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THE SUPRACHIASMATIC NUCLEI ARE INVOLVED IN DETERMINING CIRCADIAN RHYTHMS DURING RESTRICTED FEEDING

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Abstract—The circadian clock in the suprachiasmatic nuclei (SCN) responds to light and regulates peripheral circadian rhythms. Feeding regimens also reset the clock, so that time-restricted feeding (RF) dictates rhythms in peripheral tissues, whereas calorie restriction (CR) affects the SCN clock. To better understand the influence of RF vs. CR on circadian rhythms, we took advantage of the transgenic αMUPA mice that exhibit spontaneously reduced eating, and can serve as a model for CR under *ad libitum* feeding, and a model for temporal CR under RF compared with wild type (WT) mice. Our results show that RF advanced and generally increased the amplitude of clock gene expression in the liver under LD in both mouse types. However, under disruptive light conditions, RF resulted in a different clock gene phase in WT mice compared with αMUPA mice, suggesting a role for the reduced calories in resetting the SCN that led to the change of phase in αMUPA mice. Comparison of the RF regimen in the two lighting conditions in WT mice revealed that *mPer1, mClock,* and *mBmal1* increased, whereas *mPer2* decreased in amplitude under ultradian light in WT mice, suggesting a role for the SCN in determining clock gene expression in the periphery during RF. In summary, herein we reinforce a role for calorie restriction in resetting the SCN clock, and unravel a role for the SCN in determining peripheral rhythms under RF.

Key words: biological clock, circadian rhythms, caloric restriction, restricted feeding, αMUPA.

The central circadian clock located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus influences nearly all aspects of physiology and behavior, including sleep–wake cycle, cardiovascular activity, endocrine system, body temperature, hepatic metabolism, etc. (Panda et al., 2002; Reppert and Weaver, 2002). Similar clock oscillators have been found in peripheral tissues, such as the liver, intestine, and retina (Lee et al., 2001; Reppert and Weaver, 2002; Froy et al., 2006; Young, 2006; Froy and Chapnik, 2007). It is necessary to entrain the SCN clock each day to the external light/dark cycle to prevent drifting (or free-running) out of phase (Quintero et al., 2003). The SCN receives light information via the retinohypothalamic tract (RHT), interprets it, and sends signals to peripheral oscillators via neuronal connections or circulating humoral factors in order to prevent the dampening of circadian rhythms in these tissues (Gooley et al., 2001; Lucas et al., 2001; Reppert and Weaver, 2002). Complete destruction or excision of the SCN abolishes circadian rhythmicity in the periphery, as it leads to a loss of synchrony among individual cells and damping of the rhythm at the population level (Yoo et al., 2004; Welsh et al., 2004).

The clock is a cell autonomous, intracellular transcriptional–translational mechanism sharing the same molecular components in SCN neurons and peripheral cells (Schibler et al., 2003). Many clock gene products function as transcription factors, which possess PAS (PER, ARNT, SIM) and basic helix–loop–helix (bHLH) domains involved, respectively, in protein–protein and protein–DNA interactions. These factors ultimately activate or repress their own expression and, thus, constitute a self-sustained transcriptional feedback loop. Changes in concentration, subcellular localization, posttranslational modifications, and delays between transcription and translation lead to the achieved 24-h cycle (Dunlap, 1999; Panda et al., 2002; Reppert and Weaver, 2002). The core clock mechanism involves CLOCK:BMAL1 heterodimer that binds to enhancer sequences and mediates transcription of a large number of genes including the negative feedback loop *Per*s and *Cry*s. When PERs and CRYs are produced in the cytoplasm, they oligomerize and translocate to the nucleus to inhibit CLOCK:BMAL1-mediated transcription (Froy, 2007).

Recent evidence indicates that clock gene expression in the liver and other peripheral tissues is entrained to periodic meals (Stephan, 2002). Animals, which receive food *ad libitum* (AL) every day at the same time for only a few hours (3–12 h), adjust to the feeding period within a few days and consume their daily food intake during that limited time (Honma et al., 1983; Grasl-Kraupp et al., 1994; Froy et al., 2006). Limiting the time and duration of food availability with no calorie reduction is termed restricted feeding (RF) (Cassone and Stephan, 2002; Schibler et al., 2003; Hirota and Fukada, 2004). Restricting food to a particular time of day has profound effects on the behavior and physiology of animals. Two to four hours before the meal, the animals display an anticipatory behavior, which is demonstrated by an increase in locomotor activity, body temperature, corticosterone secretion, gastrointestinal motility, and activity of digestive enzymes (Saito et al., 1976; Honma et al., 1983; Comperatore and Stephan, 1987; Stephan, 2002), all are known output systems of the bio-

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Abbreviations: AL, *ad libitum*; CR, calorie restriction; DD, total darkness; FEO, food-entrainable oscillator; *Gapdh*, glyceraldehyde 3 phosphate dehydrogenase gene; LD, 12-h light/dark cycle; RF, restricted feeding; SCN, suprachiasmatic nuclei; WT, wild type.

