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NUCLEUS-SPECIFIC EFFECTS OF MEAL DURATION ON DAILY PROFILES OF PERIOD1 AND PERIOD2 PROTEIN EXPRESSION IN RATS HOUSED UNDER RESTRICTED FEEDING

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Abstract—Restricted feeding (RF) schedules provide a cycle of fasting and feeding each day and induce circadian rhythms in food-anticipatory activity. In addition, daily rhythms in the expression of circadian clock genes, such as rhythms in Period1 (PER1) or Period2 (PER2), are also shifted in many brain areas that are important for the regulation of motivation and emotion. In order to differentiate brain areas that respond to the time of food presentation from areas that are sensitive to the degree of restriction, the present study compared RF schedules that provided rats with either a 2 h-meal (2hRF) or a 6 h-meal (6hRF) each day. As expected, 2hRF was associated with less food-consumption, more weight-loss, and more food-anticipatory running-wheel activity than 6hRF. In association with these metabolic and behavioral differences, the daily pattern of PER1 and PER2 expression in the dorsomedial hypothalamic nucleus (DMH), which has been proposed to be integral to the generation and/or maintenance of food-anticipatory activities, peaked earlier in the 2hRF group and later in the 6hRF group. Because both RF groups exhibited approximately synchronous food-anticipatory activity, but phase shifted rhythms of PER1 and PER2 expression in the DMH, it suggests that the phase of food-anticipatory activity is not directly regulated by this brain area. Next, daily rhythms of PER2 expression in the limbic forebrain responded to each RF schedule in a nucleus-specific manner. In some brain areas, the amplitude of the PER2 rhythm was differentially adjusted in response to 2hRF and 6hRF, while other areas, responded similarly to both RF schedules. These findings demonstrate that daily rhythms of clock gene expression can be modulated by the motivational state of the animal, as influenced by meal duration, weight loss and food-consumption.

Key words: circadian clock gene, dorsomedial hypothalamic nucleus, oval nucleus of the bed nucleus of the stria terminalis, central nucleus of the amygdala, basolateral amygdala, dentate gyrus.

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Abbreviations: AL, *ad libitum* food access; BLA, basolateral amygdala; BNSTov, oval nucleus of the bed nucleus of the stria terminalis; CEA, central nucleus of the amygdala; DG, dentate gyrus of the hippocampus; DMH, dorsomedial hypothalamic nucleus; IR, immunoreactivity; PER1, Period1 protein; PER2, Period2 protein; Ppargc1a, peroxisome proliferative activated receptor gamma coactivator-1alpha; RF, restricted feeding; SCN, suprachiasmatic nucleus; Sirt1, Sirtuin1; ZT, zeitgeber time; 2hRF, restricted feeding: 2 h daily meal; 6hRF, restricted feeding: 6 h daily meal.

Restricted feeding (RF) schedules that provide a single meal at the same time each day induce robust circadian rhythms in behavior and physiology (Richter, 1922; Mistlberger, in press). In rodents, these rhythms in food-anticipatory activity are associated with alterations in the daily expression rhythms of circadian clock genes and proteins, such as Period1 (PER1) and Period2 (PER2), in many peripheral tissues and brain nuclei (Hara et al., 2001; Wakamatsu et al., 2001; Minami et al., 2002; Kudo et al., 2004; Mieda et al., 2006; Angeles-Castellanos et al., 2007; Waddington Lamont et al., 2007). In recent years there has been considerable interest in the role that these tissue- and nucleus-specific rhythms might play in the generation of food-anticipatory activities as well as how they might interact with local metabolic processes (Feillet et al., 2006; Shirai et al., 2007; Sonoda et al., 2007; Asher et al., 2008; Belden and Dunlap, 2008; Fuller et al., 2008; Nakahata et al., 2008; Challet et al., 2009; Escobar et al., 2009; Pendergast et al., 2009; Storch and Weitz, 2009; Karatsoreos et al., 2011). However, the factors that influence the effect of RF on clock gene expression in the periphery and brain have not been fully explored. For example, we have shown that the effect of RF on the daily rhythm of PER2 expression in the rat forebrain varies as a function of whether food is presented during the light phase or dark phase of the light-dark cycle (Verwey et al., 2007, 2008) and whether it is given at the same or different time each day (Verwey et al., 2009; Verwey and Amir, 2011). The duration of the daily meal influences food-consumption, weight-loss, hunger, arousal, and food-anticipatory activity (Honma et al., 1983; Stephan and Becker, 1989), but whether or not it is important in the modulation of the daily pattern of clock gene expression has not been determined.

