbiome could even influence HD-pathology. At a minimum, the results of our analysis confirm that the KD alters the composition of the gut microbiota in the BACHD model.

Transcriptional changes in the striatum

Since one of the features of HD is transcriptional changes in the striatum (32, 33), we sought to determine if the KD could alter the transcriptional landscape in this brain region. Prior work found evidence that the KD increased the number of rhythmic genes in the liver and gut with a sharp peak in the middle of the day (37). While we did not examine gene expression in the striatum across the circadian cycle, we did not see much evidence for a large change in transcription in the striatum at night. However, there were a few transcripts altered by diet alone including *Bdnf*, *Fos*, *Il6*, and cytokine receptor *Osmr* (**Figure 3**, **Supplementary Figure 1**, and **Table 2**). BDNF is of particular importance in HD as a variety of studies have reported reduced levels in both patients and animal models (46–48), which is likely to have implications for neuronal survival. Prior studies have found evidence of a KD-driven increase in BDNF levels (49, 50) although these findings were not universal (51). A larger number of transcripts were altered by both genotype and diet and more importantly, several exhibited a significant interaction between these two factors including, among others Adora2a, *Nos1*, *Homer1*, and *Il6ra* (**Figure 3**, **Supplementary Figure 1**, and **Table 2**). Interestingly, neural activity in Nos1 expressing neurons is thought to be critical for sleep regulation (52, 53) and levels of *Homer1* change in response to sleep deprivation (54, 55). While exploring the possible function of these transcriptional changes is beyond the scope of the present study, hopefully these transcriptional changes can be pursued in future work. In the context of this study, the transcriptional analysis does demonstrate that diet can modulate gene expression in the striatum and presumably other regions within the nervous system. Overall, the data from both the microbiome and transcriptional analyses demonstrate that the KD is biologically impactful in the BACHD model.

Sleep and activity

Disturbances in the sleep/wake cycle including prolonged latency to fall asleep, sleep fragmentation and difficulty maintaining wakefulness during the day are common in HD and often become apparent years before the onset of classic motor symptoms (56). Similarly, mouse models of HD also exhibit a disrupted circadian rest/activity cycle that mimics the symptoms observed in human patients (34, 57, 58). We have previously found that scheduled feeding (TRF) improved many of these deficits in animal models (21, 22). In the present study, we found that the KD enhanced several parameters of activity rhythms including rhythm power,% of activity in the day, and variation in activity onset such that the treated BACHD were indistinguishable from WT controls (**Figure 4** and **Table 4**). One key exception is the lengthening of the free-running circadian period seen in the BACHD mice under constant dark conditions. The failure of the KD to correct this change suggests that the molecular circadian clock at the level of the suprachiasmatic nucleus (SCN) was unlikely to be altered

by the diet. This finding is consistent with our prior work in which we found that TRF altered the phase of the PER2:LUC rhythms measured *in vivo* and *in vitro* outside of the SCN but did not impact the amplitude or phase of the rhythms measured in the SCN (21). In humans, there is also evidence that TRF alters rhythms in metabolites without perturbing clock gene expression (18).

In the present study, we found that the KD reduced the known sleep fragmentation and corrected the delay in sleep onset of the mutants (**Figure 5** and **Table 4**). Since we were using a behavioral assay, we could not determine if the diet also impacted changes in the EEG power distribution, such as changes in beta and gamma activity, which are commonly seen in HD models (59–62). In humans, the published data on KD and sleep was inconsistent although at least one study did find that this regimen did increase slow wave sleep in men (63).

Motor performance

HD is a movement disorder with hallmark symptoms of this disease including motor deficits and loss of neurons within the basal ganglia circuits. Importantly, KD did delay the reduction in motor performance as measured by rotarod and challenging beam (**Figure 6** and **Table 5**). On the other hand, grip strength was not improved by KD. We have previously found improved motor performance on these same assays in the BACHD and Q175 lines of mice at the same age using scheduled feeding (21, 22). In the R6/2 mouse model, providing food only during the activity period (12 h feed/fast cycle) did improve performance of the mice on a battery of neurological tests (SHIRPA) as well as locomotor activity levels (64). The beneficial impact of KD on motor performance could be dependent upon or independent from the improvements in circadian output. In prior work, we found that the improved circadian behavior was correlated with improved motor function when we used a scheduled feeding protocol (22). This finding is at least consistent with the possibility that the improved sleep/wake cycle driven by the KD underlies the improved motor function in the treated mice. Thus, KD joins a growing list of interventions including TRF (21, 22); sleep-inducing drugs (65, 66), stimulants (67, 68), bright light & restricted wheel access (69) and blue light (28), which can improve motor function in HD models. This body of work supports our general hypothesis that circadian-based interventions and chrono-nutrition can improve symptoms in neurodegenerative disorders (70).