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logical clock. RF drives rhythms in arrhythmic clock mutant mice and animals with lesioned SCN, independently of the light/dark cycle, and in constant darkness (Stephan et al., 1979; Mistlberger, 1994; Hara et al., 2001; Stephan, 2002; Oishi et al., 2002). In most incidents, RF affects circadian oscillators in peripheral tissues, such as liver, kidney, heart, and pancreas, with no effect on the central pacemaker in the SCN, 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 that are normally 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). As soon as food availability returns to normal, the SCN clock, whose phase remains according to the light/dark cycle, resets peripheral oscillators (Damiola et al., 2000). The location of this food-entrainable oscillator (FEO) has been elusive. Lesions in the dorsomedial hypothalamic nucleus (DMH) (Mieda et al., 2006; Gooley et al., 2006; Landry et al., 2006, 2007), the brain stem parabrachial nuclei (PBN) (Davidson et al., 2000; Gooley et al., 2006), and the core and shell regions of nucleus accumbens (Mistlberger and Mumby, 1992; Mendoza et al., 2005a) revealed that these brain regions may be involved in FEO output, but they cannot fully account for the oscillation (Davidson, 2006). However, the role of the biological clock in the anticipatory behavior has been recently demonstrated, as *mPer2* mutant mice did not exhibit wheel-running food anticipation (Feillet et al., 2006; Mistlberger, 2006). As opposed to RF, calorie restriction (CR), which restricts the amount of calories to 60%-70% compared with animals fed AL, entrains the clock in the SCN (Challet et al., 1998, 2003; Mendoza et al., 2005b; Resuehr and Olcese, 2005), indicating that calorie reduction could affect the central oscillator. This effect was apparent in photic entrainment rather than clock gene expression. Animals fed a calorically restricted diet usually consume their daily dose within a few hours. Thus, entrainment of the periphery during CR could be achieved directly, due to the temporal eating, similarly to RF, or by first resetting the SCN, as was previously suggested (Froy and Miskin, 2007).

Experimentally, it is difficult to eliminate the effect of RF, i.e. temporal food consumption, in calorically restricted animals. αMUPA transgenic mice (Miskin et al., 1990), which over-express in the brain the urokinase-type plasminogen activator (uPA), spontaneously eat less when fed AL and live longer compared with their wild type (WT) control mice (Miskin and Masos, 1997; Miskin et al., 2005). αMUPA mice exhibit additional 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, 2005; Miskin et al., 2005). αMUPA mice exhibit reduced feeding also under RF (Froy et al., 2006). Therefore, αMUPA mice can serve as a model for CR in the absence of imposed temporal food consumption under AL feeding, and a model for imposed temporal CR under RF conditions. We used αMUPA mice and WT mice to investigate the contribution of RF or CR under normal or ultradian light on circadian rhythms.

EXPERIMENTAL PROCEDURES

Animals, treatments, and tissue

Female FVB/N and αMUPA mice (Miskin et al., 1990, 1999; Miskin and Masos, 1997) 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). Mice were entrained to a 12-h light/dark cycle (LD) for 2 weeks with food available AL. Rectal body temperature was measured using a thermometer (Oregon Scientific, Neu-Isenburg, Germany). For clock gene expression, mice, having been fed AL, were anesthetized with i.p. injection of ketamine/xylazine (100/7.5 mg/kg) and liver tissues were collected every 3 h around the circadian cycle under dim red light in total darkness (DD). Animals were humanely killed at the end of the experiment. For the RF experiments, after 2 weeks of AL feeding, mice were given food between ZT3 and ZT6 for 3 weeks (ZT0 is the time of lights on). The amount of food eaten was measured, and after 3 weeks the mice were killed 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 three light-dark cycles in 24 h for 5 weeks (LDLDLD). Food intake and tissue collection were performed after the 5-week acclimation in the new light regimen. After 5 weeks, one group was fed AL, whereas the second group was RF-fed for 3 weeks under LDLDLD. All tissues were analyzed by quantitative real-time PCR. The experiments were conducted in full compliance with the strict guidelines of the Hebrew University policy on animal care and use. All experiments conformed to international guidelines on the ethical use of animals. The number of animals used and their suffering were minimized.

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, 2006). Two micrograms of DNase I-treated RNA was reverse transcribed using MMuLV reverse transcriptase (Promega) and random hexamers. One-twentieth 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-R5′-ttgtcccgacgcctctttt-3′) were tested alongside the normalizing gene glyceraldehyde 3 phosphate dehydrogenase (*Gapdh*) (mGapdh-F 5′-caagaggtggacacagtggaga-3′; mGapdh-R 5′-cggccactatattcttcaaggc-3′).

RESULTS

To test the effect of feeding regimens and light conditions on the biological clock, we used the transgenic αMUPA mice that exhibit spontaneously reduced eating under both