CHAPTER SEVEN

Response of Peripheral Rhythms to the Timing of Food Intake

Megumi Hatori*, Satchidananda Panda†,1
*School of Medicine, Keio University, Shinjuku-ku, Tokyo, Japan
†Regulatory Biology Laboratory, Salk Institute for Biological Studies, La Jolla, CA, USA
[1]Corresponding author: e-mail address: satchin@salk.edu

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Abstract

Metabolism and physiology in animals show diurnal rhythm to adapt to the daily cycles of activity–rest and the associated rhythm in feeding and fasting. Accordingly, gene expression, protein activities, and numerous metabolites show daily rhythm in abundance. The significance of these rhythms in promoting healthy lifespan and preventing disease has recently come to light. Mice with genetic disruption of circadian rhythm, mice, and humans under shift-work paradigm, and mice fed high-fat diet *ad libitum* exhibit chronic disruption of feeding–fasting rhythm and dampened daily rhythms in physiology, metabolism, and gene expression. These dampened rhythms are associated with metabolic diseases. Conversely, time-restricted feeding, in which mice are fed for certain number of hours every day, restores rhythms and can prevent obesity and metabolic diseases even when mice are fed high-fat diet. These observations seek

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mechanistic explanations, which will require careful experiments in which feeding duration, genotype, nutrient, and feeding time relative to light:dark cycle will be manipulated and molecular changes in peripheral organs and a few brain regions will be assessed. This chapter will primarily focus on the use of mouse as an experimental animal and the experimental setup so that the molecular readouts can be better interpreted.

1. INTRODUCTION

Mammals including humans exhibit circadian rhythms in activity, sleep, and food intake. Such circadian organization temporally tunes behavior, physiology, and metabolism for optimum health. Chronic disruption of circadian rhythms as in shift-work or in experimental mice carrying mutations in clock genes predisposes to chronic diseases including metabolic diseases, cancer, coronary heart diseases, and dementia (Barclay et al., 2012; Bass, 2012; Davis, Mirick, & Stevens, 2001; Fonken et al., 2010; Karlsson, Knutsson, & Lindahl, 2001). Since the general population is increasingly leading a lifestyle that is similar to that of shift workers, there is a growing interest in understanding how diurnal rhythm in behavior and metabolism impacts health.

The overt diurnal rhythms are products of interaction between the internal circadian clock and the external timing cues. The circadian oscillator is cell autonomous and is present in both neuronal tissues and nonneuronal organs, where the oscillators temporally tune neuroendocrine, immune-, and metabolic functions to maintain homeostasis (Hastings, Reddy, & Maywood, 2003). The "master circadian oscillator" present in the hypothalamic suprachiasmatic nucleus (SCN) receives ambient light information through monosynaptic input from the intrinsically photosensitive melanopsin-expressing retinal ganglion cells (referred as ipRGC or mRGCs) of the inner retina (Hatori & Panda, 2010). In nocturnal rodents, light also suppresses activity, thereby supporting daytime sleep. Consequently, majority of food intake in nocturnal mice occurs during the night when mice are awake. Daily oscillation in the mRNA or protein levels of several circadian clock components has been observed in multiple cell types of the central nervous system (CNS) and the peripheral organs of rodents. The CNS centric view of behavior had promoted the idea that light would be the dominant environmental cue that entrains the circadian oscillator in different organs to the ambient condition. However, in a series of seminal

papers using simple change in feeding time without changing the ambient light:dark cycle, it was shown that the phase of the circadian oscillator in the liver (peripheral clocks) is entrained by the timing of food intake (Balsalobre, Brown, et al., 2000; Balsalobre, Marcacci, & Schibler, 2000; Damiola et al., 2000; Stokkan, Yamazaki, Tei, Sakaki, & Menaker, 2001). Such independent effect of light and food in the central and peripheral clocks, respectively, formed the hypothesis that desynchrony between the CNS clock and the peripheral clocks under disrupted lighting or feeding condition disrupts homeostasis and predisposes to chronic diseases.

