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Effect of intermittent fasting on circadian rhythms in mice depends on feeding time

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ABSTRACT

Calorie restriction (CR) resets circadian rhythms and extends life span. Intermittent fasting (IF) also extends life span, but its affect on circadian rhythms has not been studied. To study the effect of IF alongside CR, we imposed IF in FVB/N mice or IF combined with CR using the transgenic FVB/N alphaMUPA mice that, when fed *ad libitum*, exhibit spontaneously reduced eating and extended life span. Our results show that when food was introduced during the light period, body temperature peak was not disrupted. In contrast, IF caused almost arrhythmicity in clock gene expression in the liver and advanced *mPer2* and *mClock* expression. However, IF restored the amplitudes of clock gene expression under disruptive light condition regardless whether the animals were calorically restricted or not. Unlike daytime feeding, nighttime feeding yielded rhythms similar to those generated during *ad libitum* feeding. Taken together, our results show that IF can affect circadian rhythms differently depending on the timing of food availability, and suggest that this regimen induces a metabolic state that affects the suprachiasmatic nuclei (SCN) clock.

1. Introduction

In mammals, the central circadian clock is located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus in the brain (Reppert and Weaver, 2002; Panda et al., 2002). The central SCN clock receives light information from the retina (Gooley et al., 2001; Lucas et al., 2001) and transmits neuronal or circulating synchronization cues to peripheral clocks in the liver, retina, intestine, etc., regulating cellular and physiological functions (Lee et al., 2001; Reppert and Weaver, 2002; Froy et al., 2006; Young, 2006; Froy and Chapnik, 2007). The SCN clock is self-sustained, but it is necessary to reset the circadian pacemaker everyday to the external light–dark cycle to prevent drifting out of phase (Quintero et al., 2003). In mice, the clock proteins mCLOCK and mBMAL1 (brain–muscle-Arnt-like 1) heterodimerize and bind to enhancer sequences to mediate transcription of a large number of genes including *Periods* (*mPer1*, *mPer2*, *mPer3*) and *Cryptochromes* (*Cry1*, *Cry2*), which constitute part of the negative feedback loop. When PERs and CRYs are produced in the cytoplasm, they oligomerize and translocate to the nucleus to inhibit CLOCK:BMAL1-mediated transcription. This intracellular mechanism is shared among SCN and peripheral tissues (Reppert and Weaver, 2002).

In addition to light, feeding regimens have been shown to affect clock gene expression in peripheral tissues (Stephan, 2002; Froy, 2007). Restricted feeding (RF), in which food is provided with no calorie reduction at the same time everyday for about 3–12 h, entrains peripheral clocks (Cassone and Stephan, 2002; Schibler et al., 2003; Hirota and Fukada, 2004). Animals display anticipatory behavior 2–4 h before the meal, which is typified by increased functionality of clock-controlled output systems, such as locomotor activity and body temperature (Saito et al., 1976; Honma et al., 1983; Comperatore and Stephan, 1987; Stephan, 2002). RF is dominant over the SCN and drives rhythms in arrhythmic and clock mutant mice and animals with lesioned SCN, regardless of the lighting conditions (Stephan et al., 1979; Mistlberger, 1994; Hara et al., 2001; Stephan, 2002; Oishi et al., 2002; Horikawa et al., 2005). In most incidents, RF affects circadian oscillators in peripheral tissues with no effect on the SCN clock under light–dark conditions (Damiola et al., 2000; Hara et al., 2001; Stokkan et al., 2001; Cassone and Stephan, 2002; Oishi et al., 2002; Schibler et al., 2003; Hirota and Fukada, 2004). Thus, RF uncouples the SCN from the periphery, so that many physiological activities normally

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dictated by the SCN clock, such as body temperature, locomotor activity, heart rate, etc., are phase-shifted by RF to the time of food availability (Hara et al., 2001; Mistlberger, 1994; Boulamery-Velly et al., 2005; Hirao et al., 2006). The location of this food-entrainable oscillator (FEO) has so far been elusive (Davidson, 2006). As opposed to RF, calorie restriction (CR) to 60–70% of the daily intake entrains the SCN clock (Challet et al., 1998; Challet et al., 2003; Mendoza et al., 2005; Resuehr and Olcese, 2005). In addition, CR has been shown to extend the life span of a wide range of organisms and retard aging of laboratory rodents (Smith et al., 2004; Masoro, 2005). Animals fed a calorically restricted diet usually consume their daily dose within a few hours. Thus, we have previously suggested that entrainment of the periphery during CR could be achieved directly, due to the temporal eating, similarly to RF, or by first resetting the SCN (Froy and Miskin, 2007).