Mechanisms

We do not know the mechanism through which the KD produced the benefits that we observed in the BACHD mice. Generally, the KD shifts the body into a state of elevated ketone body production and increasing serum ketone bodies, called ketogenesis or ketosis. Increasing the production of ketone bodies by the liver is dependent upon achieving a period during which glycogen stores are being depleted, such as during fasting, or by consuming a low-carbohydrate, high-fat diet, such as the one used in this study. During these states, ketone bodies are generated at higher levels, regulated by the rate limiting enzymes carnitine-palmitoyl transferase 1a (CPT1A), gating beta-oxidation, and hydroxymethyl-glutaryl CoA synthase 2 (HMGCS2), gating ketone body production, which are expressed in a circadian regulated manner (23). Notably, βHB and acetoacetate readily enter tissue and cross the blood–brain barrier through monocarboxylic transporters (38, 71). The primary fate of ketone bodies is to be converted to acetyl CoA in extrahepatic mitochondria and enter the Krebs cycle at the level of citrate, bypassing glycolysis to generate ATP. Notably, this happens with higher efficiency and lower production of ROS compared to glucose (72). While most of the βHB that is used as an energy source in the brain is synthesized by the liver, ketone bodies also undergo synthesis and release by astrocytes (73). Ketone bodies are thought to have direct effects on inflammatory molecules (74, 75), mitochondria (76), histone deacetylases (77), and BDNF expression (see above). Therefore, there are a number of important pathways through which the KD could be specifically benefiting the BACHD model as well as generally neurodegeneration (24, 25, 78).

While we do not know which biochemical pathways underlie the observed benefits of KD, there is growing evidence for sleep/wake regulation of the clearance of misfolded proteins in the brain glymphatic system, an astroglial-mediated interstitial fluid bulk flow (79). In HD and other neurodegenerative disorders, it has been proposed that sleep fragmentation drives a decline in clearance of brain waste. The activity of the glymphatic system is high during sleep and low during wakefulness (80). There are daily rhythms in both Aβ levels, as well as extracellular levels of tau (81). Sleep-deprivation increases Aβ plaque deposition, as well as tau pathology (82, 83). While sleep may be a direct driver, there is also good reason to suspect the circadian system involvement as well. For example, deletion of *Bmal1* causes severe circadian fragmentation, significantly blunts Aβ rhythms, and increases amyloid plaque deposition in a transgenic mouse model of AD (84). By improving sleep, the KD may be delaying the formation of aggregates in HD.

Limitations

We were forced to bring this study to a premature closure because of COVID-19. Due to the research stoppage, there

were some compromises, and this study presents several limitations. We did not collect data on the impact of the KD on the challenging beam task in WT mice. In addition, recently, concerns were raised on the negative impact of KD on cognition (85–87) and our study lacks cognitive measurements. Furthermore, the data on body composition and on the free-running period of circadian rhythms in locomotor activity were collected on a different cohort of mice. The study was originally designed to follow both sexes, as we have previously reported the presence of sex differences in the BACHD mice (35), with the females presenting less severe symptoms at early stages of disease. Sadly, another limitation of the present study is the usage of only male mice. Finally, we were unable to extend the study beyond 6 months of age, and age is likely another important critical factor. It should be underlined that we began the treatment at 3 months, when the BACHD young adults, largely, do not exhibit any phenotype at least for the parameters measured. In general, this line exhibits neuropathological changes such as striatal and cortical volume loss, protein aggregation and neuronal degeneration beyond 12 months (27). Intriguingly, KD was shown to reduce amyloid-β42 and β40 in APP (Amyloid Precursor Protein) mice (88), a mouse model for Alzheimer's disease, and recently it has been suggested that nutritional ketosis could improve several astrocytic functions, while reducing astrogliosis (89). Future work will need to determine whether KD can ameliorate mitochondrial dysfunction, protein aggregation, ER stress, and/or facilitates autophagy later in disease progression. Obviously, because of all the above-mentioned limitations, more work is required, and caution should be used in the interpretation of our results.

Summary

The weight of clinical and preclinical research indicate circadian and sleep dysfunction should be considered a core symptom of HD. We have previously shown that a feeding schedule benefits HD mouse models and that this treatment can drive a spike in ketones. Hence, in this study, we report that a KD effectively drove a rhythm in ketone bodies in serum of both WT and BACHD mice, but also dramatically altered their gut microbiome compositions and produced selective changes of the transcriptional landscape in the striatum. The KD strongly improved the activity rhythms as well as reducing fragmentation and the ameliorated the delayed sleep onset in the mutants. Motor performance on rotarod and challenging beam were also improved, while grip strength was unaltered. It is worth emphasizing that HD is a genetically caused disease with no known cure. Life-style changes that not only improve the quality of life but also delay disease progression for HD patients are greatly needed (90). Our study demonstrates the therapeutic potential of chrono-nutrition-based treatment strategies in a pre-clinical model of HD.

Data availability statement

The data presented in this study are deposited in the Dryad repository, University of California Curation Center, accession number https://doi.org/10.5068/D1BT3K.

Ethics statement

This animal study was reviewed and approved by UCLA Division of Animal Medicine.

Author contributions

DW, GB, CG, and CC conceived the hypothesis and experimental design of this study. DW, RB, SV, SL, and TT performed the experiments. RB and DD'A analyzed the microbiome data. DW, TT, CG, and CC analyzed the data. DW wrote the first draft. CG and CC edited, wrote, and compiled the final version manuscript with contribution from the other authors. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.1034743/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Bar charts showing the fold changes in gene expression driven by the ketogenic diet in WT and BACHD animals and corrected *p*-values (app.rosalind.bio).

SUPPLEMENTARY FIGURE 2
Ketogenic diet driven changes in gene expression in the striatum of 6–7 months of WT and BACHD mice on ND or KD for 3+ months. Mice were kept on KD until euthanasia was performed at ZT14. Selected genes were analyzed by two-way ANOVA followed by Holm Sidak's multiple comparisons test. The relative expression values obtained with the nSolver software were average (n = 5–6 animals per group) and are shown as the Mean ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. mice on ND (effect of diet); $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ between genotypes (same diet) (see also **Table 2**).

