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FOOD ENTRAINABLE CIRCADIAN OSCILLATORS IN THE BRAIN

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ABSTRACT

Circadian rhythms in mammalian behavior and physiology rely on daily oscillations in the expression of canonical clock genes. Circadian rhythms in clock gene expression are observed in the master circadian clock, the suprachiasmatic nucleus (SCN), but are also observed in many other brain regions that have diverse roles, including influences on motivational and emotional state, learning, hormone release, and feeding. Increasingly, important links between circadian rhythms and metabolism are appreciated. In particular, restricted feeding (RF) schedules which limit food-availability to a single meal each day, lead to the induction and entrainment of circadian rhythms in food-anticipatory activities in rodents. Food-anticipatory activities include increases in core body temperature, activity and hormone release in the hours leading up to the predictable mealtime. Crucially, RF schedules and the accompanying food-anticipatory activities are also associated with shifts in the daily oscillation of clock gene expression in diverse brain areas involved in feeding, energy balance, learning and memory, and motivation. Moreover, lesions of specific brain nuclei can affect the way rats will respond to RF, but have generally failed to eliminate all food-anticipatory activities. As a consequence, it is likely that a distributed neural system underlies the generation and regulation of food-anticipatory activities under RF. Thus, in the future, we would suggest that a more comprehensive approach should be taken, one that investigates the interactions between multiple circadian oscillators in the brain and body, and starts to report on potential neural systems rather than individual and discrete brain areas.

INTRODUCTION

Circadian rhythms are observed in many aspects of animal behavior and physiology, and include daily rhythms in motivated behaviors, hormone release, body temperature, cognition and mood (Hastings *et al.*, 2007; Lamont *et al.*, 2007; Mendoza, 2007; Schibler, 2007; Wirz-Justice, 2008). At the molecular level, circadian clocks rely on daily oscillations in the transcription and translation of canonical clock genes (Reppert & Weaver, 2002). In mammals, circadian oscillations in clock gene expression are observed in the master circadian clock, the suprachiasmatic nucleus (SCN), and are fundamentally linked to the generation and regulation of circadian rhythms within this structure (Fuller *et al.*, 2008; Gavrila *et al.*, 2008). Circadian rhythms in clock gene expression are also observed in many brain areas outside of the SCN, areas with diverse roles in behavior and physiology (Guilding & Piggins, 2007). Tissue-specific clock gene expression is thought to modulate many essential cellular, homeostatic and metabolic processes (Rutter *et al.*, 2002; Challet *et al.*, 2003; Pardini & Kaeffer, 2006; Hastings *et al.*, 2007). In turn, it is becoming increasingly clear that metabolic signals influence clock gene expression (Liu *et al.*, 2007; Belden & Dunlap, 2008; Nakahata *et al.*, 2008; Rodgers *et al.*, 2008; Grimaldi *et al.*, 2009). Thus, patterns of food-intake not only affect satiety and hunger, but also have the potential to influence clock gene expression throughout the brain and body.

To probe the interaction between metabolism and clock gene expression, in rodents, restricted feeding (RF) schedules have been used. RF schedules limit food-availability to a single meal each day and in response, animals

gradually develop a number of food-anticipatory activities (see Fig. 1). Specifically, increases in locomotor activity, body temperature and hormone release are observed before the predictable daily meal (Stephan, 2002). Most importantly, RF schedules also shift or entrain the daily rhythm of clock gene expression in many brain areas (Wakamatsu *et al.*, 2001; Mieda *et al.*, 2006; Abe *et al.*, 2007; Angeles-Castellanos *et al.*, 2007; Girotti *et al.*, 2009). Thus, RF provides a powerful tool with which to study the neural and molecular bases of food-anticipatory activities as well as the nature and function of daily oscillations of clock gene expression in the brain.

CIRCADIAN CLOCK GENE EXPRESSION IN THE BRAIN

The basis for self-sustained circadian rhythms is a highly conserved molecular autoregulatory feedback loop that oscillates with a ~24-h period (Reppert & Weaver, 2002; Yoo *et al.*, 2004; Kornmann *et al.*, 2007; Liu *et al.*, 2007; Asher *et al.*, 2008; Belden & Dunlap, 2008). Briefly, at the core of this loop, the genes Clock and Bmal1 encode proteins that dimerize and act as a transcription factor. CLOCK:BMAL1 heterodimers then enhance the transcription of period (Per1, Per2, and Per3) and cryptochrome (Cry1 and Cry2) genes (Gekakis *et al.*, 1998; Hogenesch *et al.*, 1998). PER and CRY expression oscillates across the day and the proteins feedback into the nucleus, and interfere with the transcriptional activity of CLOCK:BMAL1 (Kume *et al.*, 1999; Vitaterna *et al.*, 1999; Shearman *et al.*, 2000). In essence, these genes and their protein products are the gears and motors that allow circadian clocks to "tick." Thus, daily oscillations in clock gene

expression (e.g. PER1 and PER2), allow for the identification of brain areas that express crucial circadian clockwork.

