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Daily Restricted Feeding Rescues a Rhythm of Period2 Expression in the Arrhythmic Suprachiasmatic Nucleus

Elaine Waddington Lamont, Laura Renteria Diaz, Jane Barry Shaw,

Jane Stewart and Shimon Amir

Center for Studies in Behavioral Neurobiology,

Department of Psychology,Concordia University, Room SP244,

7141 Sherbrooke St. W., Montreal, Quebec, Canada H4B 1R6

Correspondence should be addressed to S. A. (shimon.amir@concordia.ca)

Abstract--Second only to light, daily restricted feeding schedules can entrain circadian rhythms in mammals (Boulos and Terman, 1980, Stephan, 2002). Contrary to light, however, such feeding schedules have been found not to affect the master circadian clock in the suprachiasmatic nucleus (SCN) (Damiola et al., 2000, Wakamatsu et al., 2001). Here, we show that in rats that are arrhythmic as a consequence of prolonged housing in constant light, a daily restricted feeding schedule not only restores behavioral rhythmicity, as previously shown (Mistlberger, 1993), but in addition, induces a rhythm of the clock protein, Period2 (PER2) in the SCN. These findings challenge the idea that the SCN is invulnerable to feeding schedules and call for a reevaluation of the role of SCN clock in the circadian effects of such schedules.

Studies of the effect of scheduled feeding on circadian rhythms have been typically carried out in rodents housed under normal lighting conditions, where the rhythms of the

SCN clock are entrained by daily light, or in constant darkness, where the rhythms are sustained but free run. Under these conditions feeding schedules in which a single meal is provided at a fixed time each day have been shown to alter daily behavioral and physiological rhythms, including rhythms of locomotor activity, body temperature and corticosterone secretion (Boulos and Terman, 1980, Mistlberger, 1994, Stephan, 2002). Furthermore, under these lighting conditions such schedules were found to gain control over the rhythms of expression of clock genes such as *Per1* and *Per2* in various peripheral organs, including liver, lung, heart and pancreas, and in some brain regions, including the cerebral cortex and hippocampus (Damiola et al., 2000, Hara et al., 2001, Stokkan et al., 2001, Wakamatsu et al., 2001, Challet et al., 2003, Schibler et al., 2003, Kobayashi et al., 2004). In contrast, these schedules have been found not to affect Per expression in the SCN of rodents (Damiola et al., 2000, Wakamatsu et al., 2001, Schibler et al., 2003). These findings suggest that the SCN is relatively invulnerable to feeding schedules and such a conclusion is consistent with evidence that the SCN clock is not required for circadian entrainment by feeding schedules (Stephan et al., 1979, Mistlberger, 1994, Hara et al., 2001). Alternatively, it may not be that the SCN is invulnerable to the effects of such feeding schedules, rather it may be that factors that promote the normal operation of the clock somehow buffer it from these effects (Castillo et al., 2004). To address this idea we studied the effect of a restricted feeding schedule in rats housed in prolonged constant light (LL), a condition that abolishes the circadian oscillations of Per in the SCN and leads to behavioral and physiological arrhythmicity (Beaule et al., 2003, Sudo et al., 2003). We assessed locomotor activity and the expression of the clock protein PER2 in the SCN and in one extra SCN structure, the oval

nucleus of the bed nucleus of the stria terminalis (BNST-OV), where we have found that the rhythm of PER2 expression is normally synchronized with that in the SCN and, like the SCN, is abolished in LL (Amir et al., 2004).

EXPERIMENTAL PROCEDURES

Animals and housing

The experimental procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee, Concordia University. Adult male Wistar rats (275-300 g) were housed individually in clear plastic cages equipped with running wheels. Each cage was place in a ventilated, sound and light tight isolation chamber equipped with a computer-controlled lighting system (VitalView Mini Mitter Co. Inc., Sunriver, OR). The rats were maintained either on a 12h:12h light/dark cycle ((LD, light: 300 lux at cage level) or LL. Wheel-running activity data were recorded with VitalView software (Mini Mitter Co.) and analyzed with Circadia, as previously described (Amir et al., 2004). After at least 4 weeks of LD or LL housing with free access to food and water, half of the rats from each lighting condition were placed on a restricted feeding schedule for 10 days. Rats from the LD condition were allowed to eat for 3 h/day, during the daytime, from zeitgeber time (ZT) to ZT7 (ZT0 denotes the onset of the light phase). Rats housed in LL were allowed to eat for 3 h/day at the same time. Control rats from each lighting condition continued to have free access to food. All rats were perfused 11 days after the start of the restricted feeding or control schedules. Rats from the LD groups were perfused at ZT0, 6, 12, or 18. Rats from the LL groups were perfused 0, 6, 12, or 18 hours after food presentation.