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Barriers to adherence in time-restricted eating clinical trials: An early preliminary review

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Time-restricted eating (TRE) has shown potential benefits in optimizing the body's circadian rhythms and improving cardiometabolic health. However, as with all dietary interventions, a participant's ability to adhere to the protocol may be largely influenced by a variety of lifestyle factors. In TRE trials that reported participants' rates of adherence, the percentage of total days with successful adherence to TRE ranged from 47% to 95%. The purpose of this review is to (1) summarize findings of lifestyle factors affecting adherence to TRE clinical trials outside of the lab, and (2) explore a recommended set of behavioral intervention strategies for the application of TRE. A literature search on Pubmed was conducted to identify clinical TRE studies from 1988 to October 5, 2022, that investigated TRE as a dietary intervention. 21 studies included daily self-monitoring of adherence, though only 10 studies reported a combination of family, social, work, and miscellaneous barriers. To maximize participant adherence to TRE and increase the reliability of TRE clinical trials, future studies should monitor adherence, assess potential barriers, and consider incorporating a combination of behavioral intervention strategies in TRE protocols.

KEYWORDS

time-restricted eating (TRE), time-restricted feeding (TRF), intermittent fasting (IF), adherence–compliance–persistence, community dwelling adults, dietary intervention, barriers and facilitative factors, behavioral intervention

1. Introduction

The last decade has seen a proliferation of clinical trials investigating the effects of time-restricted eating (TRE) on a myriad of health outcomes. TRE is an eating-fasting schedule in which the eating window is limited to a consistent 6–10 h per day, with some studies including 4–12 h eating windows (1–3). TRE is thought to facilitate nutrient homeostasis *via* the synchronization of metabolic processes to optimal times of each circadian cycle (4), and results have been promising for the improvement of various health outcomes such as weight loss, blood pressure, and glycemic control (5–8). Recent trials have also reported significant increases in insulin sensitivity (1, 9) as well as reductions in fat mass and/or body weight (10–14). Additionally, the benefits of TRE have encompassed improvements in sleep quality and quality of life (15–17). As a novel

dietary intervention, TRE is unique in that it demands minimal medical supervision, does not require calorie counting or dietary restrictions, and can be adaptable to individual needs. The methodology across TRE trials has varied, ranging from controlled laboratory settings to implementation in community-dwelling individuals with little to no supervision. This review focuses on community-dwelling individuals, where TRE was incorporated into participants' regular daily lives—which often include family, work, and social obligations.

Despite being a relatively simple dietary intervention, TRE is still a lifestyle change, so the implementation of TRE—particularly in community-dwelling individuals—has posed some challenges. Social events, family obligations, and work commutes are external barriers that commonly influence an individual's ability to maintain a consistent eating window (18). On the other hand, psychological factors (e.g., stress and boredom) can evoke erratic eating patterns induced by stress-eating and compulsive snacking (19). The capacity for individuals to adhere to TRE provides valuable insight into its feasibility as a lifestyle; yet the confluence of external and psychological barriers—and, more importantly, how to mitigate them from a research methodology standpoint—is poorly understood. As participants' adherence to dietary interventions forms the basis of conclusions we can draw from clinical trials, increasing rates of adherence will improve our overall understanding of the impacts of TRE. In light of the challenges associated with adherence to TRE, there is a clear need to (1) explore potential patterns in barriers to adherence across TRE trials, and (2) develop TRE protocols that adapt to the inherent variability of community-dwelling individuals. We have yet to truly understand whether TRE is feasible as a long-term dietary intervention and/or component of a healthy lifestyle.

Data on barriers to TRE adherence and methods of improvement are currently limited. Of the 66 published TRE clinical trials, only 10 reported details on barriers to adherence. This review will summarize findings from these studies on lifestyle factors affecting TRE adherence, summarize participants' methods of overcoming barriers, explore how a set of behavioral intervention strategies may be applied to the TRE protocol, and provide suggestions for improving adherence in future trials.

2. Reported barriers to TRE

2.1. Socio-environmental factors

A common barrier to TRE adherence across trials was socio-environmental factors (Table 1). External pressures and responsibilities such as family commitments, work schedules, and social occasions can be challenges to maintaining TRE.

2.2. Social events

Eight of 10 TRE trials in this analysis reported social commitments as a barrier to TRE (Table 1). In a 10-week TRE trial where each participant's eating windows were shortened by 3 h, social eating and drinking events were the most commonly reported barriers. Nine of 16 participants felt TRE was unsustainable beyond 10 weeks mainly due to an incompatible social and family life (20). Similarly, participants in a 4-week 8-h TRE trial reported that food served at social events during the fasting period was a challenge to TRE adherence (25). In another trial with an 8-h eating window ending at 5 pm, having a social life was one of three main barriers identified by participants (23). Importantly, this study did not allow a self-selected eating window, so participants were required to finish their last meal between 5 and 5:30 pm. As the average dinner time in the U.S. occurs at about 6:24 pm (27), the inability to eat later in the evening may have limited nighttime social activities.