Circadian rhythms in clock gene expression have been found in many brain regions outside the SCN including the hippocampus, piriform cortex, cingulate cortex, prefrontal cortex, striatum, paraventricular hypothalamic nucleus, arcuate nucleus, olfactory bulb, nucleus accumbens, and pituitary (Yamamoto *et al.*, 2001; Abe *et al.*, 2002; Gillespie *et al.*, 2003; Kriegsfeld *et al.*, 2003; Shieh, 2003; Granados-Fuentes *et al.*, 2004; Shieh *et al.*, 2005; Angeles-Castellanos *et al.*, 2007). Furthermore, we recently identified robust rhythms of PER2 expression in select regions of the limbic forebrain, areas that are important in the regulation of stress, motivation and emotion (Amir *et al.*, 2004; Lamont *et al.*, 2005b). These limbic areas include the oval nucleus of the bed nucleus of the stria terminalis (BNSTov), central nucleus of the amygdala (CEA), basolateral amygdala (BLA) and dentate gyrus (DG). Although circadian oscillations in clock gene expression in most brain areas are either directly or indirectly under the control of the SCN, rhythms in brain regions outside of the SCN are also sensitive to diverse hormonal and behavioral manipulations, including restricted feeding (Wakamatsu *et al.*, 2001; Lamont *et al.*, 2005a; Amir & Robinson, 2006; Perrin *et al.*, 2006; Segall *et al.*, 2006; Angeles-Castellanos *et al.*, 2007; Verwey *et al.*, 2007; Waddington Lamont *et al.*, 2007; Verwey *et al.*, 2008; Verwey *et al.*, 2009).

BRAIN AREAS SENSITIVE TO RESTRICTED FEEDING

There are two reasons to study circadian rhythms of gene expression under RF. The first reason is to determine which brain areas are involved in the regulation and entrainment of food-anticipatory activities, per se. The second reason is to study of the properties and functions of putative circadian oscillators outside the SCN. Our recent work has been concerned primarily with the latter issue and we have recently started to define some of the hormonal and behavioral influences that affect clock gene expression in the limbic forebrain (Amir *et al.*, 2004; Lamont *et al.*, 2005b; Amir & Stewart, 2009). To carry out these studies, we and other have studied immediate early gene expression (e.g. cFOS) and the expression of clock genes (e.g. PER1, PER2) across the circadian cycle (Angeles-Castellanos *et al.*, 2004; Mendoza *et al.*, 2005b; Angeles-Castellanos *et al.*, 2007; Verwey *et al.*, 2007).

Immediate early genes, such as cFOS, are readily expressed throughout the brain in response to diverse stimuli. Although cFOS expression might be elevated in a particular brain area, clock gene expression may be unaffected in the same region. Our own research has illustrated this point by contrasting rats on a RF schedule with rats on a restricted treat (RT) schedule (Verwey *et al.*, 2007). Specifically, a restricted daily meal of highly palatable complete meal replacement, chocolate Ensure Plus, was given to fasted (RF) and freely-fed (RT) rats. Although both groups consumed similar amounts of Ensure, only 37% of the RT group exhibited anticipatory running wheel activity in the hours leading up to the Ensure-access while 100% of the RF group anticipated the daily mealtime. Of note, in response to Ensure, both groups exhibited increases in

cFOS expression in the limbic forebrain and hypothalamus. In contrast, the daily pattern of PER2 expression in the same brain structures was only affected by RF; while the RT group and the ad libitum fed control groups were indistinguishable. Thus, this dissociation between cFOS and PER2 expression in response to RF and RT, emphasizes the importance of distinguishing conclusions based on immediate early gene expression from conclusions based on clock gene expression. Moreover, the differential effects of RF and RT on PER2 expression suggest that clock gene expression in the limbic forebrain and hypothalamus is relatively insensitive to the incentive value of food, and instead, requires the metabolic challenges associated with fasting under RF.

The limbic forebrain – The limbic forebrain contributes to the regulation of motivation and emotion, and includes brain areas such as the bed nucleus of the stria terminalis (BNST), amygdala and hippocampus. Collectively, these areas modulate neuroendocrine, autonomic and behavioral responses to different types of stress and to drugs of abuse (Loewy, 1991; Gray, 1993; Erb *et al.*, 2001; Nijsen *et al.*, 2001). Moreover, these brain areas also modulate fear and anxiety, learning and memory, reproductive and maternal behaviors, as well as ingestive behaviors (Casada & Dafny, 1991; Van de Kar & Blair, 1999; Stefanova & Ovtscharoff, 2000; Walker *et al.*, 2001; Figueiredo *et al.*, 2003; Walker *et al.*, 2003). Under ad libitum feeding conditions, PER2 is expressed with a circadian rhythm in the BNSTov, CEA, BLA and DG (Amir *et al.*, 2004; Lamont *et al.*, 2005b). On a 12h:12h light-dark schedule, daily oscillations of PER2 expression in the BNSTov and CEA peak around the time of transition from day to night