During intermittent fasting (IF), food is available *ad libitum* every other day. IF-treated mice eat on the days they have access to food roughly twice as much as those having continuous access to food (Anson et al., 2003; Descamps et al., 2005). Similarly to calorically restricted animals (Masoro, 2005), IF-fed animals exhibit increased life span in comparison with the *ad libitum*-fed control (Goodrick et al., 1990) as well as improved glucose metabolism, cardio-protection, neuro-protection (Contestabile and Ciani, 2004; Mattson, 2005; Sharma and Kaur, 2005; Ahmet et al., 2005; Mager et al., 2006; Anson et al., 2003), and increased resistance to cancer (Descamps et al., 2005). The IF-induced beneficial effects are thought to occur independently of the overall caloric intake, but the underlying mechanisms are still unknown. One suggested mechanism is stimulation of cellular stress pathways induced by the IF regimen (Anson et al., 2003; Mattson et al., 2004; Mattson, 2008). To date, the effect of IF on the oscillation of peripheral clocks or that of the central pacemaker in the SCN has not been studied.

To study the effect of IF on circadian rhythms we used the αMUPA transgenic mice (Miskin et al., 1990) and their wild type FVB/N control mice. αMUPA mice spontaneously eat less when fed *ad libitum* and live longer compared to the WT mice (Miskin and Masos, 1997; Miskin et al., 2005). αMUPA mice exhibit similarities with calorically restricted mice, such as reduced body weight, reduced levels of serum IGF-1 or glucose, enhanced capacity to conduct apoptosis in the liver, and reduced incidence of spontaneous tumors or carcinogen-induced pre-neoplastic lesions (Tirosh et al., 2003, Tirosh et al., 2005; Miskin et al., 2005). As αMUPA mice exhibit reduced feeding under different feeding regimens (Froy et al., 2006), these mice can serve as a model for CR under *ad libitum* feeding and a model for calorically restricted IF under intermittent fasting.

2. Materials and methods

2.1. Animals, treatments, and tissues

FVB/N wild type (WT) mice and transgenic αMUPA mice derived from FVB/N mice (Miskin et al., 1990; Miskin and Masos, 1997; Miskin et al., 1999) were obtained from the Weizmann Institute of Science (Rehovot, Israel) at 4 months of age. Mice were housed in a temperature- and humidity-controlled facility (23–24 °C, 60% humidity), 10 mice per large cage throughout the experiments. Mice were entrained to a light–dark cycle of 12 h light and 12 h darkness (LD) for two weeks with food available *ad libitum*. Rectal body temperature was measured using a thermometer (Huger, Ireland). For clock gene expression, mice, having been fed *ad libitum* (AL), were anesthetized with intraperitoneal injection of ketamine/xylazine (100/7.5 mg/kg) and humanely killed at 3 h intervals around the circadian cycle under dim red light in total darkness (DD). Liver tissues were collected and analyzed (mean ± SEM; $n = 3$ for each time-point and each mouse group). For the intermittent fasting experiments, after two weeks of AL feeding, mice were given food every other day for 24 h beginning at ZT0 for 3 weeks (ZT0 is the time of lights on). The amount of food eaten was measured, and after 3 weeks mice were sacrificed and their livers removed around the circadian cycle under dim red light in DD. For light disruption, the light–dark cycle was changed every 8 h, i.e., there were 3 light–dark cycles in 24 h for 5 weeks (LDLDLD). Body temperature, food intake, and tissue collection were performed after the five-week acclimation in the new light regimen. After 5 weeks, mice were IF-fed for 3 weeks under LDLDLD. All tissues were analyzed by quantitative real-time PCR. For the daytime and nighttime IF experiments, we used 3-month-old C57BL mice given food at either ZT0 or ZT12 for 24 h for 3 weeks. The experiments were conducted in full compliance with the strict guidelines of the Hebrew University of Jerusalem policy on animal care and use.

