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Restricted access to food, but not sucrose, saccharine, or salt synchronizes the expression of Period2 protein in the limbic forebrain

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Abbreviations: CEA, central nucleus of the amygdala; BLA, basolateral amygdala; DG, dentate gyrus; SCN, suprachiasmatic nucleus; PER2, Period2; BNST-OV, oval nucleus of the bed nucleus of the stria terminalis; LD, light dark; LL, constant light, RF, restricted feeding; ZT, zeitgeber time; AL, ad libitum

Abstract

Restricted feeding schedules (RF) in which daily access to food is limited to a few hours each day can entrain the rhythms of expression of circadian clock genes in the brain and periphery in rodents. The critical factors mediating the effect of RF on rhythms of clock gene expression are unknown. Previously, we demonstrated that daytime RF shifts the phase of expression of the clock protein, Period2 (PER2) in the oval nucleus of the bed nucleus of the stria terminalis in rats kept on a 12-hour light dark cycle, and restored the rhythm of PER2 expression in rats housed in constant light. We now report that RF also modifies the rhythms of PER2 expression in the central and basolateral nuclei of the amygdala and in the dentate gyrus, such that all three areas become synchronized, peaking 12 hours after the time of food presentation. Daily limited access to sucrose or saccharine in freely fed rats or scheduled access to saline in sodium-deprived rats had no effect on these PER2 rhythms. Thus, it would appear that the rhythms of PER2 in limbic forebrain structures are sensitive to signals that arise from the alleviation of a negative metabolic state associated with scheduled feeding and that access to rewarding substances in the absence of food deprivation or metabolic challenges, *per se,* are not sufficient to alter the rhythms of PER2 expression in these regions.

Keywords: Clock genes, Suprachiasmatic nucleus, Oval nucleus of the bed nucleus of the stria terminalis, Central nucleus of the amygdala, Basolateral amygdala, Dentate gyrus, Rat

The Period2 (PER2) protein plays a key role in the generation of mammalian circadian rhythms. We recently found that in addition to its rhythmic expression in the suprachiasmatic nucleus (SCN), the master circadian clock, PER2 is expressed rhythmically in limbic forebrain structures that play key roles in emotional and motivational regulation, the oval nucleus of the bed nucleus of the stria terminalis (BNST-OV), the central nucleus of the amygdala (CEA), the basolateral amygdala (BLA) and the dentate gyrus (DG) (Amir et al., 2004; Lamont et al., 2005a). Importantly, although we found that the rhythms of PER2 in these regions were under the control of, and in synchrony with, the SCN, they could be uncoupled from that in the SCN by perturbations of adrenal and ovarian hormones (Amir et al., 2004; Perrin et al., 2006; Segall et al., 2006) and by changes in the lighting conditions (Amir et al., 2004; Lamont et al., 2005b). In addition, we have found that daily restricted feeding, which disrupts normal circadian rhythms (Krieger, 1974; Honma et al., 1983; Diaz-Munoz et al., 2000; see Stephan, 2002 for a review) and which induces anticipatory bouts of locomotor activity, can entrain the rhythm of expression of PER2 in the BNST-OV (Lamont et al., 2005b). This finding is consistent with previous work showing that rhythms of clock gene expression in peripheral tissues (Damiola et al., 2000; Hara et al., 2001; Le Minh et al., 2001; Stokkan et al., 2001) and cerebral cortex (Wakamatsu et al., 2001) in rodents can be entrained by daily feeding schedules. Furthermore, it suggests that PER2 rhythms in the BNST-OV and perhaps other limbic forebrain regions, including the CEA, BLA and DG, are responsive to

factors involved in energy balance in addition to being sensitive to hormonal signals and signals from the SCN.

Two questions arise from the findings on the effects restricted-feeding schedules on clock gene expression. First, would limited daily access to a palatable substance, in the absence of food deprivation, affect rhythms of clock gene expression? This question arises from the finding that daily presentation of a palatable, nutritionally balanced meal during the daytime can induce food anticipatory behavior even when standard lab chow is freely available (Mistlberger & Rusak, 1987). A similar schedule has been shown to induce *c-Fos* in reward related brain areas (Mendoza et al., 2005a, b), suggesting that reward may be as important as deprivation in the effect of restricted feeding on circadian mechanism mediating anticipatory behavior. To explore the importance of reward, in the absence of deprivation, in the control of clock gene expression, we studied the effects of limited daytime access to two highly palatable substances, sucrose and saccharine in normally fed rats. A second question is whether scheduled access to a substance that is consumed in face of a homeostatic challenge other than that induced by food deprivation would affect rhythms of clock gene expression in a similar manner. To address this issue, we assessed the effects of restricted access to salt in sodium-depleted rats.