(dusk), a daily expression profile which is in synchrony with PER2 expression in the SCN (Amir *et al.*, 2004; Lamont *et al.*, 2005b). In contrast, peak PER2 expression in the BLA and DG is observed around the time of transition from night to day (dawn) (Lamont *et al.*, 2005b), opposite to the oscillation observed in the BNSTov, CEA and SCN (see Fig. 2). Consistent with other extra-SCN circadian oscillators (Sakamoto *et al.*, 1998), no circadian rhythm is observed in PER2 expression in the limbic forebrain after the SCN is lesioned (Amir *et al.*, 2004; Lamont *et al.*, 2005b). Additionally, daily rhythms of PER2 expression in the BNSTov and CEA are also sensitive to hormonal signals. In the rat, adrenal stress hormones (corticosterone), gonadal hormones (estrogen, testosterone) and thyroid hormones, all modulate PER2 expression in the BNSTov and CEA, though, importantly, these hormones do not modulate PER2 expression in the SCN, BLA and DG (Amir *et al.*, 2004; Lamont *et al.*, 2005b; Amir & Robinson, 2006; Perrin *et al.*, 2006). In particular, the daily rhythm of corticosterone, the main adrenal glucocorticoid in rats, is critical for daily rhythms in PER2 expression in the BNSTov and CEA (Segall *et al.*, 2006; Segall *et al.*, 2009). Interestingly, in contrast to many other manipulations that fail to modulate PER2 expression in the BLA and DG, the daily rhythm of PER2 expression in these areas is affected by RF schedules.

In our studies on the effect of RF on PER2 expression in the limbic forebrain, we found that when food is restricted to the middle of the day when rats normally do not eat (daytime RF), the daily rhythms of PER2 expression in the BNSTov, CEA, BLA and DG shift such that peak PER2 expression in all of

these structures is observed ~12 h after the daytime meal (Verwey *et al.*, 2007). In contrast, RF schedules that provide a nighttime meal (nighttime RF), a more appropriate mealtime for nocturnal rats, shift the daily rhythms of PER2 expression in the BNSTov and CEA, but not the BLA and DG (see Fig. 3). As was the case for daytime RF, peak PER2 expression was observed in the BNSTov and CEA ~12 h after the predictable mealtime, however, PER2 expression in the BLA and DG was unaffected and similar to that in ad libitum fed controls (Verwey *et al.*, 2008). Thus clock gene expression in the BLA and DG is affected only by daytime RF, whereas, because PER2 expression in the BNSTov and CEA peaks ~12 h after both daytime RF and nighttime RF, clock gene expression in these areas would appear to be food-entrained.

The changes in clock gene expression seen in response to RF vary as a function of the gene studied. Differences between PER1 and PER2 expression have been observed in the limbic forebrain. In ad libitum fed rats, PER1 expression is reportedly rhythmic in the BNST and hippocampus, but not rhythmic in the CEA or BLA (Angeles-Castellanos *et al.*, 2007), whereas, as previously noted, PER2 expression is rhythmic in all of these brain areas under ad libitum feeding (Amir *et al.*, 2004; Lamont *et al.*, 2005b). Under a daytime RF schedule, PER1 expression was reported to be unchanged in the CEA and BLA, but altered in the BNST and hippocampus (Angeles-Castellanos *et al.*, 2007). In contrast, under similar conditions, we found robust shifts in PER2 expression in all these regions (Amir *et al.*, 2002; Verwey *et al.*, 2007; Waddington Lamont *et al.*, 2007; Verwey *et al.*, 2008). These data suggest key differences in the

regulation of PER1 and PER2 expression in the limbic forebrain by RF, but these two genes have not been studied concurrently in the same experiment, so a note of caution is necessary. Indeed, whereas we quantified PER2 expression specifically in the BNSTov, Angeles-Castellanos et al. (2007) quantified PER1 immunoreactivity in the BNST as a whole. Similarly, we quantified PER2 expression specifically in the DG, whereas Angeles-Castellanos et al. (2007) examined PER1 expression throughout the hippocampus. Both the BNST and hippocampus are heterogeneous structures that contain many distinct subregions with different functions and these subregions should be analyzed individually. Nevertheless, there is also evidence that PER1 and PER2 are differentially expressed in the limbic forebrain in the mouse (Feillet *et al.*, 2008).

The suprachiasmatic nucleus (SCN) – The SCN also exhibits changes in the expression of clock genes in response to RF schedules, but compared to the large shifts observed in the limbic forebrain, these changes are usually subtle. For example, the daily rhythm of expression of Per1 mRNA in the SCN shifts in response to RF, but these changes are relatively small. The rhythms of Per1 in the SCN did not become entrained by the RF schedule (Mendoza *et al.*, 2005a). In our studies we have not observed RF-induced changes the expression of PER2 in the SCN (Verwey *et al.*, 2007; 2008). One exception to this generalization occurs when rats are housed in constant light. Under these conditions, circadian locomotor activity rhythms become disrupted and PER2 expression in the SCN does not exhibit a circadian rhythm. Remarkably, when placed on a RF schedule in these constant light conditions, both locomotor

activity and PER2 rhythms are reinstated and are entrained to the time of feeding (Lamont *et al.*, 2005a).