Studies that allowed a self-selected eating window, however, did not necessarily resolve conflicts between TRE and social events: in a 12-week long trial with self-selected 8-h eating windows, participants reported dining out, having visitors, and having drinks after work as the most frequent social barriers. This study also noted that adherence to TRE during weekends decreased over time, though the reason for this was unspecified (18). Bjerre et al. (23) highlighted the importance of having adequate social support during TRE trials, citing experiences of participants whose eating windows would end in the middle of a three-course meal, or were continually offered food by those around them after their eating window had ended. At the risk of seeming impolite, participants often chose to fit into their social context by continuing to eat during their fasting window. Self-selection of an eating window, while certainly helpful for catering to variations across participants' schedules, cannot entirely insulate each individual from social influences. Eating is often a shared experience that provides important opportunities to socialize, so having the support and understanding of one's social circle may likely increase a participant's ability to follow a TRE protocol.

2.3. Family

Another barrier in TRE trials was the conflict between eating windows and family-related commitments. Some participants expressed that family needs came first in their household; a TRE protocol requiring an early dinner would disrupt family schedules and would therefore be impractical (24). Similarly, participants in a shorter 4-week-long TRE trial reported that eating their last meal before 7 pm was difficult to integrate with both family and work commitments (19). Implementing a TRE schedule that interferes with regular family dinnertimes would likely negatively impact long-term adherence, as family dinners

in the US occur frequently in many households—an average of 4.1 times per week (28). In contrast, one trial noted that family support was critical to TRE adherence: spouses of enrolled participants adjusted their eating habits to be in synchrony with the TRE schedule (25).

2.4. Work

The average age of participants ranged from 29 to 77 years old (Table 1), so work schedules sometimes interfered with TRE. During a 4-week long trial, participants reported that changes in work schedules impeded TRE adherence (25). Similarly, busy workdays in which food was not able to be consumed rendered the TRE protocol to be difficult or entirely impossible (24). Other challenges included hunger at work and difficulties with integrating TRE protocol with shift work schedules (22). Despite work-related challenges, one study's survey results indicated that participants seemed to have a positive attitude overall toward their experiences with integrating work schedules with TRE: 78% of 63 participants felt TRE integrated well with their professional activities, while only 18% reported experiencing difficulties with work and TRE conflicts, and 3% considered it neither good nor difficult (21).

2.5. Psychological/physical factors

Of the few studies that collected participant feedback regarding specific barriers to TRE, psychological or physical factors such as stress, boredom, and hunger did not seem to significantly affect adherence (19, 22, 23). In one trial, hunger was reported as a difficulty by five out of 40 participants during the initial few weeks of the intervention period (22). Parr et al. (24) found that participants ate larger meals to avoid hunger during the fasting period. Similarly, concerns about potential hunger drove some participants to consume more food than usual during their eating window despite not feeling hungry (23). This behavior decreased as the intervention progressed, though it is unclear to what extent it negatively affected adherence. In most TRE studies, however, researchers have noted an overall decrease in calories.

2.6. Participants' reported strategies for overcoming barriers

Participants reported using a variety of strategies to overcome the aforementioned barriers.

2.6.1. Planning ahead

Some participants prepared meals ahead of time in case they did not have enough time to cook before their eating window ended, while others carried food with them for easy access. Few participants also set alarms to remind them when to begin cooking dinner, or when to stop eating (23). The benefits of planning to increase protocol adherence are compounded by food choices, as participants who did not plan reported reaching for less healthy options to adhere to their eating window (20).

2.6.2. Activities

For some, eating an earlier dinner meant creating a void in activity at night in which they felt bored. Coping methods included filling time with other usual activities (e.g., watching television, reading, and sitting at the computer), or simply going to bed earlier. In one study, some participants woke up later as a strategy to avoid early morning hunger while waiting for their eating window to begin (23). When zero-calorie beverages were permitted while fasting, participants reported that drinking water or black coffee helped to distract from hunger (18).

3. Potential strategies to increase adherence: American Health Association's Evidence-based strategies to enhance adherence to changes in diet and eating behaviors

Sixty-six TRE trials were examined for the following components: study design, daily eating window duration, intervention duration, method of tracking adherence, and adherence rate. We were specifically interested in the studies' method(s) of tracking adherence to TRE, which were categorized based on the American Health Association (AHA)/American College of Cardiology's (ACC) evidence-based strategies to enhance adherence to changes in diet and eating behaviors (29). These strategies have been used to encourage changes in dietary composition and intake—such as following a Mediterranean diet, or reducing alcohol and sugar intake—but have not yet been applied to TRE. Strategies include goal setting, self-monitoring, tailoring the regimen, ongoing contact, reinforcement, and social support.

3.1. Goal setting

A critical aspect of goal-setting in the context of TRE is setting realistic expectations at the beginning of the intervention. The reality of any dietary intervention is that some level of sacrifice and compromise needs to be made. For example, the design of TRE makes it nearly impossible for someone to be

TABLE 1 Reported barriers to adherence in TRE trials.