Daily PER2 rhythms, which are responsive to RF, have been reported in regions of the limbic forebrain that are important in the regulation of motivational and emotional state (Amir et al., 2004; Lamont et al., 2005a,b; Waddington Lamont et al., 2007; Amir and Stewart, 2009). These regions include the oval nucleus of the bed nucleus of the stria terminalis (BNSTov), the central nucleus of the amygdala (CEA), the basolateral amygdala (BLA) and the dentate gyrus (DG). Furthermore, the daily pattern of PER1 and PER2 expression in the dorsomedial hypothalamic nucleus (DMH), an area that has been linked to the regulation of feeding and arousal, and to food-anticipatory activity in some studies, is also affected by RF (Mieda et al., 2006; Verwey et al., 2007, 2008; Fuller et al., 2008). Therefore, to study the impact of meal duration on clock gene expression in these brain regions, we provided rats

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with either a 2 h-meal (2hRF) or a 6 h-meal (6hRF) at the same time each day. The results show that meal duration plays a key role in the regulation of food-anticipatory activity and circadian rhythms of clock gene expression in the forebrain, and underscore the importance of motivational factors in the entrainment of behavioral and molecular circadian rhythms by RF in rats. Preliminary results have been presented in abstract form (Verwey and Amir, 2010).

EXPERIMENTAL PROCEDURES

Animals, housing and restricted feeding

All experimental procedures were approved by the Animal Care Committee at Concordia University (Montréal, QC, Canada) and followed the guidelines set out by the Canadian Council on Animal Care. Male Wistar rats (72 rats; 250–275 g at the start of each experiment) were individually housed in cages equipped with running wheels, and kept in light-proof and sound-attenuated chambers. Running wheel activity was continuously recorded by computer (Vitalview, Minimitter, OR, USA) and inspected with circadia software. All rats were kept in a regular 12 h-light (~300 lx at cage level):12 h-dark cycle, and had free access to food (Rodent diet #5075, Charles River Laboratories, St. Constant, QC, Canada) and water. After a two-week acclimation period, rats in the restricted feeding groups received either a single 2 h meal/day (2hRF) or a 6 h meal/day (6hRF) for 10 days. In both groups the meals began at zeitgeber time 4 (ZT4), 4 h after the environmental lights turned on (ZT0 denotes time of light on). Accordingly, the 2hRF group had access to food from ZT4–6, while the 6hRF group had access to the food from ZT4–10. The *ad libitum* (AL) fed group had free access to food throughout the experiment.

Immunohistochemistry

At the end of the restricted feeding schedules, rats were injected with an overdose of euthanyl (~150 mg/kg, CDMV, St. Hyacinthe, QC, Canada) and perfused transcardially around the clock (ZT 1, 5, 9, 13, 17, 21; n=4/timepoint) with 300 ml of cold saline (4 °C; 0.9% NaCl in distilled water) followed by 300 ml of cold paraformaldehyde solution (4 °C; 4% paraformaldehyde in 0.1 M phosphate buffer). Brains were removed and post-fixed for ~24 h in paraformaldehyde solution. Coronal brain sections (50 μm) were sliced on a vibratome, and brain sections containing the regions of interest were collected and stored at −20 °C, in Watson's cryoprotectant, until staining (Watson et al., 1986). Sections containing the suprachiasmatic nucleus (SCN), BNSTov, CEA, BLA, DG and the DMH were immunostained for PER2 protein. Whereas, a second set of brain sections that contained only the DMH was stained for the PER1 protein.