2.2. RNA extraction and quantitative real-time PCR

For clock gene expression analyses, RNA was extracted from liver using TRI Reagent (Sigma, Israel). Total RNA was DNase I-treated using RQ1 DNase (Promega, USA) for 2 h at 37 °C, as was previously described (Froy et al., 2003; Froy et al., 2006). 2 μg of DNase I-treated RNA was reverse transcribed using MMuLV reverse transcriptase (Promega, USA) and random hexamers. 1/20 of the reaction was then subjected to quantitative real-time PCR using the Sybr Green Master kit (Applied Biosystems, USA) and the ABI Prism 7300 Sequence Detection System. Primers for *mPer1*, *mPer2*, *mCry1*, *mClock*, and *mBmal1* (mPer1-F 5'-ccgaatacacacttcgaaaccag-3'; mPer1-R 5'-tcccgtttgcaacgcag-3'; mPer2-F 5'-cgggctatgaagcgcctag-3'; mPer2-R 5'-ggttgttgtgaagatcctcttctca-3'; mCry1-F 5'-agccagctgatgtatttccca-3'; mCry1-R 5'-agtttagtgatgttccattccttgaa-3'; mClock-F 5'-cctagaaaatctggcaaaatgtca-3'; mClock-R 5'-ccttttccatattgcattaagtgct-3'; mBmal1-F 5'-caagaatgcaagggaggcc-3'; mBmal1-R 5'-ttgtcccgacgcctctttt-3') were tested alongside the normalizing gene Glyceraldehyde 3 phosphate dehydrogenase (*Gapdh*) (mGapdh-F 5'-caagaggtggacacagtggaga-3'; mGapdh-R 5'-cggccactatattcttcaaggc-3').

3. Results

3.1. Food intake and body temperature under IF in αMUPA mice vs. WT mice

To study the effect of IF on circadian rhythms, we used two mouse types of the same genetic background, FVB/N WT mice and transgenic αMUPA mice (Miskin et al., 1990). αMUPA mice exhibit spontaneously reduced eating compared to their WT control mice and could serve as a model for CR under *ad libitum* (AL) feeding and CR-IF under IF conditions. Food was introduced every other day at ZT0 (when lights turn on) for 24 h in the course of 3 weeks (Fig. 1). Analysis of daily food intake in WT mice and αMUPA mice revealed that total food consumption of αMUPA mice was relatively low, 70% (2.8 g/mouse/day), compared with that of WT mice (4 g/mouse/day) (Fig. 1A). Body weight values were on average 24.14 ± 0.6 g ($n = 18$) and 21.86 ± 0.53 g ($n = 10$) for WT mice and αMUPA mice, respectively (Student's t-test $p = 0.019$) (Fig. 1B). Since αMUPA mice weigh less, we corrected food consumption to body mass. αMUPA mice ate less then WT mice (0.16 and 0.12 g/g, respectively, ratio 0.77), reiterating that αMUPA mice are indeed calorically restricted. Under IF, it took WT mice and αMUPA mice about a week to reach a maximum in their food intake (Fig. 1A). After 3 weeks, there was 2.0–2.2-fold increase in food consumption in WT mice (8.5 g/mouse/day vs. 4 g/mouse/day) and αMUPA mice (5.6 g/mouse/day vs. 2.8 g/mouse/day). Thus, both mouse types maintained their average daily calorie consumption, and αMUPA mice consistently exhibited a state of reduced eating (62–70%) (Fig. 1A). Analysis of circadian food intake revealed that IF decreased the nocturnal peak of food consumption, but added a diurnal spike (Fig. 2), reflecting the day of fast that preceded food availability.

We previously showed that the rhythmicity and acrophase of body temperature, another clock-controlled output system, was similar in WT mice and αMUPA mice under LD conditions (Froy et al., 2006). Therefore, we analyzed body temperature at two time points, the mid-light and mid-dark phase, the trough and peak of body temperature, respectively. As expected, rectal body temperature correlated with feeding behavior in both mouse types, high during the dark period and low during the light period (Student's t-test $p < 0.05$) (Fig. 3). The difference in mid-day vs. mid-night body temperature was maintained under IF conditions (Fig. 3), suggesting that the animals remained mainly nocturnal. Notably, αMUPA mice maintained low body temperature compared with WT mice (Student's t-test $p < 0.05$), as expected from calorically restricted animals.