Some of the most robust effects on clock gene expression in the SCN have been observed in response to caloric restriction (Challet *et al.*, 1996; Challet *et al.*, 1998; Andrade *et al.*, 2004; Mendoza *et al.*, 2005d). In mice, hypocaloric feeding schedules can entrain SCN-driven circadian rhythms and affect clock gene expression in this brain area. Importantly, this effect has been attributed to the hypocaloric feeding, per se, rather than the reorganization of circadian activities and the development of food-anticipatory activities. Specifically, delivering a single hypocaloric meal each day leads to the development of food-anticipatory activities. However, the delivery of 6 meals/day do not lead to the same development of coordinated food-anticipation and yet, still affect clock gene expression in the SCN (Mendoza *et al.*, 2008). This work clearly demonstrates that certain changes in clock gene expression can be brought on simply by reduced caloric intake rather than food-entrainment, per se.

The dorsomedial hypothalamic nucleus (DMH) – The DMH is an important relay in the transmission of signals from the SCN to the rest of the brain and body. This structure also appears to integrate metabolic signals with inputs it receives from the SCN (Elmquist *et al.*, 1998), and provides important outputs to brain areas that regulate sleep, such as the ventrolateral preoptic nucleus, and arousal, such as the lateral hypothalamus (Chou *et al.*, 2003). Under ad libitum feeding conditions, the DMH does not exhibit robust daily oscillations in clock gene expression, but under RF, large amplitude circadian rhythms in Bmal1,

PER1 and PER2 expression emerge (Mieda *et al.*, 2006; Verwey *et al.*, 2007; Fuller *et al.*, 2008; Verwey *et al.*, 2008; Moriya *et al.*, 2009). These daily oscillations in clock gene expression in the DMH depend on the time of day when meals are provided. Specifically, under a daytime RF schedule, peak PER2 expression is observed in the DMH soon after the mealtime. However, under a nighttime RF schedule, peak PER2 expression is observed in the DMH several hours after the mealtime (Verwey *et al.*, 2007; 2008). This interaction between the time when food is given and the time of peak expression suggests that although clock gene expression in the DMH is affected by RF schedules, it may not be exclusively food-entrained. To date, studies of clock gene expression in the DMH have been carried out in SCN-intact rats and mice and the DMH has strong connections to the SCN (Chou *et al.*, 2003). An intriguing question, therefore, is whether clock gene expression in the DMH could be food-entrained if the SCN were lesioned.

THE CIRCADIAN BASIS OF FOOD-ANTICIPATORY ACTIVITIES

The characteristics of food-anticipatory activities are consistent with an underlying food-entrained circadian oscillator. For example, when rats are fasted for 2-4 days after being on a RF schedule, food-anticipatory activities continue to appear each day around the predicted mealtime (Boulos *et al.*, 1980). An hourglass model, whereby rats anticipate subsequent food-arrival a fixed number of hours after the last meal, would not account for daily food-anticipation under a 72-h fast. Also consistent with a circadian mechanism, food-anticipatory

activities show limits of entrainment. Although rodents accurately predict 24-h food-intervals, 18-h intervals do not lead to characteristic food-anticipatory activities (Stephan *et al.*, 1979a; Stephan *et al.*, 1979b). If food anticipatory behaviors were based on an hourglass mechanism, 18-h and 24-h schedules should represent a similar challenge. Based on these findings, putative circadian oscillators throughout the brain and body are thought to contribute to the regulation and entrainment of food-anticipatory activities. Although some aspects of food-anticipation persist when canonical circadian clock genes are mutated or knocked out, deficits are also observed (Feillet *et al.*, 2006; Fuller *et al.*, 2008; Storch & Weitz, 2009).

Light-entrained and food-entrained circadian oscillators are viewed as separate and relatively independent systems (Mistlberger, 1994; Stephan, 2002). Consistent with this hypothesis, there are generally subtle effects of RF on clock gene expression in the SCN, and SCN lesions do not interfere with the development of food-anticipatory activities (Stephan *et al.*, 1979a; Stephan *et al.*, 1979b). However, because no single brain lesion eliminates all food-anticipatory activities, the specific areas and tissues of the brain and body that are involved in circadian food-entrained anticipatory activities remain unclear. Food-entrained and light-entrained oscillators affect the expression of overlapping canonical clock genes in many brain areas and so the focus of experiments should start to shift towards uncovering networks of RF-sensitive brain areas.