References	Study design	Participants (total; completed)	Baseline characteristics	Age (mean or range)	TRE intervention (eating window duration)	Duration	Barrier categories	Frequency of barriers (# of participants)	Description of barriers
Tinsley et al. (12)	RCT	T: $n = 28$; C: $n = 18$	Healthy	22 (SD = 2.4)	4 h (6p−10p)	4 days/week for 8 weeks	Social	Not reported	Social eating opportunities
Antoni et al. (20)	NRXT	T: $n = 16$; C: $n = 13$	Healthy	29–54	Shortened eating window by 3 h (delayed the first meal and advanced last meal by 1.5 h each)	10 weeks	Social	Not reported	Social eating/drinking events
Parr et al. (19)	RXT	T: $n = 14$; C: $n = 11$	Overweight/obese	38.5 (SD = 5)	8 h (meals at 10 am, 1 pm, and 5 pm)	5 days each arm w/10-day washout	Family, work, and social	Work: 11 of 11; social life: 8 of 11; family life: 5 of 11	The main barriers to TRE were work schedules ($n = 11$), social life ($n = 8$), and family life schedules ($n = 5$)
Kesztyüs et al. (21)	Pre-post	T: $n = 63$; C: $n = 61$	Healthy, employed	47.8 (SD = 10.5)	8–9 h (self-selected)	12 weeks	Work	11 of 61	18% of participants did not report good compatibility of TRE with work schedules
Kesztyüs et al. (22)	Pre-post	T: $n = 40$; C: $n = 38$	At least 1 component of metabolic syndrome (63% on daily medication)	49.1 (SD = 12.4)	8–9 h (self-selected)	12 weeks	Work and physical/ psychological	Work: 6/38; Hunger: 13/38	25 participants combined TRF very well or well with their daily work routine, 6 badly or very badly, and 6 were in between. Daily hunger was reported by 3, several days per week by 10, and once a week or less by 22 participants
Bjerre et al. (23)	Interviews from RCT	T: $n = 17$; C: $n = 17$	BMI $\geq$ 30 and eating window of $\geq$ 12 h and at least 1 day/week $\geq$ 14 h	46–68	10 h (between 6 am and 8 pm); self-selected	12 weeks	Work and social	Not reported	The largest barriers were social evening activities and societal constraints such as normal working and operating hours of establishments
Parr et al. (24)	Pre-post	T: $n = 24$; C: $n = 19$	Type-II diabetes	50 (SD = 9.0)	9 h (10 am−7 pm)	4 weeks	Family, work, social, and physical/ psychological	Not reported	Hunger and eating before 7 pm were difficult to integrate with social, work, and family commitments. Nighttime snacking due to stress or boredom

(Continued)

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TABLE 1 (Continued)

References	Study design	Participants (total; completed)	Baseline characteristics	Age (mean or range)	TRE intervention (eating window duration)	Duration	Barrier categories	Frequency of barriers (# of participants)	Description of barriers
Przulj et al. (18)	Pre-post	T: $n = 52$; C: $n = 50$	Obesity with BMI $\geq$ 30 kg/m^2 or $>$28 kg/m^2 with comorbidities	50.1	8 h (self-selected)	12 weeks	Social	Not reported	The most common barriers were social occasions including eating out, hosting visitors, and having drinks after work
Lee et al. (25)	Pre-post	T: $n = 10$; C: $n = 9$	Overweight and sedentary	77.1	Gradual adjustment to 8 h TRE (self-selected)	4 weeks	Social and work	Not reported	Barriers included social eating events during the fasting window and changes in work schedules
Vidmar et al. (26)	RCT	T: $n = 50$; C: $n = 45$	Adolescents with BMI $\geq$ 95th percentile	14–18	8 h (self-selected)	12 weeks	Social, family, and work	7 of 45	Participants reported barriers including incompatibility with work or sleep schedule, conflict with social events, and difficulty explaining dietary intervention to family

able to eat breakfast before a regular work shift (at or before 9 am) while also grabbing drinks in the evening. Similarly, participants with families may not be able to have both breakfast and dinner with their spouses and/or children on weekdays. Thus, study coordinators should work with participants to make a judgment call as to how their current lifestyle may fit in with the suggested TRE protocol, evaluate the aspects of their current eating patterns that are the most important to them, and prioritize accordingly.

3.2. Self-monitoring

Self-monitoring, as defined by AHA, is "systematically observing and recording one's behavior." In the context of TRE, it refers to any form of self-reported data from participants regarding their adherence. The advantages of incorporating self-monitoring in TRE trials are two-fold: researchers can gather data on adherence rates, and participants may feel an increased sense of accountability when their daily choices are being monitored. In a 4-week TRE trial where meals were recorded on a smartphone app, Parr et al. (19) suggested that self-monitoring allowed participants to identify patterns/relationships between their eating habits and their health, particularly between dietary choices and blood glucose, which increased overall feelings of self-awareness and accountability.

A total of 21 studies utilized daily self-monitoring, in which participants recorded either (1) all food intake on a phone app, or (2) the timing and/or content of their first and last calorie in a written diary or electronically (Table 2). As with any form of self-reporting, a drawback of self-monitoring is the possibility of inaccurately reported data and increased user burden. Using a smartphone application (e.g., Easy Diet Diary or myCircadianClock) that automatically generates time stamps with food entries can increase the accuracy of eating window data. myCircadianClock, the only TRE research app created by a research team, is functional on both Android and iPhones (38) and allows participants to record food intake with a brief note and a picture. Study coordinators are also able to remotely check participants' eating histories and daily progress on protocol adherence. However, meals eaten can still be omitted by the participant—intentionally or not—which highlights the need for self-monitoring to be used as part of a multi-faceted approach to managing adherence.