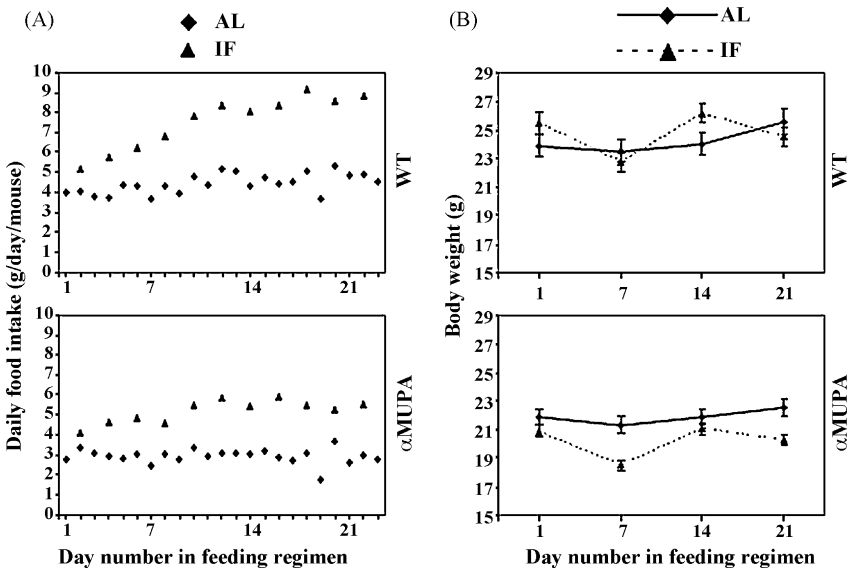

Fig. 1. Daily food consumption (A) and body weight (B) during *ad libitum* (AL) and intermittent fasting (IF) of αMUPA mice and WT mice. Food was given every other day for 24 h at ZT0 for 3 weeks. Every feeding day the amount of food consumed was measured (n = 10 for each mouse group). Body weight of WT mice and αMUPA mice (n = 10 for each mouse group; mean ± SEM) was measured throughout the experiment.

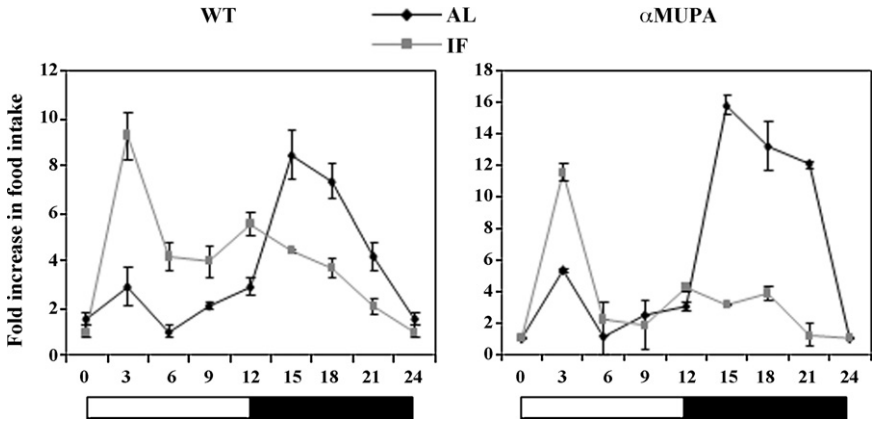

Fig. 2. Food consumption of αMUPA mice and WT mice during *ad libitum* (AL) and intermittent fasting (IF). Food consumption was measured every 3 h around the circadian cycle (n = 10 for each mouse group; mean ± SEM). Values were normalized to the lowest point (ZT6 for WT/αMUPA, AL; ZT0 for WT/αMUPA, IF), which was determined as baseline 1. The white and black bars designate the light and dark cycles, respectively. Food consumption exhibited a peak during the dark period (one-way variance, $p < 0.0001$).

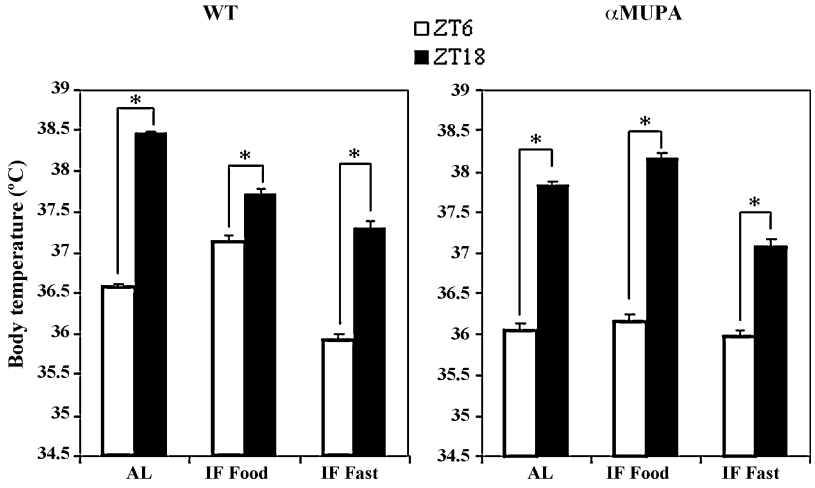

Fig. 3. Body temperature of αMUPA mice and WT mice during *ad libitum* (AL) and intermittent fasting (IF) on the day the animals were fasted (IF Fast) and the day they were fed (IF Food). Body temperature was measured at ZT6 and ZT18 (n = 10 for each mouse group; mean ± SEM). * $p < 0.05$.