THE DMH AND FOOD-ANTICIPATORY ACTIVITY

Clock gene expression in the DMH has been suggested to be necessary for the expression of food anticipatory activities (Gooley *et al.*, 2006; Fuller *et al.*, 2008). Specifically, mutant mice with a targeted disruption of the Bmal1 gene are reported to exhibit deficient circadian rhythms and disruptions in their ability to show food-anticipatory increases in core body temperature (Fuller *et al.*, 2008). Moreover, food-anticipation has been reported to be restored when exogenous Bmal1 is transfected to the DMH (Fuller *et al.*, 2008). Lesions of the DMH have also been reported to interfere with the expression of food-anticipatory activities (Gooley *et al.*, 2006). Clearly, these studies suggest that the DMH could be important in the expression of certain food-anticipatory rhythms. However, there are also a number of other studies that contradict this conclusion (Landry *et al.*, 2006; Landry *et al.*, 2007; Moriya *et al.*, 2009; Storch & Weitz, 2009). For example, it has recently been reported that some food-anticipatory behaviors persist in Bmal1 mutant mice (Pendergast *et al.*, 2009; Storch & Weitz, 2009). Furthermore, there are a number of reports of robust food-anticipatory activities in DMH-lesioned rats (Landry *et al.*, 2006; Landry *et al.*, 2007). These discrepancies have led to active debate in the literature (Gooley *et al.*, 2006; Landry *et al.*, 2006; Fuller *et al.*, 2008; Mistlberger *et al.*, 2008) and it is clear that a more comprehensive understanding of the control of food anticipatory rhythms will depend on further experimentation and replication.

Accurate food-anticipatory activities are not required for robust circadian rhythms in clock gene expression in the DMH. Variable restricted feeding (VRF) schedules provide an unpredictable meal each day, and inherently prohibit

accurate food-anticipation (Escobar *et al.*, 2007; Verwey *et al.*, 2009). When the mealtime is varied within either the 12 h of light or the 12 h of darkness, large amplitude rhythms in PER1 expression are clearly observed in the DMH, despite a lack of food-entrained running wheel activity (Verwey *et al.*, 2009). In contrast, when food availability is varied throughout the entire 24-h cycle, PER1 expression in the DMH is elevated across the day as compared to controls, though no daily rhythm is observed (Verwey *et al.*, 2009). Thus, robust circadian rhythms in PER1 expression can be induced by RF schedules even without the entrainment of food-anticipatory activity. Conversely, food-anticipatory activities have also been observed in the absence of circadian rhythms in clock gene expression in the DMH. Food-anticipatory activities have been reported in rats receiving a daily chocolate treat (Mistlberger & Rusak, 1987; Mendoza *et al.*, 2005c), but in those rats anticipating the daily treat, PER2 expression in the DMH is similar to ad libitum fed controls (Verwey *et al.*, 2007; 2008). Collectively, these findings demonstrate that the food-entrainment of running wheel activity and PER expression in the DMH are dissociable. However, the induction and entrainment by RF of daily rhythms in clock gene expression in the DMH certainly represents an interesting and important area of study, whether or not it is crucial to all food-anticipatory behaviors.

POTENTIAL MECHANISMS

In the anticipation of food, canonical clock proteins do not operate in isolation. Homeostatic signals, learning, and as yet undiscovered circadian mechanisms all

may contribute to the expression of food-anticipatory rhythms. Furthermore, in spite of the observation that certain food-anticipatory activities persist in mice with circadian gene mutations, extra-SCN clock gene expression remains an important area of study in the context of restricted feeding. Indeed, several lines of evidence, mostly from work in the periphery, have made it abundantly clear that clock gene expression has an important influence on tissue-specific metabolism and physiology (Fu *et al.*, 2002; Oster *et al.*, 2006; Chen-Goodspeed & Lee, 2007; Winter *et al.*, 2007). Fundamentally, understanding the regulation and consequence of clock gene expression in different regions of the brain is not only important for the elucidation of mechanisms underlying RF, but also essential to understanding the interaction between the circadian system and physiology.

Several pathways influence circadian clock gene expression throughout the brain and body. Daily rhythms of clock gene expression in the limbic forebrain and hypothalamus are selectively sensitive to RF and not RT. Thus, rather than pathways associated with reward and feeding, per se, metabolic pathways associated with fasting are likely involved in the regulation of clock gene expression in these areas. One metabolic pathway whereby RF schedules could influence circadian oscillators in the brain involves two interacting proteins that relate directly to cellular metabolism and clock gene expression, PGC1a and SIRT1 (Rodgers *et al.*, 2005; Liu *et al.*, 2007; Asher *et al.*, 2008; Nakahata *et al.*, 2008). Specifically, the expression of PGC-1a in peripheral tissues such as liver and muscle follows a circadian rhythm (Liu *et al.*, 2007). In turn, PGC-1a has

been found to induce and regulate the rhythmic expression of clock genes in peripheral tissues and, significantly, to affect behavioral and physiological circadian rhythms (Canaple *et al.*, 2006; Liu *et al.*, 2007). In contrast, SIRT1 is directly sensitive to food-availability, and deacetylates PER2, thus also affecting the circadian feedback loop (Asher *et al.*, 2008; Nakahata *et al.*, 2008). These mechanisms have been well-studied in the periphery (Liu *et al.*, 2007; Asher *et al.*, 2008; Nakahata *et al.*, 2008), but additional research is needed to demonstrate their importance in the brain. PGC-1a mRNA is expressed in many brain areas, including the olfactory bulb, cerebral cortex, septal nucleus, striatum, hippocampus, and substantia nigra (Tritos *et al.*, 2003; Cowell *et al.*, 2007). Furthermore, the expression of PGC-1a in cortical cells has been shown to be regulated by neuronal activity (Meng *et al.*, 2007), raising the intriguing possibility that PGC-1a may mediate the effects of signals arising from daily restricted feeding and from other perturbations of physiology and behavior on the expression of clock genes in the brain.