It is difficult to examine how incorporating self-monitoring may have affected adherence to TRE in prior studies, as many studies did not monitor or report on rates of adherence. This also is a likely contributor to differences in outcomes between studies. Nonetheless, it is clear from other behavioral interventions, such as caloric restriction, that incorporating daily self-monitoring is necessary to track participant adherence throughout a study. A caloric restriction requires calorie

TABLE 2 Adherence rates and methods used to encourage adherence.

References	Study design	Eating window	Intervention duration	Method of tracking and encouraging adherence	Evidence-based method to increase adherence	Adherence to TRE
Chow et al. (30)	RCT	8 h (self-selected start time)	12 weeks	Daily food logging in myCircadianClock app	Self-monitoring, ongoing contact, and reinforcement	66%
Przulj et al. (18)	Pre-post	8 h (self-selected start time)	12 weeks	Clinic visits at 1, 6, and 12 weeks. Phone calls at weeks 2, 3, 4, and 5. Participants were also given a diary card to log their daily eating window and keep track of their TRE adherence/hunger ratings	Ongoing contact and self-monitoring	71%
Parr et al. (19)	Pre-post	9 h (10 am—7 pm)	4 weeks	Self-monitoring smartphone app (EasyDietDiary) to record all food entries. Photos of each food/beverage were taken using their phone	Self-monitoring	72%
Kesztyüs et al. (21)	Pre-post	8–9 h (self-selected start time)	12 weeks	The daily food journal of first and last calorie	Self-monitoring	72%
Kesztyüs et al. (16)	Secondary analysis of 2 pre-post studies	8–9 h (self-selected start time)	12 weeks	The daily food journal of first and last calories	Self-monitoring	77%
Gabel et al. (31)	Single arm with matched historical control with weight loss trial	8 h (10 am—6 pm)	12 weeks	Daily adherence log (first and last calorie). And 7-day food journal during baseline and week 12 of intervention	Self-monitoring	80%
Gabel et al. (11)	Pre-post	8 h (10 am—6 pm)	12 weeks	Daily adherence log (first and last calorie) and 7-day food journal during baseline and week 12 of intervention	Self-monitoring	80%
Anton et al. (32)	Pre-post	8 h (12 h for the first week)	4 weeks	The daily food journal of first and last calories. Contacted *via* phone at the end of weeks 1, 2, and 3 to review protocol and discuss any adverse events	Self-monitoring and ongoing contact	84%
Martens et al. (33)	RCT	8 h (start time between 10–11 am)	6 weeks	A daily electronic survey administered *via* email; is sent at 7 pm daily. Participants reported their first and last eating events	Self-monitoring	84%
Kesztyüs et al. (22)	Pre-post	8–9 h (self-selected start time)	12 weeks	The daily food journal of first and last calories. Telephone call 2–3 weeks after fasting initiation to discuss coping with fasting, whether they noticed changes, how they felt about their health, and if any problems arose	Self-monitoring and ongoing contact	86%
Cienfuegos et al. (1)	RCT (3 arm)	4-h (3–7 pm), 6-h (1–7 pm)	8 weeks	Daily adherence log (first and last calorie) and weekly review with the study coordinator, who would emphasize the importance of eating within the window at the end of each meeting	Self-monitoring, ongoing contact, and reinforcement	89%

(Continued)

TABLE 2 (Continued)

References	Study design	Eating window	Intervention duration	Method of tracking and encouraging adherence	Evidence-based method to increase adherence	Adherence to TRE
Wilkinson et al. (6)	Pre-post	10 h (self-selected start time)	12 weeks	Daily food logging in myCircadianClock app. Participants received educational tips and reminders *via* in-app push notifications. Participants who were non-adherent or not logging sufficiently were contacted	Self-monitoring, ongoing contact, and reinforcement	94%
Brady et al. (34)	RCT	8 h (self-selected start time)	8 weeks	Daily food log of eating windows. Full food diaries at baseline, 4 weeks, and 8 weeks	Self-monitoring	95%
Lowe et al. (35)	RCT	8 h (12 pm–8 pm)	12 weeks	A self-monitoring smartphone app that asked participants if they were compliant with TRE every day	Self-monitoring	84%
Prasad et al. (8)	Pre-post	10 h (self-selected start time)	90 days (12.8 weeks)	Daily food logging in myCircadianClock app. Study coordinators monitored participants and sent reminders to log in/use the app *via* text or in-app push notifications	Self-monitoring and ongoing contact	47%
Vidmar et al. (26)	RCT	8 h (self-selected start time)	12 weeks	Self-recorded start/stop times of daily food intake and reported these times to study staff each week. 24 h dietary recall was conducted at three-time points during the study	Self-monitoring and ongoing contact	74%
Zhang et al. (36)	RCT	6 h (eTRE: 7 am–1 pm, lTRE: 12 pm–6 pm)	10 weeks	Daily adherence log used to record start/stop times of eating	Self-monitoring	eTRE: 89% lots: 78%
Kleckner et al. (37)	RCT	10 h (self-selected start time)	2 weeks	Daily food diary of first and last calories. 24 h dietary recall was conducted at Baseline and day 14	Self-monitoring, Ongoing contact	90%
Manoogian et al. (38)	RCT	10 h (self-selected start time)	12 weeks	Daily food logging in myCircadianClock app. Participants received educational information and reminders *via* in-app push notifications to encourage engagement and adherence. Study coordinators monitored participants 2–3 times/week and contacted participants who were non-adherent or not logging sufficiently	Self-monitoring, ongoing contact, and reinforcement	71%
Haganes et al. (39)	RCT	<10 h	7 weeks	Participants reported their daily eating window. All participants were contacted *via* phone/e-mail by study investigators every week to provide support and encouragement	Self-monitoring and ongoing contact	86%
Jamshed et al. (40)	RCT	8 h (7 am–3 pm)	14 weeks	Participants reported their daily eating window through surveys administered *via* REDCap software. Eating within their assigned window within 30 mins was considered adherent	Self-monitoring	86%