As circadian clock components are transcriptional regulators, and the existing technology allows comprehensive assessment of transcripts, several studies have shown a large fraction of the expressed genome in the peripheral organs show daily oscillations (Akhtar et al., 2002; Hogenesch, Panda, Kay, & Takahashi, 2003; Hughes et al., 2007, 2009; McCarthy et al., 2007; Miller et al., 2007; Panda et al., 2002; Sato et al., 2004; Ueda et al., 2002). Molecular studies have linked direct or indirect regulation of transcriptional oscillation by the clock components (Cho et al., 2012; Koike et al., 2012; Rey et al., 2011; Vollmers et al., 2012). Accordingly, in genetic model of circadian rhythm disruption either in the entire body or in liver, daily transcriptional oscillations in peripheral organs are dampened and the animals succumb to metabolic diseases (Barclay et al., 2013; Lamia, Storch, & Weitz, 2008; Marcheva et al., 2010; Rudic et al., 2004, 2005; Sadacca, Lamia, deLemos, Blum, & Weitz, 2011). In wild-type (WT) animals, feeding during light in nocturnal rodents or feeding during night in diurnal flies uncouples the optimal phase relationship between the central and peripheral clocks, which adversely affect animal health (Gill & Panda, 2011). Such meal mistiming is thought to contribute to metabolic diseases among shift workers. These observations have led to experiments exploring the effect of eating pattern on peripheral clocks.

The quality of nutrition can also change eating behavior and affect the peripheral clock. High-fat diet (diet with >30% calories from fat) fed *ad libitum* dampens the feeding–fasting rhythm of mice so that the animals consume more food during the daytime (Kohsaka et al., 2007). The dampened feeding rhythm is associated with dampened gene expression rhythm in the liver of WT mice fed high-fat diet *ad libitum* (Vollmers et al., 2009). When fed a normal chow *ad libitum*, the WT mice maintain a good feeding–fasting rhythm and nearly 3,000 liver transcripts show diurnal oscillations (Hughes et al., 2009). However, an additional small set of transcripts show a 12-h rhythm in oscillation, which parallels the two large nocturnal eating bouts

in these mice. Such profound effect of eating pattern on peripheral rhythms implied the diurnal transcriptome in the peripheral organs is pliable and is likely a product of peripheral clock and eating pattern. In fact, mice with no access to food for 24 h show oscillations in only a few hundred hepatic transcripts, while experimental cohorts who consume the same amount of food as *ad libitum* fed counterparts show robust rhythm in ~5,000 transcripts (Vollmers et al., 2009). $Cry1^{-/-}$;$Cry2^{-/-}$ mice, which completely lack a functional circadian oscillator, exhibit erratic eating pattern, and no significant circadian transcriptional oscillation is found in their liver. Surprisingly, when these genetically circadian-deficient mice are subject to a feeding–fasting regimen, several hundreds of hepatic transcripts (Vollmers et al., 2009) and several metabolites (Adamovich et al., 2014) regain diurnal oscillations.

Since appropriate oscillations have been hypothesized to promote health span and eating pattern is a powerful agent to shape oscillations, whether genetic factors regulate eating pattern and whether certain imposed eating pattern can counteract the adverse effects of genes and nutrition have become a translationally relevant question. Recently, a mutation in *Per1* gene affecting phosphorylation status of the PER1 protein has been shown to advance the animals' eating time by few hours to the daytime (Liu et al., 2014), while an analogous mutation in *Per2* was previously shown to advance the sleep onset time (Toh et al., 2001). The *Per1* mutants, not the *Per2* mutants, are predisposed to obesity. However, surprisingly, imposing a nighttime feeding pattern can prevent obesity in the *Per1* mutant mice (Liu et al., 2014). Similarly, the high-fat diet-induced obesity can be prevented by maintaining a strict feeding–fasting rhythm without reducing caloric intake (Hatori et al., 2012). In summary, the past decade of circadian rhythm research in peripheral organs has highlighted the importance of eating pattern on peripheral gene expression oscillations, identified genetic and nutrition factors that modulate eating pattern, and demonstrated the proof of concept that eating pattern intervention can counteract the obesogenic effect of genes and nutrition. Therefore, in studies assessing the effect of genes, nutrition, and calories on metabolism and physiology, it is important to monitor the temporal pattern of eating, as it can have profound effect on the temporal pattern of molecular changes in peripheral organs and will have measurable effect on the physiological outcomes. This chapter will focus on the experimental procedures for the studies assessing the interaction of eating pattern on the circadian clock system in the mouse model animal.