Immunohistochemistry was performed as previously described (Amir et al., 2004; Verwey et al., 2009). Briefly, polyclonal antibodies for either PER1 (1:24000; made in rabbit; generous gift from Dr. S. M. Reppert, University of Massachusetts Medical School, Worcester, MA, USA) or PER2 (1:800; made in goat; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used. Both primary antibody solutions were made with trizma-buffered saline (TBS; 50 mM trizma buffer with 0.9% NaCl) and contained triton (0.3%). The PER1 antibody solution was also milk-buffered and contained 2% normal goat serum (Vector Laboratories, Burlington, ON, Canada), whereas the PER2-containing solution was not milk-buffered and contained 2% normal horse serum (Vector Laboratories, Burlington, ON, Canada). After rinsing the free-floating tissue in fresh TBS, brain sections were placed into a secondary incubation solution containing either biotinylated anti-rabbit IgG made in goat (in the case of PER1-staining; 1 h, 4 °C; 1:200; Vector Laboratories) or biotinylated anti-goat IgG made in horse (in the case of PER2-staining; 1 h, 4 °C; 1:400; Vector Laboratories). Brain sections were rinsed in fresh TBS, and then incubated in an avidin-biotin solution for 2 h (4 °C; Vectastain Elite ABC Kit; Vector Laboratories). Next, to visualize the immunoreactive cells, sections were rinsed in a 0.5% 3,3-diaminobenzidine solution (10 min), followed by a solution containing 0.5% 3,3-diaminobenzidine, 0.01% H_2O_2 and 8% $NiCl_2$ (10 min). All sections were then mounted on gelatin-coated slides, underwent serial alcohol dehydration and cleared with citrisolv. Glass coverslips were then glued in place with permount (Fisher Scientific, Ottawa, ON, Canada).

Microscopy and data analysis

Slides were examined under a light microscope (Leitz Laborlux S) using a 20× objective. 400×400 μm^2 (SCN, BNSTov, CEA, BLA, DMH) or 400×200 μm^2 (DG) images were captured and analyzed using a Sony XC-77 camera (Sony, Tokyo, Japan), a Scion LG-3 Frame Grabber (Scion Corporation, Frederick, MD, USA) and Image SXM software (v.1.6, SD Barrett, http://www.imagesxm.org.uk). The mean number of stained nuclei in each brain region was determined by computing the average count from the six sections containing the greatest number of stained nuclei out of all the images taken of a given structure. Differences in clock gene expression between groups were determined with an analysis of variance (ANOVA) where the alpha level was set at 0.05. Running-wheel activity, food consumption and body weight were analyzed with two-way repeated measures ANOVAs, and followed with post-hoc Tukey's tests.

RESULTS

Food consumption, body weight and running wheel activity

When all rats were freely fed (day 1–5), there was no significant difference in food consumption between the groups. As expected, during RF (days 6–14), the 2hRF and 6hRF groups ate less food than the AL group ($P<0.01$; Fig. 1, top graph). Moreover, during this period of RF, the 2hRF group ate less food than the 6hRF group ($P<0.01$). For example, on the 9th day of the RF schedule (day 14), the 2hRF group ate only 49% of their baseline food consumption, while the 6hRF group ate 71% on the same day. As a consequence of these differences in food-consumption, at the end of the 10-day schedules, the 2hRF group had lost 21% of their initial body weight while the 6hRF group had only lost 10% (Fig. 1, Bottom graph). In contrast, the AL group did not change their daily food consumption and, from day 6 to day 15, gained an additional 10% in body weight (Fig. 1).

Representative actograms, which illustrate the daily patterns of running-wheel activity before and after the initiation of the RF schedules for an individual in each group, can be seen in Fig. 2. Based on all of the running-wheel activity records, both RF groups developed food-anticipatory activity, at a time-of-day when AL controls were normally inactive (Fig. 3). The total daily activity was increased in both RF groups, as compared to the AL group ($P<0.01$; Fig. 4, top graph), and the 2hRF group ran more than the 6hRF group ($P<0.01$). The 2hRF group also exhibited more food-anticipatory running-wheel activity, from ZT1–4, than the 6hRF group ($P<0.01$; Fig. 4, middle graph). Accounting for the differences in total activity (Fig. 4, bottom graph), the 2hRF group still exhibited a higher