Materials and Methods

Animals and housing. All procedures were carried out in accordance with the Canadian Council on Animal Care guidelines and were approved by the Animal

Care Committee of Concordia University. Adult male Wistar rats (300-400g; Charles River, St. Constant, QC, Canada) were individually housed in clear plastic cages equipped with running wheels. Each cage was enclosed in a light and soundproof ventilated chamber. Wheel-running activity was recorded continuously and displayed in 10-min bins using VitalView software (Mini Mitter Co. Inc., Sunriver, OR). Double plotted actograms were used to display running-wheel activity rhythms at each stage of the experiments.

All rats, except for those kept in constant light (see below), were housed on a 12h:12h light-dark schedule throughout the experiments. Rats received standard lab chow *ad libitum* (AL) throughout the experiment, except during scheduled restricted feeding (RF). Tap water was continuously available, except in the case of the scheduled salt-access experiment. All animals were weighed regularly throughout the experiment either before or after experimental manipulations. Sucrose (32%), saccharine (0.2%), and saline (2%) solutions were made with distilled water. Furosemide (Lasix, CDMV, St. Hyacinthe, QC, Canada) was diluted from a 50 mg/ml stock solution to 10 mg/ml using 0.9% saline.

Scheduled restricted-feeding experiments. For rats in the scheduled-feeding condition, food was removed from the overhead hoppers on the evening before the start of the experiment. Restricted feeding (RF) consisted of placing standard lab chow pellets in a small container inside the cage for 3 hours a day, for 10 days, at zeitgeber time (ZT) 4-7 (where ZT0 is lights on and ZT12 is light off). For AL fed rats, the sound attenuating boxes were opened at times corresponding to presentation and removal of food. A separate group of rats was

housed in constant light for 2 months before the beginning and throughout the experiment. During the experiment food was presented for 3 hours each day at the same time each day as described for the rats on the 12h:12h light-dark schedule.

Scheduled-treat experiments. Rats in the scheduled-treat experiments had their standard water bottle replaced with a bottle containing solutions of sucrose or saccharine, or water during the day (ZT 5-7) for 14 days.

Scheduled salt-access experiments. To induce acute salt appetite, rats were given a daily injection of furosemide (10 mg/kg, s.c.) one hour before presentation of a 2% hypertonic saline solution. Rats in the control condition received an injection of physiological saline. To induce chronic salt appetite Alzet osmotic mini-pumps (Cupertino, CA) containing furosemide (10 mg/kg/day) were implanted subcutaneously under isoflurane anesthesia. Control rats were implanted with mini-pumps containing saline. All rats were given 1-hour access to a 2% saline solution daily at ZT6 for 10 days.

Tissue preparation. Rats were injected with an overdose of sodium pentobarbital (100 mg/kg) on Day 11 (scheduled-feeding experiment and salt-access experiments) or Day 15 (scheduled-treat experiment) at the beginning (ZT1) or middle (ZT7) of the light phase, or the beginning (ZT13) or middle (ZT19) of the dark phase of the 12h:12h light-dark schedule. They were perfused transcardially with cold saline (0.9% sodium chloride at 4ºC) followed by cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3). Brains were removed and postfixed overnight in 4% paraformaldehyde at 4ºC. Serial coronal sections (50

μm) through the BNST, SCN, amygdala, and anterior hippocampus were taken for each brain using a Vibratome (St. Louis, MO).