2. ANIMAL STRAIN AND AGE

Animal models and strains that have been widely used in circadian rhythm and metabolism research are most effective in dissecting the interaction between nutrition and rhythms. Although several inbred mouse strains have been used in circadian rhythm research, BALB/cJ mice show large interindividual variation in circadian activity rhythm, while C57B6 shows stable periodicity and is a preferred strain in many laboratory (Schwartz & Zimmerman, 1990; Shimomura et al., 2001). Similarly, SWR/J and A/J strains of mice are resistant to obesity, while C57B6 and AKR/J are preferred strains to study diet-induced obesity (DIO) (West, Boozer, Moody, & Atkinson, 1992). These baseline strain characteristics for metabolism and circadian rhythm help determine the strain choice that is appropriate for the goal of the study. Most of the published studies on interaction between circadian and metabolism typically used male C57B6 strain due to stability of free running rhythm and susceptibility of the male mice to DIO.

If the goal of the experiment is to test the effect of eating pattern on peripheral circadian clocks and its consequence on energy balance, it is better to start the experiment at an age when mice have already completed most of their normal growth. For C57B6 mice, it is desirable to start the experiment in mice that are at least 12 weeks old. Between weaning and 10 weeks, change in eating pattern even under high-fat diet has little impact on the rate of body weight gain.

3. ANIMAL ROOM AND EQUIPMENT

All experiments should be carried out after approval from the IACUC or equivalent regulatory committee to ensure that the instruments, method, lighting, and fasting periods conform to the regulation of the oversight committee and funding agencies. Rodents in most vivariums are fed *ad libitum* under 12 h light:12 h dark (LD) cycle under constant temperature and only in some cases under constant humidity condition. The light levels in regular holding room are typically not regulated, and occasional nighttime access to the room and dim lights from indicator lamps in the room can be effective in disrupting circadian rhythm in mice (Evans, Carter, Freeman, & Gorman, 2012; Evans, Elliott, & Gorman, 2011). Therefore, for carrying out any study to monitor any aspect of

diurnal rhythm, it is preferable to have experimental groups housed in light tight isolation chambers with independent light controls.

Daily pattern of food intake, activity, and sleep are intimately related with each other and are under genetic control. They are also influenced by ambient temperature and lighting conditions. Therefore, it is important to ensure that the environmental parameters are controlled and monitored. The experiment room should ideally have independent lighting control, and the mice inside the room may be held in isolation chamber with independent lighting control (Siepka & Takahashi, 2005). These chambers may be fabricated in a workshop adhering to the guidelines of the vivarium with respect to material used, air circulation rate, white noise, light level, type of lights, etc., or can be purchased from a commercial vendor. This allows the isolation chamber(s) to be set in different conditions of LD (light–dark) cycle, DD (constant dark), or LL (constant light). The chambers can also be set to a DL (dark–light) cycle so that the mice are in the dark phase during the astronomical daytime. In addition to white light, both the room and the isolation chambers should also have dim red light to allow animal handling in the dark. Most vivariums are set at a relatively constant temperature ~22 °C. However, it is a good practice to have an analog or digital 24 h temperature logger in the room or in the isolation chamber where mice are housed. Specifically, if the mouse cages are held inside a light–tight isolation chamber, depending on the light source, heat sink, or air recirculation rate, the temperature inside such chambers can fluctuate by as much as 4 °C between light and dark. Such a change in ambient temperature can affect metabolic rate between light and dark phases and affect molecular changes, thus confounding interpretation.

It is also ideal to monitor activity and food intake in parallel so that the effect of change in eating pattern on peripheral or central clock can be accurately assessed. Emerging interest in the use of rodents in metabolic research has prompted several commercial vendors to offer longitudinal monitoring systems that track home cage activity or wheel running activity along with food intake pattern. If no manipulation of feeding time is involved, feeding and activity can be monitored in a subset of mice using an indirect calorimeter system available in many vivariums.

4. FACILITIES TO ACCOMMODATE FEEDING SCHEDULE

If access to food is to be controlled, so that the mice are fed daily at a specified period of few hours over several days or weeks, meticulous

planning and investment in equipments or modification to the experimental room are to be considered well in advance. There are expensive caging systems that continuously monitor mouse and food weight and computer-controlled solenoid gates control access to food at certain time or for predefined weight of food consumed. These systems are expensive in setup and running cost, but offer precise control over feeding pattern. A good workshop can also fabricate solenoid-controlled food access chutes to allow mice access to food at preprogrammed time. A simple, yet labor-intensive approach followed in our lab is to transfer mice between feeding and fasting cages at specific time. Switching the mice between feeding and fasting cages is preferred over adding and removing food from the food hopper, as the mice chew and drop food in the bedding, which can be consumed during fasting period. If manual cage change approach is used, the researchers must consult the animal facility and regulatory committee whether the mice transfer—at least twice daily—can be done on a sanitized table or to be done under a hood. In either case, this step will involve storing extra cages with labels and an ergonomically good working area so that numerous cage changes can be done under dim red light without much inconvenience to the staff and with minimal risk of mixing up cages or mice.