The adherence rate refers to the percentage of days during intervention when participants were compliant with their TRE protocol. Studies that were shorter than 4 weeks, did not include some form of daily monitoring of compliance or did not report their participants' protocol adherence rates were not included. Studies' methods of tracking and encouraging adherence included self-monitoring (SM), ongoing contact (OC), and reinforcement (R).

tracking at baseline and throughout the intervention, and thus the timing of dietary intake must be tracked throughout TRE interventions as well.

3.3. Tailoring the regimen

One method of facilitating a smoother implementation of TRE is allowing each individual to self-select an eating window. As the timing of caloric consumption is the focus of time-restricted eating, it will benefit participants to be judicious in selecting an eating window that fits their needs. Potential considerations include sleep/wake patterns, work schedules, and care responsibilities. Tailoring the regimen can also include making minor adjustments to an individual's TRE protocol throughout the study. For example, if a participant is experiencing intense caffeine withdrawal in the mornings, they might be allowed to shift their eating window earlier by 1 h for the rest of the study. Or, if the family dinner is at 6:30 pm, then they could set an eating window that encompasses this event.

3.4. Ongoing contact

TRE interventions can last several months; therefore, contacting participants throughout is key. Ongoing contact can vary in terms of content, frequency, and method of contact. Past studies contacted participants to check in to assess any potential side effects, send reminders for protocol adherence, or answer questions. It may be especially important to contact participants during the first few weeks of intervention to ensure clear comprehension of the protocol (e.g., calorie vs. no-calorie foods that break a fast) (25). Getting through holidays or vacations can also require extra support, both during and after. Ongoing contact can also help to address socio-environmental barriers by identifying challenging aspects of participants' environments and either implementing behavioral change strategies and/or modifying the TRE protocol accordingly.

The frequency of contact can depend on numerous factors including the length of intervention, participant compliance, and participant preference. Participants exhibiting difficulties with protocol compliance may need to be contacted regularly for reminders and encouragement, while busier participants may not wish to be contacted so frequently.

The third consideration is the method of contact. Phone calls and emails can provide in-depth forms of exchange, which can be used in combination with text messages and push notifications *via* smartphone applications that provide a brief method of contact for non-urgent matters. Preference for the method of contact may also differ for each participant and should be accommodated when possible.

3.5. Reinforcement

Reinforcement refers to any positive feedback given to participants on their progress. In the context of TRE, positive feedback can be given to participants to reinforce good behaviors such as consistently recording their meals and adhering to their eating window. In longer trials, sending reinforcement can be a great way to acknowledge participants for their efforts while encouraging them to persist. Similarly, reinforcement can also be used to encourage compliance with TRE while emphasizing any positive elements of the participant's participation to prevent negative feelings (e.g., guilt or shame) toward their progress. Ongoing contact and reinforcement frequently overlap as study coordinators often contact participants *via* phone, email, or push notifications to send encouragement and reminders for protocol adherence.

3.6. Social support

Finding support in work, social, and home settings is essential for encouraging adherence to TRE. Support can mean finding individuals that share similar goals—following a similar TRE schedule, for example—or simply finding individuals that support the participant's change in eating habits. Receiving support from family and social circles can mean fewer temptations to break a fast and encountering less friction in response to altered behavior (e.g., not eating the last course at a dinner party, avoiding alcohol at a social event, leaving an event earlier, etc.). Similar to having healthy food choices available at home and removing items of temptation, having everyone in the household on the same eating schedule is an important environmental factor for adopting and maintaining TRE.

4. Suggestions for future studies

4.1. Protocol flexibility

To address these barriers in future trials, two of the studies suggested possible alterations in protocol to enhance the flexibility, and therefore feasibility, of TRE. As most social events take place at night, particularly on weekends, allowing occasional off days as part of the TRE protocol could potentially enhance its feasibility as a long-term intervention. Specifically, Przulj et al. (18) and Parr et al. (24) suggested implementing TRE for 5 out of 7 days per week to encourage long-term adherence. However, it should be noted that without official off days, participant adherence is typically 5/7 days (Table 2). Thus, if only 5 days of TRE adherence are required, adherence may decrease to 3–4 days instead. Although most trials that report adherence to TRE as 5/7 days still conclude the efficacy of the intervention, the lack of consistency in establishing a circadian routine should

be avoided as it could contribute to circadian disruption (41). Overall, further research is required to better understand how the frequency of eating outside of the eating window impacts the positive health outcomes associated with TRE. Another potential accommodation would be to allow participants to eat outside their designated window for special occasions or social events up to once or twice a week as necessary.