Immunocytochemistry. Free floating sections were rinsed (3 X 10 min) in cold 50 mM Trisma buffered saline (TBS; pH 7.6) then incubated in a hydrogen peroxide (3% H_2O_2 in TBS) quenching solution for 30 min at room temperature (RT). Sections were again rinsed in TBS, pre-blocked in a solution of 0.3% Triton X 100 in TBS (Triton-TBS), 3% normal goat serum (NGS), and 5% milk buffer (MB), then transferred directly into a rabbit polyclonal PER2 antibody (ABI, San Antonio, TX) diluted 1:800 with a solution of 0.3% Triton-TBS, 3% NGS and 5% MB and incubated for approximately 48 hours at 4º C. Blocking experiments carried-out by adding the PER2 peptide (1mg/ml in PBS pH7.4 with 0.02% sodium merthiolate, diluted 1:100) to the primary incubation solution prevented PER2 immunostaining, providing evidence for antibody specificity (Amir et al., 2004). After incubation with the primary antibody, sections were rinsed for 3 X 10 min in cold TBS and incubated for one hour (4º C) in a secondary antibody solution made from a biotinylated anti-rabbit IgG made in goat (Vector Labs, Burlington, ON, Canada), diluted to a concentration of 1:200 with 0.3% Triton-TBS and 3% NGS, rinsed in TBS, and transferred to a tertiary phase consisting of an avidin biotin peroxidase complex in TBS (Vectastain Elite ABC Kit, Vector Labs) for 2 hours at 4 ºC. Finally, sections were rinsed with TBS, and then rinsed again with cold 50 mM Tris-HCl (pH 7.6) for 10 min. Sections were then incubated for 10 min with 0.05% DAB in 50 mM Tris-HCl, then a further 10 min in DAB/50mM Tris HCl with 0.01% H2O2 and 8% NiCl on an orbital shaker. After

this final incubation, sections were rinsed in cold TBS, wet-mounted onto gel coated microscope slides, dehydrated in a series of alcohols, soaked in Citrisolve (Fisher) for 30 min, and coverslipped with Permount (Fisher).

Immunocytochemical data analysis. Brain sections containing the BNST-OV, SCN, CEA, BLA and DG were examined under a light microscope (Leitz Laborlux S) and digitized using a Sony XC-77 Video Camera connected to a Scion LG-3 frame grabber using Image SXM (version 1.73). The number of PER2-positive cells in BNST-OV, SCN, CEA, BLA was counted within a 400 X 400 μm template and the average was calculated for the 6 unilateral images showing the highest number of labeled nuclei. A 200 x 400 μm template was used to count cells in the DG. Data were analyzed by one-way and two-way analyses of variance (ANOVA). One-way ANOVAs were used to determine differences in PER2 expression as a function of time of day (test for rhythms) in each group. Two-way ANOVAs were used to test for effects of Treatment as well as Treatment by Time interactions.

Results

Restricting feeding to the middle of the day uncouples limbic forebrain PER2 rhythms from the SCN and synchronizes them with each other

The expression of PER2 in the CEA, BLA and DG was assessed in freely fed rats housed under a 12h:12h light-dark schedule (LD) and in similarly housed rats that were placed on a 10-day restricted-feeding schedule in which food was

given for 3 hours each day, from ZT4 to ZT7. Examples of PER2 expression in the CEA, BLA and DG of freely fed control rats killed at ZT1 or ZT13 are shown in Fig. 1a. As previously reported (Lamont et al., 2005b) PER2 expression in CEA is high at ZT13 and low at ZT1 whereas in BLA and DG PER2 is high at ZT1 and low at ZT13. Fig. 1c shows the mean levels of expression of PER2 in each sampled region (Fig. 1b) as function of time of day for both control rats and in rats maintained on the 10-day restricted-feeding schedule. One-way ANOVAs carried out on each group separately revealed significant main effects of time in each region (Control group: CEA, $F[3,11] = 16.07$, $p<.0002$; BLA, $F[3,11] = 32.49$, $p<.0001$; DG, $F[3,11] = 88.96$, $p<.0001$; Restricted-feeding (RF) group: CEA, $F[3,12] = 21.65$, $p<.0001$; BLA, $F[3,12] = 15.46$, $p<.0001$; DG, $F[3,12] = 7.32$, $p<.004$). It can be seen, however, that in the RF group, the peak of PER2 expression in all three regions was shifted and became synchronized at ZT19, 12 hours after the daily food presentation. Two-way ANOVAs revealed significant Treatment by Time interactions, indicating that the time of the peaks differed (CEA, $F[3,23] = 26.06$, $p<.0001$; BLA, $F[3,23] = 10.92$, $p<.0001$; DG, $F[3,23] = 19.29$, $p<.0001$). Thus, not only did restricted feeding shift the time of the peak in all three areas, but as can be seen in Fig. 1, the rhythms in BLA and DG, which are normally in antiphase with that in the CEA, became synchronized with the PER2 rhythm in CEA.