Tissue Preparation

Rats were injected with an overdose of sodium pentobarbital (100 mg/kg) and were perfused intracardially with 300 ml of cold saline (0.9% NaCl) followed by 300 ml of cold, 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.3). Following perfusion, brains were postfixed in 4% paraformaldehyde and stored at 4° C overnight. Serial coronal brain sections (50 μm) were collected from each animal using a vibratome.

Immunocytochemistry

Free floating sections were washed in cold 50 mM Tris buffered saline (TBS; pH 7.6) and incubated at room temperature for 30 minutes in a quenching solution made of TBS and 3% w/w hydrogen peroxide (H_2O_2). Following the quenching phase, sections were rinsed in cold TBS and incubated for 1 hour at room temperature in a pre-blocking solution made of 0.3% Triton X 100 in TBS (Triton-TBS), 3% normal goat serum and 5% milk buffer. Following the pre-blocking phase, sections were transferred directly into an affinity purified rabbit polyclonal antibody raised against PER2 (Alpha Diagnostic International, San Antonio, TX) diluted 1:1000 with a solution of Triton-TBS with 3% normal goat serum in milk buffer. Sections were incubated with the primary antibody for 48h at 4°C. Following incubation in the primary antibody, sections were rinsed in cold TBS and incubated for 1 hour at □°C with a biotinylated anti-rabbit IgG made in goat (Vector Labs, Burlingame, CA), diluted 1:200 with Triton-TBS with 2% normal goat serum. Following incubation with secondary antibody, sections were rinsed in cold TBS and incubated for 2 hours at □°C with an avidin-biotin-peroxidase complex (Vectastain

Elite ABC Kit, Vector Labs). Following incubation with the ABC reagents, sections were rinsed with cold TBS, rinsed again with cold 50 mM Tris-HCl (pH 7.6), and again for 10 minutes with 0.05% 3,3'-diaminobenzidine (DAB) in 50 mM Tris-HCl. Sections were then incubated on an orbital shaker for 10 minutes in DAB/Tris-HCl with 0.01% H_2O_2 and 8% $NiCl_2$. After this final incubation, sections were rinsed in cold TBS, wet-mounted onto gel-coated slides, dehydrated through a series of alcohols, soaked in Citrisolv (Fisher), and cover-slipped with Permount (Fisher).

Data Analysis

Stained brain sections were examined under a light microscope and images were captured under X20 magnification using a Sony XC-77 video camera, a Scion LG-3 frame grabber, and NIH Image (v1.63) software, as previously described (Amir et al., 2004). Cells immunopositive for PER2 were counted manually on the captured images. The number of PER2 immunoreactive cells in the SCN and BNST-OV was calculated for each animal from the counts of 6 unilateral images showing the highest number of labeled nuclei. Differences between groups were revealed with analysis of variance (ANOVA).

RESULTS AND DISCUSSION

In freely fed control rats housed under a 12h:12h LD cycle, running wheel activity was confined to the dark phase (Fig. 1-a, top), and, as previously described (Amir et al., 2004), PER2 expression in the SCN and BNST-OV was rhythmic and synchronous, peaking in the beginning of the dark phase, at ZT12 (Fig. 1-a, bottom). When rats housed

in LD were placed on a restricted feeding schedule in which food was given for 3 hours each day, from ZT4 to ZT7, the normal pattern of nighttime activity was altered, and a second, distinct bout of activity of 2- to 3-h emerged in all rats before and during daytime food presentation (Fig. 1-b, top). Furthermore, this restricted feeding schedule uncoupled the rhythm of PER2 expression in the BNST-OV from that in the SCN. The peak of PER2 expression in the BNST-OV was delayed by 6 h relative to that in the SCN and peaked 12 h after food access (Fig. 1-b, bottom). The PER2 rhythm of the SCN was not affected by the RF schedule in these rats and expression remained entrained to the light cycle, peaking at ZT12 (Fig. 1-b, bottom).