A second pathway whereby RF schedules could influence clock gene expression in the limbic forebrain is through the action of glucocorticoids. Glucocorticoids modulate clock gene expression in the periphery, and daily rhythms in glucocorticoid release are needed to sustain at least certain brain oscillators (Segall *et al.*, 2006). The daily rhythm of glucocorticoid release is also changed by RF, and a food-anticipatory release of this stress hormone is well-documented (Ahlers *et al.*, 1980). Although glucocorticoids do not appear to influence PER2 expression in the limbic forebrain under RF (Segall *et al.*, 2008),

the RF modulation of glucocorticoid release could have a role in the modulation of clock gene expression in the brain, especially PER1, which contains a glucocorticoid responsive element in its promoter region (Yamamoto *et al.*, 2005).

A third pathway whereby RF schedules could influence clock gene expression is through body temperature. The daily rhythm of body temperature is entrained by RF and has been studied extensively in the context of the DMH. Circadian rhythms in cerebral temperature also occur (Boudreau *et al.*, 2008), but their importance to clock gene expression remains unknown. In the periphery, circadian rhythms in body temperature and the associated induction of heat shock proteins, appear to have some influence on clock gene expression (Kornmann *et al.*, 2007). Through a combination of inputs, some of which have been discussed here, RF schedules are able to influence clock gene expression in a region-dependent manner. Elucidating the mechanisms whereby RF is able to alter clock gene expression in some brain areas and not others, and uncovering the consequences of these effects, should be a major focus of future research.

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FIGURE CAPTIONS

FIGURE 1

Actograms illustrating the daily pattern of running-wheel activity for two representative rats that each received ad libitum food-access (AL; first 9 days of the record) followed by restricted feeding (RF; last 10 days of the record). Each horizontal line plots 24 h and sequential days are arranged from top to bottom. Rats were housed in a 12:12 light-dark cycle, illustrated by the empty (light phase) and shaded (dark phase) areas in each actogram. Numbers above the actograms indicate zeitgeber time (ZT). Under RF, rats received 2 h of access to food each day (ZT 4-6), illustrated by the shaded rectangle. As expected, RF produced characteristic food-anticipatory running-wheel activity in the few hours leading up to the predictable mealtime.

FIGURE 2

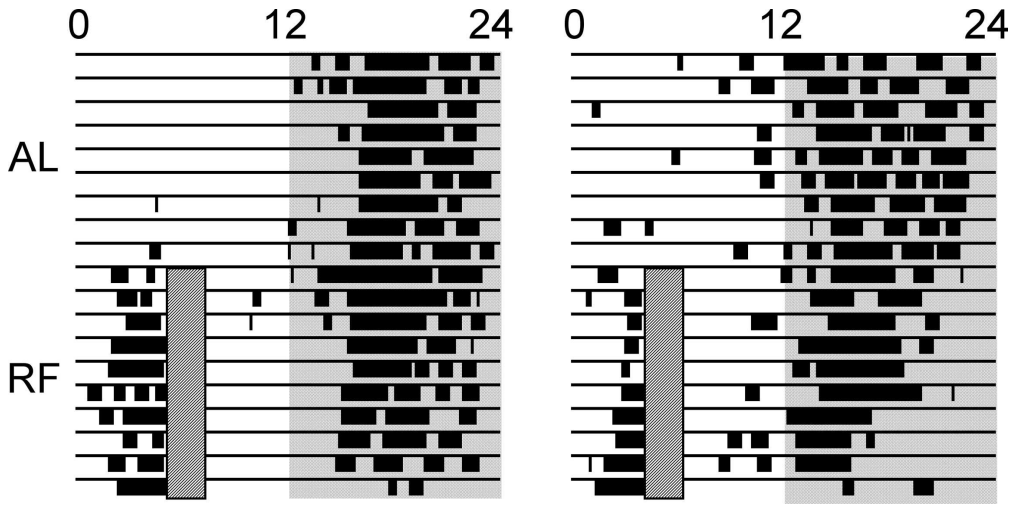
Daily patterns of PER2 expression in the SCN, BNSTov, CEA, BLA, DG and DMH of ad libitum fed rats. Each line illustrates the estimated mean of PER2-immunoreactivity in a particular structure, plotted according to zeitgeber time (ZT; ZT0=Lights on, ZT12=Lights off). The 12h:12h light-dark cycle is also illustrated by the shaded and unshaded areas of the graph. With the exception of the DMH, all structures exhibit robust daily rhythms in PER2 expression. Peak PER2 expression is observed in the SCN, BNSTov, and CEA at ZT13, whereas, peak PER2 expression is observed in the BLA and DG at ZT1 (Redrawn from data published in Verwey *et al.,* 2007).