Ideally, singly housed mice allow precise monitoring of activity, sleep, and eating pattern with higher precision. However, this adds significant cost to the experiment if multiple mouse strains, diet types, or feeding period are to be tested. Therefore, mice of same genotype, gender, and age may be cohoused in cages within the guideline of the oversight committee. Environment enrichment by placing huts or toys in the cage reduces fighting. However, certain enrichments such as access to a wheel can also impact daily activity–sleep pattern and consequent feeding pattern and have been shown to impact predisposition to metabolic disease. Therefore, the extent of cage enrichment must be factored in the study goal and interpretation of results.

5. DIET

Food quality and texture influence the total daily food intake and eating pattern. Food quality also influences the molecular response to feeding. Therefore, it is important to note the supplier, product number, composition of the mouse chow, lot number, date of production, and expiration date and to strictly follow the vendor-suggested storage condition. Many specialized diets including high-fat, high-fructose, high-fat + high-sucrose diets with increased levels of fat or sugar typically require cold storage and have

a shelf life of <6 months. Furthermore, such specialized food may become soft after a few days on the food hopper at room temperature. Mice typically do not prefer soft food and the food can even become sticky, "melt," and drop into the bedding, further making it difficult to accurately measure weekly food consumption. Specifically diet with high level of fructose or high-fat diet fed at a higher ambient room temperature often becomes very soft after 3–4 days and needs to be replaced twice a week. For other food types, weekly change of food may be sufficient. Therefore, for a large experiment involving dozens of mice or experiment spanning more than few weeks, it is important to plan food procurement, shipment size, storage space, and storage condition prior to starting an experiment.

6. MONITORING EATING PATTERN

Just like the ambient lighting condition profoundly affects activity–rest cycle and molecular rhythms in the SCN, daily food intake pattern shapes the peripheral oscillations. Therefore, in any study on peripheral oscillation, even under constant darkness, it is a better practice to have some measure of the animals' eating pattern. The standard laboratory mouse (*Mus musculus*) has a nocturnal preference for food intake, but under *ad libitum* access to food, they do not exclusively eat during the dark phase of the light:dark cycle. A crude estimate of day:night eating pattern can be done by placing known quantity of food in mouse cages and weighing the remaining amount at light and dark onset for a few days. Male C57B6 adult mice with *ad libitum* access to normal chow typically consume 65–80% of food during the night phase of a 12 h light:12 h dark regime, while mice with *ad libitum* access to high-fat diet consume 50–65% during nighttime (Kohsaka et al., 2007). Therefore, change in nutrient type can accompany a change in eating pattern, which in turn can affect metabolism and physiology. Many strains of mice lacking a functional circadian clock typically lose the nocturnal preference for food intake, and they roughly consume equivalent amount of food during day and night. Therefore, such a simple measurement of day:night food intake can be a preliminary test whether a food type or genotype affects daily eating pattern and total food intake.

Using an indirect calorimeter or specialized cages with continuous monitoring of food intake is more accurate method to measure food intake pattern. Such methods are based on singly housed animals, and some of the specialized food hoppers for such instruments impose restriction of food pellet size, hopper shape, and access port. Therefore, it is important to test the

manufacturer's specifications and plan accordingly. Furthermore, the animals might take time to adjust to the new cage condition, so the first 2–3 days of data may not reflect long-term eating pattern. Such method is sensitive to detect subtle change in eating pattern that can affect temporal gene expression pattern in peripheral organs. For example, C57B6 male mice typically eat a large meal with 2–3 h of dark onset, reduce their food intake toward the middle of the night, and increase feeding before dawn. The two nocturnal feeding bouts transiently augment expression of feeding-induced genes so that the transcripts show ~12 h oscillation in gene expression.