In rats housed in prolonged LL with free access to food, activity rhythms and rhythms of PER2 expression in SCN and BNST-OV were abolished (SCN: $F[3,22]=1.68$, $p=.19$; BNST-OV: $F[3,22]=1.76$, $p=.18$) (Fig. 2-a). In another group of LL housed rats, the schedule of restricted feeding led to the emergence of a distinct daily pattern of locomotor activity synchronized to the time of daily food presentation (Fig. 2-b). Importantly, in these rats we found a significant circadian rhythm of PER2 expression both in the SCN ($F[3,20]=14.33$, $p<.0001$) and in the BNST-OV ($F[3,20]=14.09$, $p<.0001$). Expression of PER2 in these regions was synchronous peaking 12 hours after food access (Fig. 2-b).

These results show that the SCN is sensitive to daily restricted feeding and that this sensitivity depends on the state of its endogenous oscillations. When the rhythms of the SCN are entrained by the light cycle the SCN is invulnerable to the effects of

scheduled feeding, as previously shown (Damiola et al., 2000, Wakamatsu et al., 2001), whereas when the SCN becomes arrhythmic, daily restricted feeding induces a circadian rhythm of PER2 expression in the SCN. Contrary to what was found for the SCN, the expression of PER2 in the BNST-OV was affected by daily scheduled feeding in both rhythmic and arrhythmic rats. Specifically, in normally entrained rats, the rhythm of PER2 expression in the BNST-OV was shifted by the feeding schedule, peaking 12 hours after daily feeding, and was uncoupled from that in the SCN which remained locked to night onset. Interestingly, however, in arrhythmic rats the PER2 rhythms induced in the BNST-OV was synchronous with that in the SCN, both peaking 12 hours after daily feeding. It is tempting to speculate that restoration of the PER2 rhythm of the SCN of arrhythmic rats is mediated by a signal from oscillators outside the SCN such as those in the BNST-OV, that are directly responsive to the metabolic or behavioral effects of daily feeding schedules (Stephan, 1986, Wakamatsu et al., 2001). Alternatively, signals related to daily feeding could reach the SCN from brain regions involved in the regulation of food intake or locomotor activity (Moga and Moore, 1997), independently of such peripheral oscillators. In either case, our results are consistent with findings demonstrating that the activity of the SCN neurons can be directly affected by behavioral and homeostatic states such as locomotor activity and sleep-wake state (Schaap and Meijer, 2001, Deboer et al., 2003, Vansteensel et al., 2003).

In summary, we find that in arrhythmic rats a daily restricted feeding schedule not only restores and entrains an activity rhythm but also induces synchronized PER2 rhythms in the SCN and BNST-OV that peak 12 hours after daily feeding. Thus

scheduled meals can play an important function in entrainment of the SCN clock in situations where its oscillators become uncoupled leading to disruption of physiological and behavioral rhythms.

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REFERENCES

Amir, S., Lamont, E. W., Robinson, B. and Stewart, J., 2004. A circadian rhythm in the expression of PERIOD2 protein reveals a novel SCN-controlled oscillator in the oval nucleus of the bed nucleus of the stria terminalis. J Neurosci. 24, 781-790.

Beaule, C., Houle, L. M. and Amir, S., 2003. Expression profiles of PER2 immunoreactivity within the shell and core regions of the rat suprachiasmatic nucleus: lack of effect of photic entrainment and disruption by constant light. J Mol Neurosci. 21, 133-147.

Boulos, Z. and Terman, M., 1980. Food availability and daily biological rhythms. Neurosci Biobehav Rev. 4, 119-131.

Castillo, M. R., Hochstetler, K. J., Tavernier, R. J., Jr., Greene, D. M. and Bult-Ito, A., 2004. Entrainment of the master circadian clock by scheduled feeding. Am J Physiol Regul Integr Comp Physiol. 287, R551-555.

Challet, E., Caldelas, I., Graff, C. and Pevet, P., 2003. Synchronization of the molecular clockwork by light- and food-related cues in mammals. Biol Chem. 384, 711-719.

Damiola, F., Le Minh, N., Preitner, N., Kornmann, B., Fleury-Olela, F. and Schibler, U., 2000. Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. Genes Dev. 14, 2950-2961.

Deboer, T., Vansteensel, M. J., Detari, L. and Meijer, J. H., 2003. Sleep states alter activity of suprachiasmatic nucleus neurons. Nat Neurosci. 6, 1086-1090.

Hara, R., Wan, K., Wakamatsu, H., Aida, R., Moriya, T., Akiyama, M. and Shibata, S., 2001. Restricted feeding entrains liver clock without participation of the suprachiasmatic nucleus. Genes Cells. 6, 269-278.