4.2. Eating window customization

In light of challenges faced by participants in prior TRE trials, future trials may wish to tailor their protocol to address barriers from multiple angles. Potential considerations include the participants' family/home life (Do they have family meals often?), social life (How frequently do they eat out? When do they usually socialize?), and work schedule (Are they currently employed—and if so, is their schedule able to accommodate TRE?). Additionally, it is important to consider how culture and religion may play a role in the timing of food intake. It is worth noting that most TRE trials have been conducted in limited cultural environments and thus future research would benefit from exploring the full implications of cultural and religious influences. While a singular eating window length that is optimal for health has not yet been identified, previous trials have shown that a longer eating window of 12 h does not yield the same health benefits (42), while a shorter eating window of 4 h doesn't show additional benefits compared to 6-h TRE (1). Given the current body of literature, eating windows ranging from 6 to 10 h seems to be ideal as they yield benefits while allowing participants to eat within a reasonable time frame (5, 6, 43). Moreover, some studies have shown that an earlier window may be more beneficial than an eating window that ends later in the day (9, 44), yet many benefits are still seen from TRE studies with a later eating window (45). The importance of aligning food intake with the active phase of an individual is well-studied and should be taken into consideration when selecting an eating window.

4.3. Easing implementation

Some potential protocol alterations to address barriers that have not been extensively studied include implementing (1) scheduled off-days from TRE, and (2) a gradual reduction in the eating window to reach targeted fasting hours. Participant self-monitoring can be a great way to track dietary habits and increase self-accountability, while other methods targeting behavior change can reinforce positive progress while proactively preventing deviations from the protocol. Being proactive with these methods rather than waiting for issues to arise before contacting participants may also encourage adherence.

4.4. Education

In clinical trials aimed at exploring TRE for a particular illness/disease, it may also be useful to evaluate participants' perceptions of the severity and risk of leaving the illness/disease untreated. The Health Belief Model (HBM) suggests that a person's likelihood of changing their behavior can be predicted by their perceived severity of illness along with perceptions of the effectiveness of the proposed health intervention, so leveraging aspects of belief (i.e., perceptions of severity, risk, and benefits) may be worthwhile (46).

4.5. Daily monitoring

Daily monitoring of dietary intake is a key component of assessing adherence and should thus be a core component of all TRE trials.

4.6. Behavioral science insights

Behavioral science indicates that ongoing support and interaction are essential components of ensuring adherence to any lifestyle intervention, including TRE. Thus, future research should emphasize the importance of continuous assessment and support to help participants maintain behavioral changes.

5. Limitations

Studies that collected participant feedback on barriers often had small sample sizes, and there is currently no standardized method of collecting feedback on TRE barriers. As a result, there is wide variation in the type of data collected and how it is reported, making it difficult to identify salient patterns across many trials. Most studies that collected data on TRE barriers did not report the exact number of participants experiencing each barrier. As TRE is a relatively new dietary intervention, more data is needed to conclude (a) overall adherence rates in TRE trials, (b) the types of barriers participants face and the frequencies at which they occur, and (c) the effectiveness of behavioral change strategies outlined in this review.

6. Conclusion

TRE has shown promise in clinical trials as a simple yet effective dietary intervention. As a straightforward protocol that solely focuses on the timing of food intake, participants are not required to restrict or change any aspect of their regular diet. Nonetheless, the long-term feasibility

of this protocol is less understood, which merits a closer examination of barriers faced by participants during trials. To increase protocol adherence—and therefore feasibility—of TRE, future trials may consider exploring a combination of health behavioral change strategies, tracking adherence closely, and soliciting participant feedback on TRE barriers.

Author contributions

MO'N, KL, and NG performed the literature review and compiled data. NG conceptualized the paper and completed the initial literature review and contributed to the writing. MO'N wrote the draft of the manuscript. MO'N, KL, EM, and SP edited the manuscript. EM and SP provided feedback and mentorship at all stages. All authors contributed to the article and approved the submitted version.

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Conflict of interest

SP is the author of the Circadian Code and the Circadian Diabetes Code.

The remaining 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.

Publisher's note

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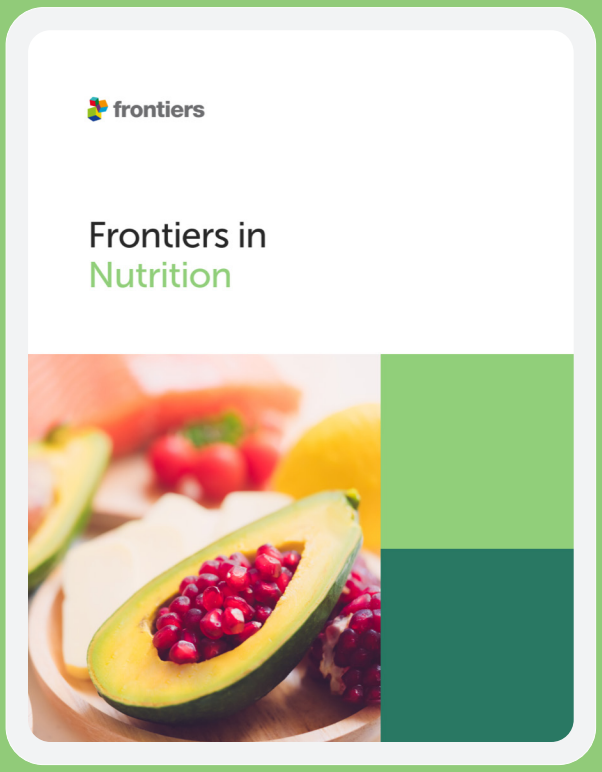

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