Scheduled restricted feeding induces synchronized rhythms in PER2 expression in rats made arrhythmic by constant light

Long-term exposure to constant light produces behavioral arrhythmicity and a

loss of the rhythm of PER2 expression in the SCN and BNST-OV (Beaule et al., 2003, Sudo et al., 2003, Amir et al., 2004, Lamont et al., 2005b, Munoz et al., 2005, Ohta et al., 2005). We reported previously, that restricted feeding restores both the circadian rhythm of activity and the PER2 rhythm in the BNST-OV and, surprisingly, the SCN, in LL-housed arrhythmic rats (Lamont et al., 2005a). As shown in Fig. 2, in freely fed rats housed in constant light, the rhythms of PER2 in the CEA, BLA, and DG were abolished (One-way ANOVAs: CEA, $F[3,8] = 2.70$, $p = .11$; BLA, $F[3,8] = 0.23$, $p = .87$; DG, $F[3,8] = 1.45$, $p = .29$). In contrast, in similarly housed rats given limited access to food for 3 hours at the same time each day, PER2 expression was rhythmic in all regions and peaked 12 hours after feeding (CEA, $F[3,8] = 13.61$, $p<.001$; BLA, $F[3,8] = 4.06$, $p<.05$; DG, $F[3,8] = 6.71$, $p<.02$). Two-way ANOVAs revealed significant main effects of Treatment on PER2 expression in CEA ($F[1,16] = 24.8$, $p<.0001$) and BLA ($F[1,16] = 13.39$, $p<.005$) and a Treatment by Time interaction in DG ($F[3,16] = 4.05$, $p<.05$).

Daily restricted access to sucrose or saccharine is not sufficient to synchronize the rhythms of PER2 expression

The goal of this study was to evaluate the effect of limited daily access to nutritive (sucrose) and non-nutritive (saccharine) sweet substances on the rhythms of PER2 expression in the limbic forebrain in the absence of food deprivation. Rats that had free access to food and water and that were given limited daily access to either sucrose or saccharine at ZT 5-7 for 14 days, drank substantial amounts of these liquids (sucrose, mean intake = 19.11 ± 0.63 ml; saccharin mean intake = 9.17 ± 0.32 ml) compared to those that had their water

bottle replaced with fresh water in the same period (mean intake =3.56 ± 0.16 ml). In none of the conditions did rats show the anticipatory running seen in the rats kept on the restricted-feeding schedule (Fig. 3). Furthermore, PER2 remained rhythmic in SCN, BNST-OV, CEA, BLA and DG and it can be seen that there was no effect of limited access to sucrose or saccharine on the peak of PER2 expression in any of the regions (Fig. 4); two-way ANOVAs revealed a significant effect of Time in all regions (SCN, $F[3,46] = 102.08$, $p<.0001$; BNST-OV $F[3,46] = 51.74$, $p<.0001$; CEA, $F[3,46] = 30.92$, $p<.0001$; BLA, $F[3,46] = 19.21$, $p<.0001$; DG, $F[3,46] = 32.71$, $p<.0001$). Only in the BNST-OV was there any effect of Treatment on overall levels of PER2 ($F[2,46]=4.7$, $p<.05$). These data show that although these substances are highly palatable and readily consumed (and in the case of sucrose, nutritive), daily limited access in the absence of food deprivation is insufficient to induce changes in the timing of the peak of the rhythm of PER2 expression.

Daily limited access to salt in sodium-deprived rats does not synchronize PER2 rhythms

Providing sodium-deprived rats with scheduled access to hypertonic saline would appear to parallel scheduled feeding in food-deprived rats (Johnson and Thunhorst, 1997). We hypothesized that scheduled access to hypertonic saline in sodium-deprived rats might mimic the effect of restricted feeding on PER2 oscillations in the limbic forebrain. In the first experiment, acute salt appetite induced by daily injections of the diuretic substance, furosemide, enhanced drinking of 2% hypertonic saline during the 1-hour daily presentation (furosemide