7. PHYSIOLOGICAL READOUT OF EATING PATTERN

It may be desirable to monitor a physiological readout of eating pattern. The use of an indirect calorimeter (Even, Mokhtarian, & Pele, 1994) fitted with additional sensors allows simultaneous measurements of such parameters including activity as measured by infrared beam breaks, O_2 consumption, CO_2 production, and temperature as sensed from the cage or from temperature sensor surgically implanted in the animals. Since each of these parameters can have wide variations from animal to animal, multiple animals of each cohort should be monitored at least for 3–5 days.

Feeding and fasting influence body temperature and oxygen consumption. Depending on the ambient temperature, postprandial thermogenesis raises body temperature in mice by 0.5–1 °C, which is detectable by implanted temperature sensor or by measurement of cage temperature in sophisticated metabolic cages. Such temperature rhythm roughly parallels feeding rhythm. Oxygen consumption also changes with feeding pattern. During feeding, animals typically use readily available carbohydrate from the food as energy source, while after few hours of fasting, they gradually switch to glycogen and then to fatty acids as energy fuel. Since oxidation of fat and carbohydrate involve different amount of O_2 consumption, accurate ratio of CO_2 exhaled and O_2 inhaled (respiratory exchange ratio or RER) is a powerful method to monitor fuel use. Theoretically, carbohydrate consumption is reflected in an RER value of 1, while fatty acid used as energy source drives the RER to 0.7 (Even et al., 1994). In WT mice fed normal chow *ad libitum*, the RER shows some oscillation between these two values. If their food access is restricted to a few hours, the RER strongly oscillates between 1 and 0.7 with characteristic transitions. During feeding the RER is close to 1, which slowly declines after cessation of food intake

toward 0.7 over 4–7 h of fasting. If food is introduced, the RER quickly switches back to 1.0 within a few minutes. *Ad libitum* feeding of high-fat diet dampens the feeding–fasting cycle and the RER remains almost flat and some rhythm in RER is restored under time-restricted feeding (TRF) condition (Hatori et al., 2012). These temporal features of fuel use indicators under different feeding regimens influence expression or activity of gene products in metabolic organs of mice. Conversely, it is also conceivable that mice carrying specific mutations in fuel metabolism regulators might show different kinetics of fuel use switch between fasting and feeding. Therefore, close attention to these noninvasive physiological readouts can yield powerful information for devising downstream invasive or terminal experiments to assess the response of peripheral molecular rhythms to eating pattern.

8. FEEDING PARADIGMS

The response of peripheral rhythms to time of food intake may be monitored at different timescale depending on the study goal. Typically, most experiments fall into three different categories: (1) how rapidly the peripheral circadian clock readjusts to a change in time of eating (Damiola et al., 2000; Stokkan et al., 2001), (2) amplitude and phase of expression of clock components under different feeding patterns (Hatori et al., 2012; Vollmers et al., 2009), and (3) assessing the contribution of circadian clock and feeding pattern on a given set of genes or on global gene expression or metabolite accumulation pattern (Adamovich et al., 2014). Experiments manipulating feeding time should be done under a fixed light:dark cycle to avoid any confounding effect of change in light:dark regimen on the peripheral clock through the SCN oscillator.

8.1. Assessing the pace of resetting of peripheral clock to a change in eating pattern

These experiments are most relevant in modeling the metabolic jet-lag that occurs when individuals abruptly change their eating time due to travel to a different time zone or due to shift work. Initially, the mice may be fed *ad libitum* or have access to food only during the nighttime. After 1–2 weeks, the time of food availability is shifted by 8–12 h and the number of days the animals take to establish a stable phase of oscillation of an oscillator component in the liver is assessed. Longitudinal measurement of body temperature or RER before the shift and few days after the shift can be used as a readout of physiological adaptation to new eating pattern. Since there was no

noninvasive method to monitor peripheral circadian rhythms, these experiments involved a relatively large number of animals. Every day after the food access time is changed, mice are sacrificed at defined interval over 24 h and the cycling of oscillator components is assessed by analyzing RNA or protein. Such analysis has led to the identification of poly ADP ribosylation as an important process that sets the pace of peripheral clocks phase adjustment to new feeding time (Asher et al., 2010). Recently, development of ingenious liver-specific luciferase-based circadian clock reporters and the use of sensitive imaging techniques on live freely moving animals have allowed precise and noninvasive methods to directly monitor the effect of feeding and potentially other manipulations on the peripheral circadian clock (Saini et al., 2013). Detailed description of the equipment, imaging condition, and sensitivity of the system has been elucidated in a recent manuscript (Saini et al., 2013). This method has shown an important role of the SCN oscillator in opposing rapid resetting of peripheral clocks to a change in feeding time.