3.2. Clock gene expression under IF in αMUPA mice vs. WT mice

To study the biological clock of αMUPA mice and WT mice under AL and IF, we tested the phase and amplitude of the clock genes *mPer1*, *mPer2*, *mCry1*, *mClock*, and *mBmal1* at the RNA level in mouse liver removed in total darkness (DD) around the circadian cycle. Quantitative real-time PCR analyses revealed that all clock genes oscillated under LD AL (one-way ANOVA, $p < 0.001$) (Fig. 4). As was previously reported (Froy et al., 2006), *mPer1*, *mPer2*, and *mCry1* exhibited higher amplitude in αMUPA mice compared with WT mice (Student's *t*-test $p < 0.05$) (Fig. 4). Interestingly, IF abolished circadian expression of *mPer1*, *mCry1*, and *mBmal1* in WT mice and αMUPA mice. *mPer2* and *mClock* still exhibited circadian expression under IF, but with decreased amplitude and a phase-advance (Fig. 4).

To study whether the reduced calories of αMUPA mice affect gene expression due to a possible effect on the SCN, we used

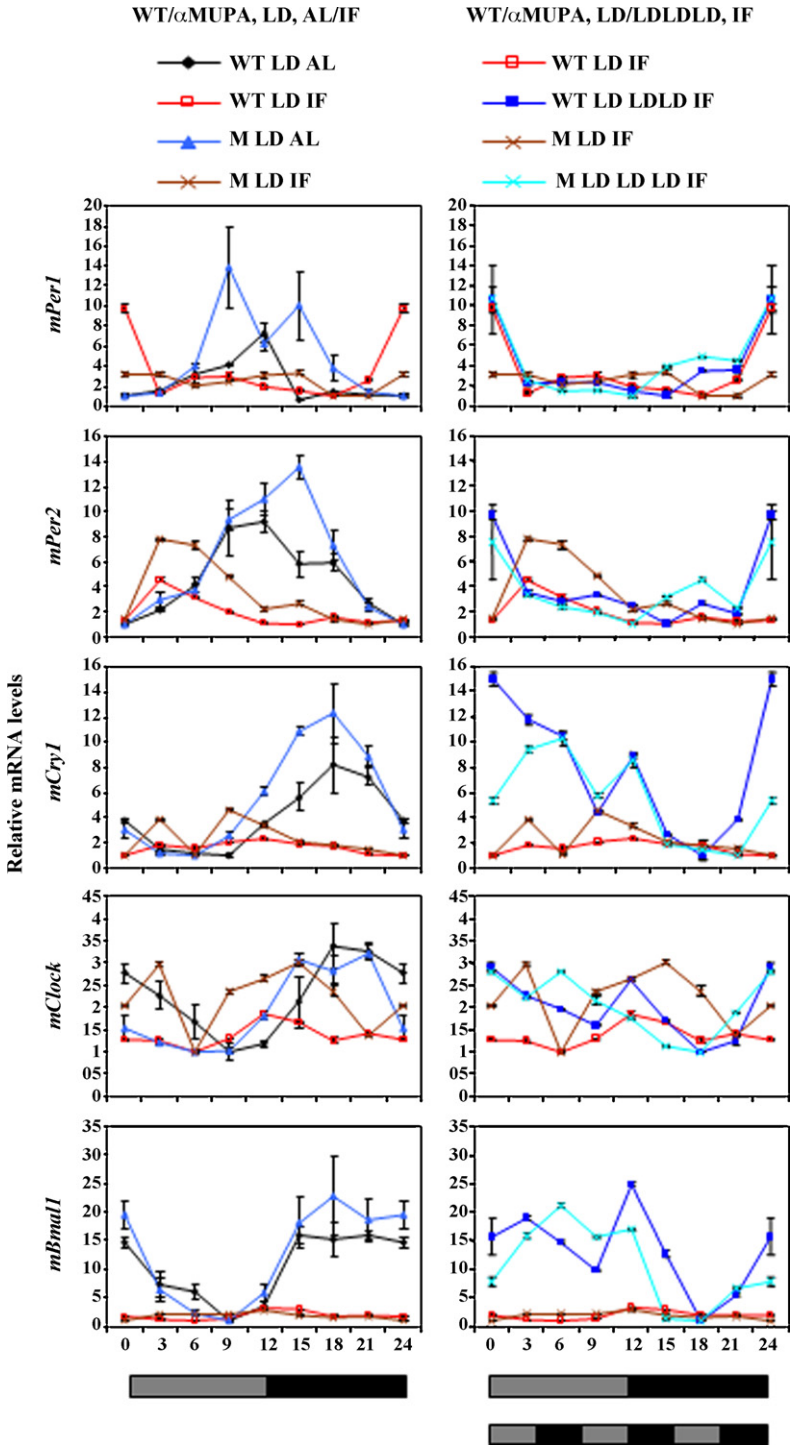

Fig. 4. Expression levels of *mPer1*, *mPer2*, *mCry1*, *mClock*, and *mBmal1* in the liver of either αMUPA mice (M) or WT mice during *ad libitum* (AL) and intermittent fasting (IF). Total RNA extracted from liver tissue collected every 3 h around the circadian cycle (mean ± SEM; $n = 3$ for each time-point and each mouse group) was reverse transcribed and analyzed by quantitative real-time PCR. Clock gene levels were normalized using *Gapdh* as the reference gene. The gray and black bars designate the subjective light and dark cycles, respectively.

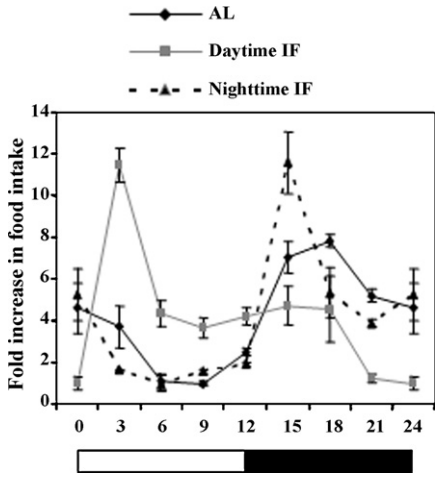

Fig. 5. Food consumption of C57BL mice during *ad libitum* (AL) and day and night intermittent fasting (IF). Food consumption was measured every 3 h around the circadian cycle (n = 10 for each mouse group; mean ± SEM). Values were normalized to the low point. The white and black bars designate the light and dark cycles, respectively.