group, 6.96 ± 0.62 ml; control group, 3.81 ± 0.26 ml), but had no effect on the rhythm of PER2 expression in any of the brain regions examined (Fig. 5). The two-way ANOVAs revealed significant main effects of Time in all regions (SCN, $F[3,16] = 37.49$, $p<.0001$; BNST-OV $F[3,16] = 23.59$, $p<.0001$; CEA, $F[3,16] = 9.13$, $p<.001$; BLA, $F[3,16] = 5.50$, $p<.01$; DG, $F[3,16] = 63.65$, $p<.0001$), but no significant Treatment or Treatment by Time interactions. In the second experiment, chronic salt appetite, induced by continuous administration of furosemide via osmotic mini-pumps, similarly enhanced salt intake (furosemide group, 7.91± 0.60 ml; control group 4.61±0.35). Under these chronic conditions there was no effect on PER2 rhythms in the SCN, BNST-OV, CEA, or DG (Fig. 6), but a significant reduction in PER2 expression in the BLA around the time of salt access was noted (main effect of Treatment $F[1, 28] = 5.63$, $p = .025$). A significant effect of Time was found in all regions (SCN, $F[3,28] = 92.89$, $p<.0001$; BNST-OV $F[3,28] = 25.34$, $p<.0001$; CEA, $F[3,28] = 8.77$, $p<.001$; BLA, $F[3,28] = 3.99$, $p<.02$; DG, $F[3,28] = 68.21$, $p<.0001$), but no significant Treatment by Time interactions was noted. As shown previously (Rosenwasser et al., 1985, Rosenwasser et al., 1988), there was little evidence of behavioral anticipation prior to salt access in either experiment (Fig. 7).

Discussion

Here we show that daily restricted feeding in rats synchronizes PER2 rhythms in the CEA, BLA and DG and uncouples them from the rhythm in the SCN. In contrast, daily limited access to sucrose or saccharin solutions in freely

fed rats or to a salt solution in sodium-deprived rats had no effect on PER2 rhythms. Thus, neither scheduled rewarding events nor homeostatic challenges, *per se*, are sufficient to entrain and synchronize rhythms of PER2 expression in the limbic forebrain.

We showed previously that daily rhythms of PER2 expression in the BNST-OV of rats housed under a 12h:12h LD cycle become synchronized by scheduled daytime restricted feeding and uncoupled from the rhythm of PER2 expression in the SCN (Lamont et al., 2005a). The findings of the present experiments show that rhythms of PER2 expression in the CEA, BLA and DG can be similarly synchronized by daytime restricted feeding. Furthermore, they show that, whereas under *ad libitum* feeding conditions the rhythms of PER2 in BLA and DG are opposite in phase from those in BNST-OV and CEA, under the conditions of restricted feeding, the PER2 rhythms in all regions become synchronous and peak 12 hours after the end of the daily feeding. In view of the fact that the PER2 rhythm in the SCN is not affected by scheduled restricted feeding in LD housed rats, these findings show, in addition, that scheduled restricted feeding not only synchronizes the rhythms of PER2 in these brain regions, but uncouples them from the rhythm of PER2 expression in the SCN. Such uncoupling of clock gene expression from that in the SCN has also been observed in peripheral tissues of rats and mice placed on restricted-feeding schedules (Damiola et al., 2000, Le Minh et al., 2001).

In previous studies we found that prolonged housing in LL not only disrupts the rhythm of PER2 in the SCN and of locomotor activity (Beaule et al.,

2003), but also abolishes the PER2 rhythm in the BNST-OV (Amir et al., 2004). Furthermore, we found in another study that scheduled restricted feeding can restore locomotor activity and PER2 rhythms in the SCN and BNST-OV (Lamont et al., 2005a). In the present study, we report that daily restricted feeding also restores and synchronizes the rhythms of PER2 in CEA, BLA and DG in rats housed under LL conditions. These findings extend our previous studies and underscore the importance of daily feeding schedules in the control of clock gene expression in the brain.

We asked whether limited daily access to a highly palatable substance would affect rhythms of PER2 in the BNST-OV, amygdala, and hippocampus in the absence of food deprivation. More specifically, would scheduled daily access to a motivationally significant rewarding event be sufficient to alter the rhythms of PER2 expression in these brains regions. It is known that diverse motivationally and emotionally significant events have access to these regions (Savander et al., 1995, Johnson et al., 1999, Pikkarainen et al., 1999, Dong et al., 2001a, Dong et al., 2001b, Petrovich et al., 2001), can modify the local expression of immediate early genes (Carr et al., 1998, Carr and Kutchukhidze, 2000, Day et al., 2001). Furthermore, these regions play key roles in behavioral, hormonal, and autonomic responses to such stimuli (Rolls and Rolls, 1973, Nachman and Ashe, 1974, Jacobson and Sapolsky, 1991, Zardetto-Smith et al., 1994, Treit et al., 1998, Johnson et al., 1999, Erb et al., 2001a, Erb et al., 2001b, Everitt et al., 2003, Pape and Stork, 2003, Pare, 2003, Petrovich and Gallagher, 2003, Nakao et al., 2004). In the present study we found that daily access to palatable sweet