FIGURE 3

Daily patterns of PER2 expression in the SCN, BNSTov, CEA, BLA, DG and DMH of rats under restricted feeding (RF) and ad libitum (AL) feeding conditions. Mean PER2-immunoreactivity is plotted for each structure, according to zeitgeber time (ZT; ZT0=Lights on, ZT12=Lights off). The daily meal of Ensure, was provided either in the middle of the day (RF-day; ZT4-6; Illustrated by the open rectangle) or middle of the night (RF-night; ZT16-18; Illustrated by the shaded rectangle). The daily pattern of PER2 expression in the BNSTov, CEA and DMH was affected by both RF schedules. In contrast, the daily pattern of PER2 expression in the BLA and DG was only shifted under RF-day conditions. Finally, PER2 expression in the SCN was unaffected by either RF schedule (Redrawn from data published in Verwey *et al.,* 2007; Verwey *et al.,* 2008).

ABBREVIATIONS

BLA	Basolateral amygdala
BNST	Bed nucleus of the stria terminalis
BNSTov	Oval nucleus of the bed nucleus of the stria terminalis
CEA	Central nucleus of the amygdala
CRY1	Cryptochrome1
CRY2	Cryptochrome2
DG	Dentate Gyrus of the Hippocampus
DMH	Dorsomedial hypothalamic nucleus
Ensure	Chocolate ensure plus
PGC-1a	Peroxisome proliferator activated receptor gamma coactivator 1a
PER1	Period1 protein
PER2	Period2 protein
RF	Restricted feeding
RT	Restricted treat
SCN	Suprachiasmatic nucleus
SIRT1	Sirtuin1



Actograms illustrating the daily pattern of running-wheel activity for two representative rats that each received ad libitum food-access (AL; first 9 days of the record) followed by restricted feeding (RF; last 10 days of the record). Each horizontal line plots 24 h and sequential days are arranged from top to bottom. Rats were housed in a 12:12 light-dark cycle, illustrated by the empty (light phase) and shaded (dark phase) areas in each actogram. Numbers above the actograms indicate zeitgeber time (ZT). Under RF, rats received 2 h of access to food each day (ZT 4-6), illustrated by the shaded rectangle. As expected, RF produced characteristic food-anticipatory running-wheel activity in the few hours leading up to the predictable mealtime.
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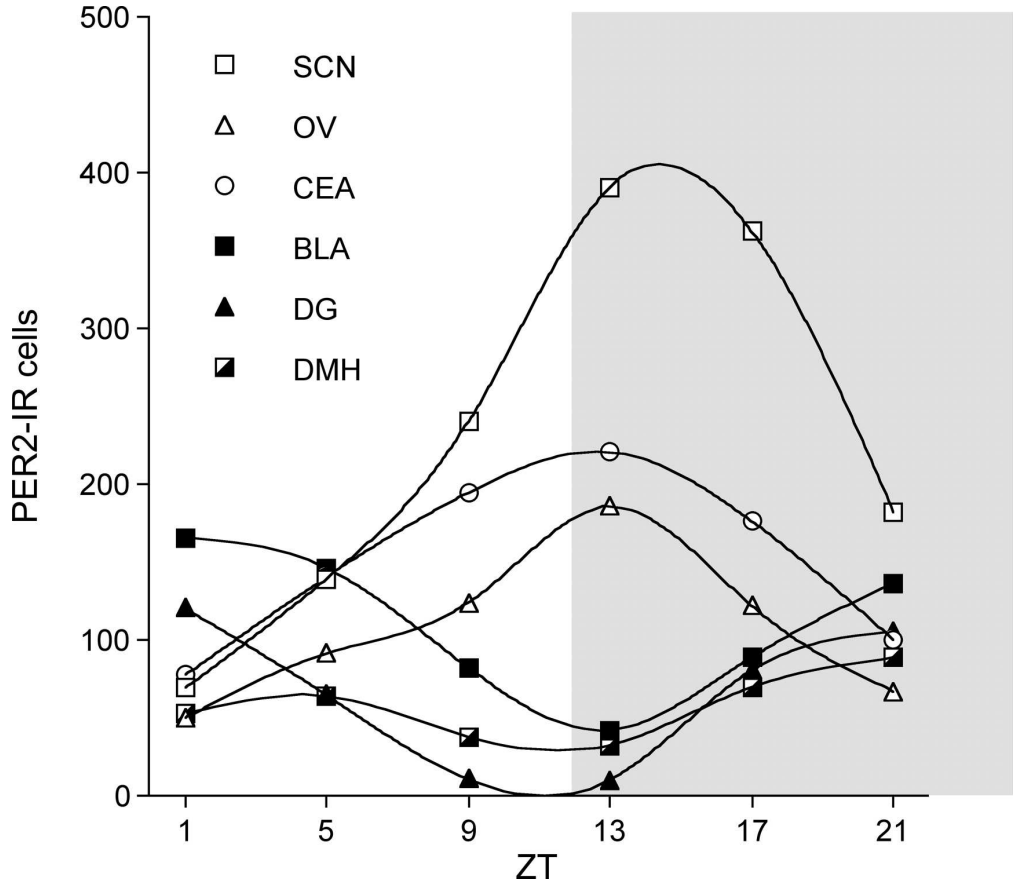