Kobayashi, H., Oishi, K., Hanai, S. and Ishida, N., 2004. Effect of feeding on peripheral circadian rhythms and behaviour in mammals. Genes Cells. 9, 857-864.

Mistlberger, R. E., 1993. Effects of scheduled food and water access on circadian rhythms of hamsters in constant light, dark, and light:dark. Physiol Behav. 53, 509-516.

Mistlberger, R. E., 1994. Circadian food-anticipatory activity: formal models and physiological mechanisms. Neurosci Biobehav Rev. 18, 171-195.

Moga, M. M. and Moore, R. Y., 1997. Organization of neural inputs to the suprachiasmatic nucleus in the rat. J Comp Neurol. 389, 508-534.

Schaap, J. and Meijer, J. H., 2001. Opposing effects of behavioural activity and light on neurons of the suprachiasmatic nucleus. Eur J Neurosci. 13, 1955-1962.

Schibler, U., Ripperger, J. and Brown, S. A., 2003. Peripheral circadian oscillators in mammals: time and food. J Biol Rhythms. 18, 250-260.

Stephan, F. K., 1986. Coupling between feeding- and light-entrainable circadian pacemakers in the rat. Physiol Behav. 38, 537-544.

Stephan, F. K., 2002. The "other" circadian system: food as a Zeitgeber. J Biol Rhythms. 17, 284-292.

Stephan, F. K., Swann, J. M. and Sisk, C. L., 1979. Entrainment of circadian rhythms by feeding schedules in rats with suprachiasmatic lesions. Behav Neural Biol. 25, 545-554.

Stokkan, K. A., Yamazaki, S., Tei, H., Sakaki, Y. and Menaker, M., 2001. Entrainment of the circadian clock in the liver by feeding. Science. 291, 490-493.

Sudo, M., Sasahara, K., Moriya, T., Akiyama, M., Hamada, T. and Shibata, S., 2003. Constant light housing attenuates circadian rhythms of mPer2 mRNA and mPER2 protein expression in the suprachiasmatic nucleus of mice. Neuroscience. 121, 493-499.

Vansteensel, M. J., Yamazaki, S., Albus, H., Deboer, T., Block, G. D. and Meijer, J. H., 2003. Dissociation between circadian Per1 and neuronal and behavioral rhythms following a shifted environmental cycle. Curr Biol. 13, 1538-1542.

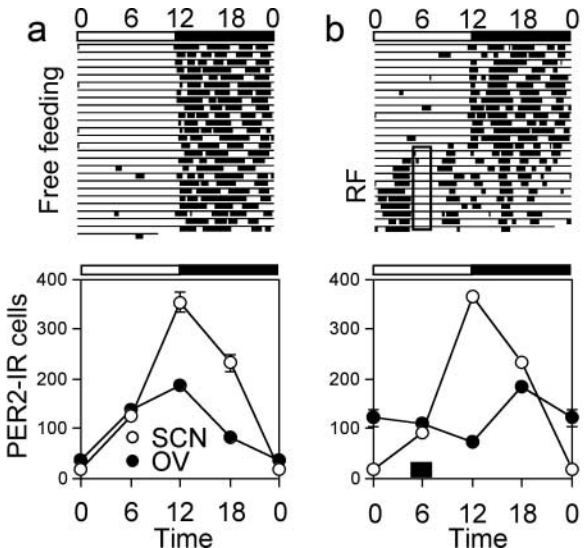
Wakamatsu, H., Yoshinobu, Y., Aida, R., Moriya, T., Akiyama, M. and Shibata, S., 2001. Restricted-feeding-induced anticipatory activity rhythm is associated with a phase-shift of the expression of mPer1 and mPer2 mRNA in the cerebral cortex and hippocampus but not in the suprachiasmatic nucleus of mice. Eur J Neurosci. 13, 1190-1196.

Figure Captions

Fig. 1. Representative actograms of locomotor activity and mean (±sem) number of PER2 immunoreactive nuclei measured in the SCN and BNST-OV as a function of time in (a) freely fed rats housed in LD (n=5-6/group), (b) restricted-feeding rats housed in LD (n=7-10/group). In each actogram the vertical marks indicate periods of activity of at least 10 wheel revolutions/10 min. Successive days are plotted from top to bottom. The vertical rectangle and black box in (b) mark the time of food presentation during the restricted feeding schedule (RF).