ultradian light to disrupt circadian rhythms. The loss of clock control would allow us to study the effect of the feeding regimen as the major external cue. Ultradian light (LDLDLD) was used since mice cannot normally entrain to this short cycle, as others and we have shown (Zheng et al., 2001; Froy and Miskin, 2007). Indeed, under these conditions, daily food intake and clock gene expression were disrupted in both αMUPA mice and WT mice (Froy and Miskin, 2007). IF restored rhythms and yielded the same phase in both WT mice and αMUPA mice counteracting the disruptive effect of ultradian light (Fig. 4). Taken together, it seems that IF disrupts clock gene expression, but restores rhythms that have been disrupted by ultradian light, regardless whether the animal is calorically restricted or not.

3.3. Effect of daytime IF vs. nighttime IF

To study whether the disruptive effect of IF under LD conditions was due to the time of food availability, we introduced food during the light period (at ZT0) or the dark period (at ZT12) for 24 h every other day. We used C57BL mice, a mouse strain commonly used in biological clock experimentation. As expected, circadian food consumption exhibited a spike during the light period under daytime IF (Fig. 5), as was shown for WT and αMUPA mice (Fig. 2). Nighttime IF maintained the nocturnal feeding behavior seen under AL conditions (Fig. 5). Analysis of circadian clock gene expression in mouse liver by real-time PCR revealed that although daytime IF disrupted rhythms, as seen with WT and αMUPA mice (Fig. 4), nighttime IF yielded similar amplitudes as under AL conditions (Fig. 6). It is noteworthy that nighttime IF induced slightly shifted rhythms, presumably resulting from the timed food availability at ZT12. These results suggest that the timing of food availability is important for normal expression of clock genes and circadian food intake under the IF regimen.