8.2. Assessing the contribution of circadian clock and feeding pattern on peripheral molecular rhythms

Circadian rhythm in activity and sleep also imposes a rhythm in feeding during the active phase and fasting during sleep. In peripheral metabolic organs involved in substrate interconversion, storage, and substrate utilization, several metabolic pathways respond to feeding and fasting to tune metabolism so that organism-level energy homeostasis is maintained. Therefore, to dissect the contribution of a functional clock and the feeding–fasting cycle on peripheral rhythm, it is desirable to assess rhythms under *ad libitum* condition, under constant fasting and under a defined feeding–fasting cycle (TRF).

Age- and gender-matched mice may be adapted to *ad libitum* or TRF condition for at least 2 weeks prior to tissue collection. For simplicity, food access in the TRF group may be allowed for 8–12 h during the dark phase of a typical 12 h light:12 h dark LD cycle starting from ZT12. Since animals tend to spill food from the hopper that can potentially be consumed during fasting period, it is desirable to transfer the animals between feeding and fasting cages at specific times. The *ad libitum* fed mice may also be transferred between cages with food to control for any unintended effect of handling. More importantly, to avoid any unintended effect of caloric restriction on TRF cohort, food intake should be monitored by weighing the food remaining in the hopper every 2–7 days. Typically, after 1 week of TRF, rodents with access to food for 8 h or longer starting from ZT12 learn to

consume equivalent amount of calories as their *ad libitum* counterpart (Hatori et al., 2012; Vollmers et al., 2009). However, we have noticed the age at which the mice are introduced to TRF, diet quality, and strain background can affect the duration of food access during which mice can consume at least 90% calories of their *ad libitum* counterparts. So, this window may be empirically determined. If the lab has access to an indirect calorimeter with real-time measurement of food intake, it may be desirable to monitor the feeding pattern and O_2 consumption in a subset of *ad libitum* and TRF cohorts for at least 5 days. Such monitoring will show the daily pattern of eating, activity, and O_2 consumption and illustrate the whole animal energy consumption pattern, which can be useful in interpreting molecular oscillations.

If a constant fasting group (fasting for up to 36 h) is to be analyzed, either *ad libitum* or TRF mice can be subject to constant fasting. Using a subset of TRF mice for constant fasting is preferred, as it reduces the animal number used in experiment. For constant fasting, after at least 6 h of transfer to fasting cages, tissue samples may be collected at 2–3 h interval for up to 24 h. Tissue samples from *ad libitum* and TRF cohorts are also collected at equivalent sampling frequency. However, as the fuel usage from stored fat to readily available carbohydrate in food occurs rather quickly, sampling the fasting and TRF groups at 1 h interval from ZT11 till ZT14 allows high-resolution dissection of food-induced changes. Depending on the IACUC Committee's directive and the goal of the study, 24 or 48 h sampling may be done.

8.3. Amplitude and phase of expression of peripheral clock under timed feeding or *ad libitum* condition

There is growing interest in eating pattern and peripheral rhythms as eating pattern manipulation without changing caloric intake has been shown to affect the repertoire of rhythmic transcripts and their phase of expression. Certain eating patterns have been shown to protect, while others predispose to metabolic diseases. Several eating patterns have been explicitly described in the literature, which include daytime restricted feeding (for simplicity, we will refer to it as dTRF), shift-work eating pattern (when mice have dTRF during weekday and *ad libitum* feeding in the weekend), and 3–6 meals/day at regular interval throughout the 24 h. Additionally, several caloric restriction studies in rodents also inadvertently involve a profound change in eating pattern. Specifically in caloric restriction studies where the control mice have *ad libitum* access to food and the caloric restriction mice are given a hypocaloric diet at a specific time of the day, or they are given access to food for 24 h in every other day, there is a clear difference in eating pattern

between cohorts. Since TRF without caloric restriction has been shown to maintain desirable health parameters, in the coming years there will be growing interest in dissecting the beneficial effect of eating pattern and caloric restriction.

In these experiments, the *ad libitum* and timed-fed groups are adapted to the feeding condition for at least 2 weeks. It may be desirable to monitor their whole body energy usage, activity, rest, and food consumption pattern by placing a subset of animals in indirect calorimeter. Tissue samples may be collected at 2–3 h interval over 24 h.