Fig. 2. Representative actograms of locomotor activity and images showing PER2 expression in the SCN and BNST-OV and mean (±sem) number of PER2 immunoreactive nuclei measured in the SCN and BNST-OV as a function of time in (a) freely fed rats housed in LL (n=6-7/group) and (b) restricted-feeding rats housed in LL (n=6-7/group). The vertical rectangle and black box in (b) mark the time of food presentation during the restricted feeding schedule (RF). P-values indicate the significance of differences between time points (ANOVA).

Figure 1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PER2-IR cells
400
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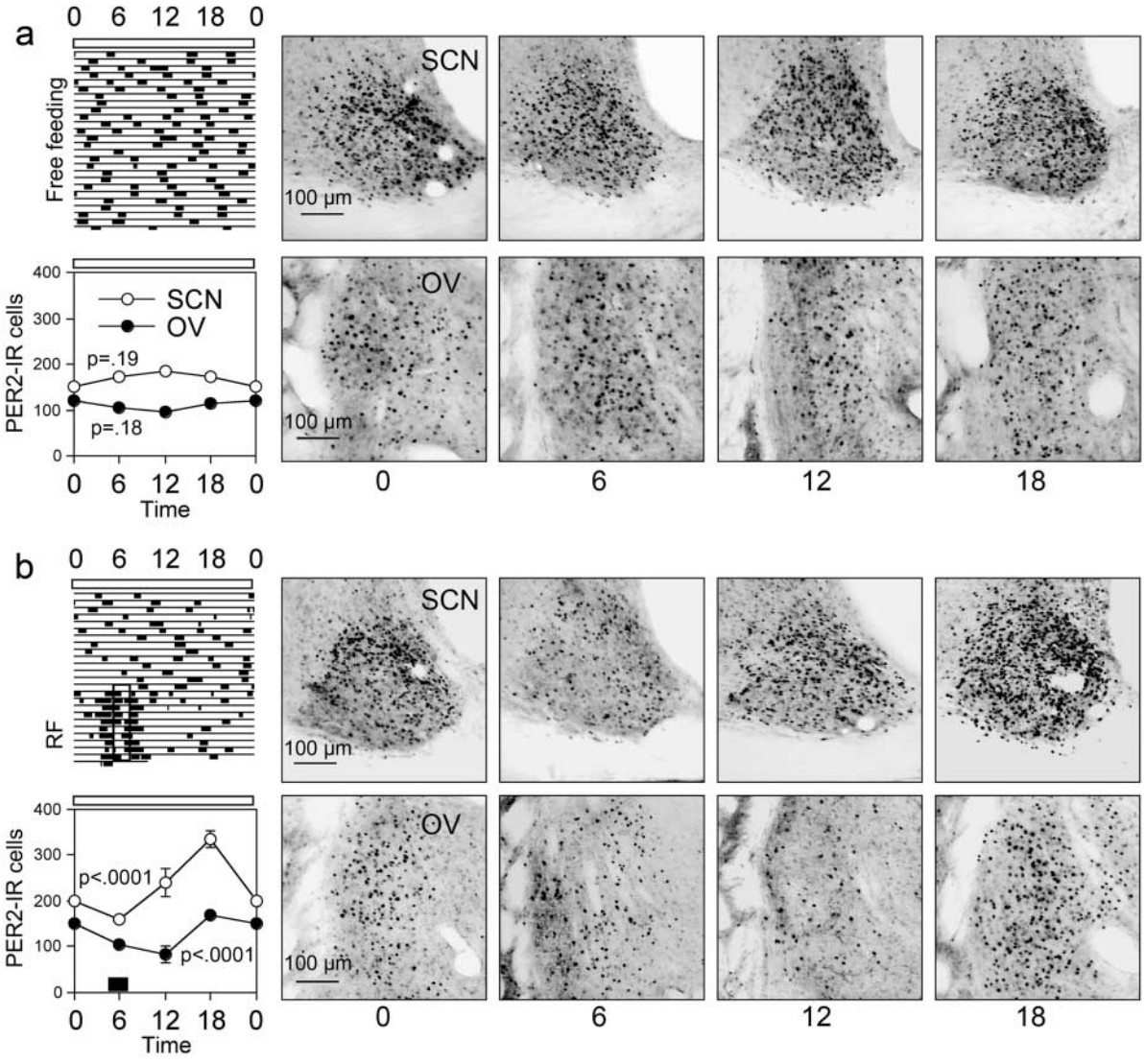
Figure 2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