Daily patterns of PER2 expression in the SCN, BNSTov, CEA, BLA, DG and DMH of ad libitum fed rats. Each line illustrates the estimated mean of PER2-immunoreactivity in a particular structure, plotted according to zeitgeber time (ZT; ZT0=Lights on, ZT12=Lights off). The 12h:12h light-dark cycle is also illustrated by the shaded and unshaded areas of the graph. With the exception of the DMH, all structures exhibit robust daily rhythms in PER2 expression. Peak PER2 expression is observed in the SCN, BNSTov, and CEA at ZT13, whereas, peak PER2 expression is observed in the BLA and DG at ZT1 (Redrawn from data published in Verwey et al., 2007).
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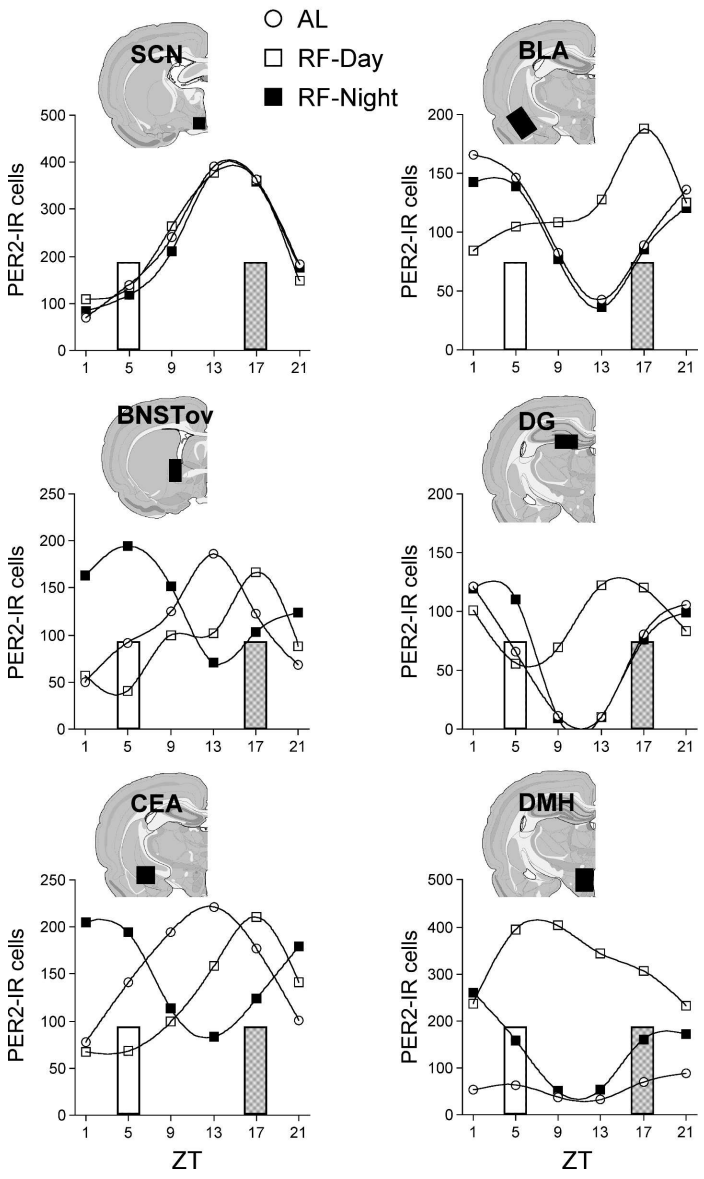

Daily patterns of PER2 expression in the SCN, BNSTov, CEA, BLA, DG and DMH of rats under restricted feeding (RF) and ad libitum (AL) feeding conditions. Mean PER2-immunoreactivity is plotted for each structure, according to zeitgeber time (ZT; ZT0=Lights on, ZT12=Lights off). The daily meal of Ensure, was provided either in the middle of the day (RF-day; ZT4-6; Illustrated by the open rectangle) or middle of the night (RF-night; ZT16-18; Illustrated by the shaded rectangle). The daily pattern of PER2 expression in the BNSTov, CEA and DMH was affected by both RF schedules. In contrast, the daily pattern of PER2 expression in the BLA and DG was only shifted under RF-day conditions. Finally, PER2 expression in the SCN was unaffected by either RF schedule (Redrawn from data published in Verwey et al., 2007; Verwey et al., 2008).
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