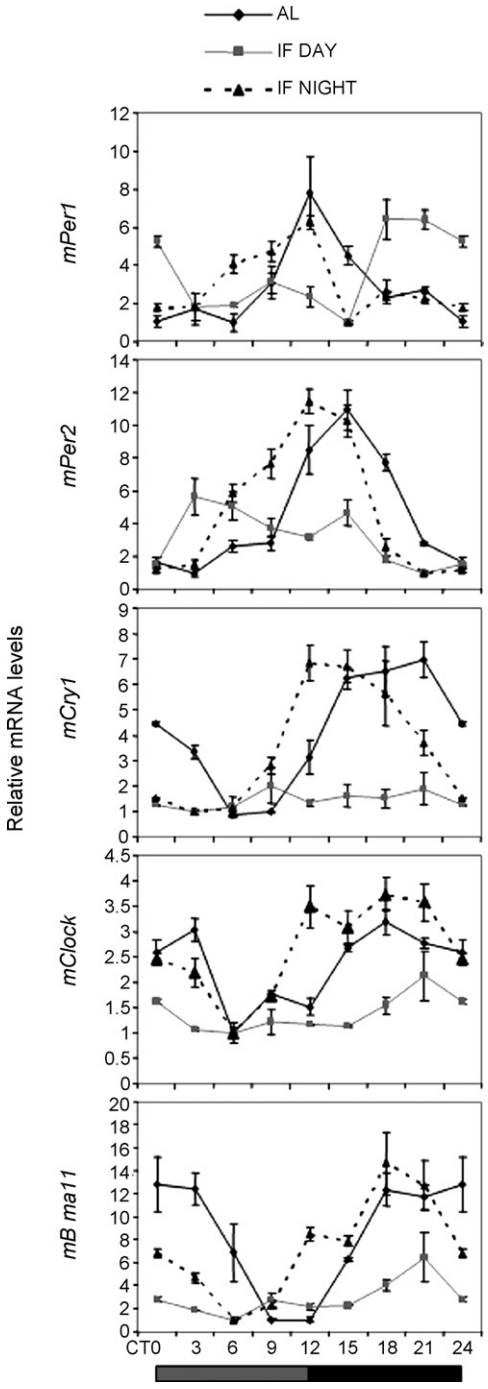

Fig. 6. Expression levels of *mPer1*, *mPer2*, *mCry1*, *mClock*, and *mBmal1* in the liver of C57BL mice during *ad libitum* (AL) and day and night intermittent fasting (IF). Total RNA extracted from liver tissue collected every 3 h around the circadian cycle (mean ± SEM; n = 3 for each time-point and each mouse group) was reverse transcribed and analyzed by quantitative real-time PCR. Clock gene levels were normalized using *Gapdh* as the reference gene. The gray and black bars designate the subjective light and dark cycles, respectively.

4. Discussion

4.1. Effect of IF on circadian rhythms

In this study, we demonstrated that IF can modify circadian rhythms depending on the timing of food availability. Under daytime IF, as food was available for 24 h every other day, mice remained nocturnal as reflected by the high body temperature during the dark period and low during the light period (Fig. 3) and the nocturnal peak of food intake (Fig. 2). In contrast, the effect of daytime IF on the expression of clock genes in the liver was detrimental in a way that their oscillation was almost abolished (Fig. 4). These results are in agreement with recent findings, in which short-term fasting and re-feeding modulated the circadian rhythms of clock genes to different extents in peripheral tissues (Kawamoto et al., 2006). The effects of IF reported herein are in

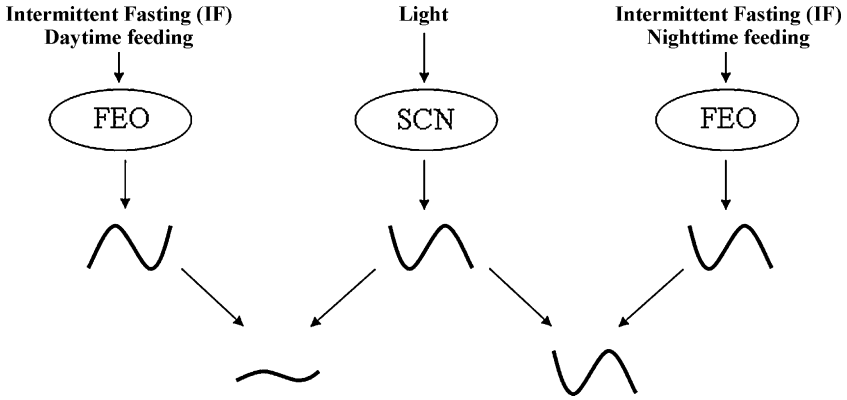

Fig. 7. The contribution of IF and lighting conditions to clock gene expression in the liver. Light is absorbed through the eye and is transmitted to the SCN *via* the retinohypothalamic tract. The SCN then dictates rhythms in the periphery apparently *via* humoral or neuronal cues. IF affects the SCN to yield rhythms in the periphery, but also the FEO to yield rhythms to match with food availability. Nighttime IF yields rhythms that are in sync with those generated by the SCN and FEO, whereas daytime IF leads to dampened rhythms, as rhythms dictated by the FEO clash with those generated by the SCN. SCN, suprachiasmatic nuclei; FEO, food-entrainable oscillator.