9. MOUSE TISSUE COLLECTION

At the end of the experiment, mouse tissues are collected every 2–3 h interval over at least 24 h to monitor gene, protein, or metabolite changes. Although many published experiments are primarily focused on molecular changes in the liver, several peripheral organs and even some CNS regions involved in ingestion behavior are likely affected by changes in the time of eating. Therefore, it is important to collect multiple tissue samples from these animals.

Depending on the experimental goal, tissue collection may be done on mice under LD, DD, or LL condition. At least three mice may be sacrificed for every time point as experimental replicates. As mice are coprophagous, if gastrointestinal sample or gut/cecal or fecal microbiome is to be analyzed, mice from different cages at each time point serve as better experimental replicates. Mouse sacrifice is done according to a method approved by the respective IACUC. If samples are to be collected in the dark, the eyes are removed by a curved scissors so that subsequent tissue collection can be done under light. Eye removal also punctures the retro-orbital vein, so the blood may be collected in appropriate tubes for plasma or serum analyses. If multiple samples are to be collected from the brain and peripheral organs, it may be ideal to train several lab members on tissue collection so that consistency in sample collection and processing is maintained. Tissues are flash frozen under liquid nitrogen or by dry ice before long-term storage at −80 °C.

For larger tissues such as white adipose tissue and liver, to avoid freeze thaw, the tissues can be divided into several tubes. These organs are typically ground into a fine powder under liquid nitrogen using pestle and mortars. The frozen powder can be aliquoted for further RNA, protein, and metabolic assays. Small organs and tissues, such as adrenal glands and hypothalamic brain punches are frozen and used directly for specific downstream assays.

10. TRANSCRIPT, PROTEIN, AND METABOLOME EXPRESSION ANALYSIS

The effect of eating pattern on transcripts, proteins, and metabolites can be monitored by specific extraction protocols and detection methods. Total RNA from mouse tissues is extracted following any of the silica-based or phenol-based commercial extraction methods following vendor's protocol. RNA extraction from protein–rich tissues might require additional proteinase treatment prior to RNA extraction. DNase treatment of RNA samples is preferred while measuring the pre-mRNA or intron-less RNA. To obtain enough RNA from small-size tissues, such as SCN or pineal gland, low-bind tubes (Axygen Scientific, MAXYMum Recovery™ Microtube, MCT-175-L-C), and tips (Axygen Scientific, MAXYMum Recovery™ Filter tips) may be used. RNA from individual SCN, pineal, or pituitary gland may be eluted in 20 μl of RNase-free water, half of which may be used for cDNA synthesis using Superscript III (Invitrogen, CA, USA) or qScript cDNA SuperMix (Quanta Biosciences, MD, USA). After up to 30× dilution of the cDNA, up to 100 different transcripts can be quantified by q-RT-PCR. The RNA can also be used in RNA-seq experiments to monitor global response to feeding time. RNA quantification methods using gene-specific primers or RNA-seq methods as adapted by the lab or the sequencing cores may be followed.

Protein and metabolite extraction and analyses largely depend on the protein of interest, its posttranslational modification, detection method (Western blotting, ELISA, multiplexed beads, etc.). Similarly, metabolite extraction methods, choice of solvents, and detection methods are metabolite specific and it is customary to consult with a metabolomics core or a vendor at the outset of the experiment so that appropriate number of replicates can be included to offer enough statistical power, and the samples can be collected, stored, and shipped to the analytical lab in appropriate condition.

11. CONCLUSION

Over past several decades, the effect of diet on physiology was largely assessed by letting animals eat *ad libitum* either a balanced standard diet or diet rich in a specific macro- or micro-nutrient. Very little attention was paid to the daily eating pattern. This approach has been useful in understanding

mechanism of nutrient metabolism and development of metabolic diseases under nutrient challenges, such as high-fat DIO. However, the major impact of maintaining a consistent pattern of feeding and fasting on switching between anabolic and catabolic metabolism that is associated with prevention of DIO will hopefully draw some attention to eating pattern while interpreting the result of any metabolic or circadian studies in metabolic and neuroendocrine organs. The content of the chapter is primarily intended to discuss the factors that affect the eating pattern in mice and the methodologies to monitor or manipulate eating pattern leading to change in gene expression in peripheral organs. We hope this will facilitate better design and interpretation of eating pattern on metabolism, by identifying genes and pathways that integrate circadian rhythm, metabolic regulators, and nutrient sensing pathways to setting eating pattern.

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