solutions, sucrose and saccharine, did not affect PER2 rhythms in BNST-OV, amygdala or hippocampus. Importantly, although under these conditions rats avidly drank the solutions upon presentation, a clear sign of their motivational significance, they did not show anticipatory wheel-running. This latter finding might lead one to conclude that there is a direct link between anticipatory wheel-running and changes in PER2 rhythms in these areas. We have shown in another study, however, that as is the case for sucrose and saccharine solutions, daily limited access to a highly palatable meal (Chocolate Ensure) does not affect PER2 rhythms in the limbic forebrain in spite of the fact that it induces anticipatory wheel-running (Verwey et al, 2005; see also Mistlberger and Rusak, 1987). Interestingly, and consistent with the work of Mendoza et al. (2005a), we also found that daily limited access to Chocolate Ensure in freely fed rats synchronized the expression of FOS protein in the BNST-OV and CEA, pointing to a dissociation between the effects on PER2 and FOS rhythms of expression in these regions.

The result from the sucrose and saccharine experiments show that the incentive effects of scheduled rewarding stimuli, in themselves, are not sufficient to entrain and synchronize PER2 expression in these brain regions, leading to the conclusion that a deprivation state is required. To address this issue, we asked whether limited daily access to salt in sodium-deprived rats, that show a strong appetite for salt solutions, would be effective. Again, we found that under these conditions the rhythms of PER2 were unaffected. Thus, we conclude that the daily temporary restoration of energy balance associated with scheduled

restricted feeding provides the critical stimulus for clock gene entrainment and synchronization within the BNST-OV, amygdala and hippocampus. Neither daily timed consumption of a palatable rewarding substance nor a daily alleviation from a specific deprivation state, such as that induced by sodium depletion, is sufficient to affect PER2 expression in these regions.

The mechanism responsible for the synchronization of PER2 rhythms and their uncoupling from that in the SCN that occurs during scheduled daytime restricted feeding remains unidentified. One possibility is that the synchronization depends on the rise in the adrenal glucocorticoid hormone, corticosterone, that occurs shortly before the scheduled daytime meal (Krieger, 1974, Morimoto et al., 1977, Honma et al., 1983, Davidson and Stephan, 1999, Damiola et al., 2000, Diaz-Munoz et al., 2000). We have shown that in freely fed rats, circulating corticosterone plays a key role in the control of PER2 rhythms in BNST-OV and CEA (Amir et al., 2004, Lamont et al., 2005b, Segall et al., 2006). Although we found no evidence for a role of glucocorticoids in the control of PER2 rhythms in BLA and DG, it is possible that in food deprived rats glucocorticoids are involved in the regulation of PER2 in these regions as well.

Alternatively, it is more likely that the mechanism mediating the effect of restricted feeding on clock gene expression involves post-prandial signals that occur during the restoration of energy balance. This is consistent with the finding that daily limited access to rewarding substances in non-deprived animals does not entrain the rhythms of PER2 expression in the limbic forebrain. Such post-prandial signals may be communicated from the gut via the parabrachial nuclei

previously shown to be important in restricted feeding-induced anticipatory behavior (Davidson et al., 2000) and/or via hormones such as thyroxin, insulin, ghrelin, CCK and leptin acting directly on the brain (Gibbs and Smith, 1982, Havel, 2001, Bodosi et al., 2004). Although there is evidence that at least some of these hormones are not involved in the effect of restricted feeding on clock gene expression in peripheral tissue (Davidson et al., 2002, Oishi et al., 2004), their effects in the brain have not been studied.

In conclusion, our studies show that the rhythms of expression of PER2 in limbic forebrain structures can be synchronized and uncoupled from that in the SCN only by daily scheduled restricted access to food in food-deprived animals, but not by daily scheduled access to sweet solutions in non-deprived animals or by daily scheduled access to salt in sodium deprived animals. Thus, it would appear that in addition to being responsive to signals from the SCN, rhythms of PER2 in the BNST-OV, CEA, BLA, and DG are highly sensitive to signals that arise from the alleviation of a negative metabolic state associated with scheduled restricted feeding. Accordingly, the powerful influence that these signals provide, not only competes with the SCN, but in the absence of consistent signals from the SCN can provide an alternative, stable environmental entraining influence.