contrast with those of restricted feeding (RF) that dictates peripheral rhythms in arrhythmic and mutant mice and animals with lesioned SCN regardless of the lighting conditions (Stephan et al., 1979; Mistlberger, 1994; Hara et al., 2001; Stephan, 2002; Oishi et al., 2002; Horikawa et al., 2005). Thus, our findings show quite explicitly that IF is not as dominant as RF in dictating peripheral rhythms. However, IF exhibited some similarities with RF as reflected by the anticipatory feeding behavior that proceeded the day of fast (Fig. 2) and restoration of circadian rhythms under arrhythmic conditions (Fig. 4), due most likely to the effect on the food entrainable oscillator (FEO). On the other hand, although CR is capable of entraining the clock in the SCN (Challet et al., 1998; Challet et al., 2003; Mendoza et al., 2005; Resuehr and Olcese, 2005), the effect of IF on circadian rhythms in αMUPA mice was similar to that of WT mice, even though they were calorically restricted. We have recently shown that RF affected peripheral rhythms differently in WT mice and αMUPA mice, suggesting a role for the CR-influenced SCN in resetting peripheral rhythms in αMUPA mice (Froy et al., 2008). As both αMUPA mice and WT mice reacted similarly to the IF regimen, these observations suggest that IF, similarly to CR, may affect the SCN clock. This effect could possibly be mediated through a metabolic state generated by the day of fast in IF and the reduced calories in CR. Thus, under daytime IF, hepatic clock gene expression would be controlled by the SCN through the light–dark cycle and IF, as well as by the FEO due to the diurnal anticipatory behavior, as reflected by the spike in food intake (Fig. 2). Activation of both the FEO and the SCN would yield rhythms at two opposite phases leading to an overall arrhythmicity (Fig. 7). In contrast, under nighttime IF, normal rhythms are generated, as both the FEO and the SCN work in synchrony to dictate peripheral rhythms (Fig. 7).

4.2. Role of circadian rhythms in IF-mediated beneficial effects on health and longevity

Similarly to CR (Smith et al., 2004; Masoro, 2005), IF can extend life span and promote health conferring cardio- and neuroprotection (Goodrick et al., 1990; Contestabile and Ciani, 2004; Mattson, 2005; Sharma and Kaur, 2005; Ahmet et al., 2005; Mager et al., 2006; Anson et al., 2003). Recently, we have suggested that the capacity of CR to reset the SCN clock (Challet et al., 1998; Challet et al., 2003; Resuehr and Olcese, 2005) and affect photic responses (Mendoza et al., 2005) poses the biological clock as a possible major factor determining longevity of calorically restricted mice (Froy et al., 2006; Froy and Miskin, 2007). This suggestion was based on better reset rhythms previously associated with improved health and extended life span (Hurd and Ralph, 1998; Hofman and Swaab, 2006), as we have recently found in long-lived, calorically restricted αMUPA mice (Froy et al., 2006; Froy and Miskin, 2007). Here we show, for the first time, that IF can also modify biological rhythms. However, as seen here, food given during the daytime disrupted clock gene expression, whereas food given during the nighttime yielded normal rhythms (Fig. 6). These results suggest that IF can be beneficial when food is given during the activity period of the animal, as explained above. Indeed, neuro- and cardio-protection alongside increased fatty acid oxidation and improved stress resistance have been induced after weeks of IF treatment when food was introduced at the beginning of the activity period (Heilbronn et al., 2005a; Heilbronn et al., 2005b; Mager et al., 2006; Aksungar et al., 2007). It is noteworthy that cardio- and neuro-protection and life span extension were also seen when food was introduced during the day, but after many months of IF treatment (Goodrick et al., 1990; Anson et al., 2003), so that the animals could adjust after such a prolonged treatment. In light of these findings, we assume that the effect of the IF on the SCN through the metabolic change mentioned above alongside the timed feeding might affect the SCN to yield better-reset rhythms. Thus, IF can affect biological rhythms, as shown here, and it promotes health and life span extension (Goodrick et al., 1990), whereas clock disruption leads to accelerated aging and reduced longevity (Penev et al., 1998; Hurd and Ralph, 1998; Klarsfeld and Rouyer, 1998). Therefore, we suggest that SCN resetting by IF could be involved in the health benefits conferred by this regimen.

Acknowledgements

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