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Figure captions

Figure 1

Restricted feeding synchronizes PER2 expression in the limbic forebrain. (a) Examples of PER2 expression in the CEA, BLA and DG in freely fed rats killed at ZT1 or ZT13 (scale bars=200μm). (b) Brain maps showing location of regions under study (adapted from Swanson, 1992). The empty black square in each map indicates the area scanned for quantification of PER2 immunoreactivity (CEA, BLA: 400x400-μm, DG: 200x400-μm). (c) Graphs showing mean (±SEM) number of PER2-immunoreactive nuclei in the CEA, BLA and DG as a function of ZT in freely fed (AdLib) and restricted-feeding (RF) rats housed in LD (n=3-5/group). Vertical rectangles inside the graphs indicate the time of food presentation. Asterisks indicate significant difference from corresponding RF groups (Student-Newman-Keuls, $p<.05$). Abbreviations: BMAa, basomedial nucleus of the amygdala, anterior part; CA1, field CA1, Ammon's horn; CA3, field CA3, Ammon's horn; CEAl, CEA lateral part; CEAm, CEA medial part; cing, cingulum bundle; GP, globus pallidus; LA, lateral nucleus of the amygdala; LD, lateral dorsal nucleus of the thalamus; LP, lateral posterior nucleus of the thalamus; MEA, medial nucleus of the amygdala.

Figure 2

Restricted feeding restores and synchronizes PER2 expression in the limbic forebrain of LL-housed rats. Mean (±SEM) number of PER2-immunoreactive

nuclei measured in the CEA, BLA and DG as a function of time in freely fed (AdLib) rats and restricted feeding (RF) rats housed in LL (n=3/group). The rectangles indicate the time of food presentation. Asterisks indicate significant difference from corresponding AdLib groups (Student-Newman-Keuls, $p<.05$).

Figure 3

Daily limited access to sucrose or saccharine has no effect on circadian wheel-running activity rhythms. Representative actograms of wheel running activity in freely fed rats housed in LD and given limited access to water, sucrose (32%) or saccharine (0.2%) solution. In each actogram each vertical mark indicates a period of activity of at least 10 wheel revolutions/10 min. Successive days are plotted from top to bottom. The rectangle marks the time of fluid presentation during the limited access schedule.

Figure 4

Daily limited access to sucrose or saccharine has no effect on PER2 rhythms in the SCN and limbic forebrain. Mean (±SEM) number of PER2-immunoreactive nuclei measured in the SCN, BNST-OV, CEA, BLA and DG as a function of ZT in freely fed rats given daily limited access to sucrose (32%) or saccharine (0.2%) solutions (N=4-6/group). The rectangles indicate the time of daily access.

Figure 5

Daily limited access to a salt solution in acutely sodium-depleted rats has no effect on PER2 rhythms. Mean (±SEM) number of PER2-immunoreactive nuclei measured in the SCN, BNST-OV, CEA, BLA and DG as a function of ZT in rats given daily injections of saline or furosemide (10mg/kg, SC) followed by 1-h access to hypertonic saline (2%) (N=3-4/group). The rectangles indicate the time of daily saline access.

Figure 6

Daily limited access to a salt solution in chronically sodium-depleted rats has no effect on PER2 rhythms. Mean (±SEM) number of PER2-immunoreactive nuclei measured in the SCN, BNST-OV, CEA, BLA and DG as a function of ZT in control rats (Saline, n=3/group) or furosemide-treated rats (continuous administration via osmotic mini-pumps, n=5-7/group) given daily 1-h access to hypertonic saline (2%). The rectangles indicate the time of daily saline access.

Figure 7

Daily limited access to a salt solution has no effect on circadian wheel running activity rhythms in sodium depleted rats. Representative actograms of wheel running activity in control rats (Saline-SC, Saline-pump) and in acutely (Furosemide-SC) or chronically (Furosemide-pump) sodium-depleted rats housed in LD and given a daily 1-h access to hypertonic saline (2%). The rectangles mark the time of saline presentation during the limited access schedule.

Figure 1

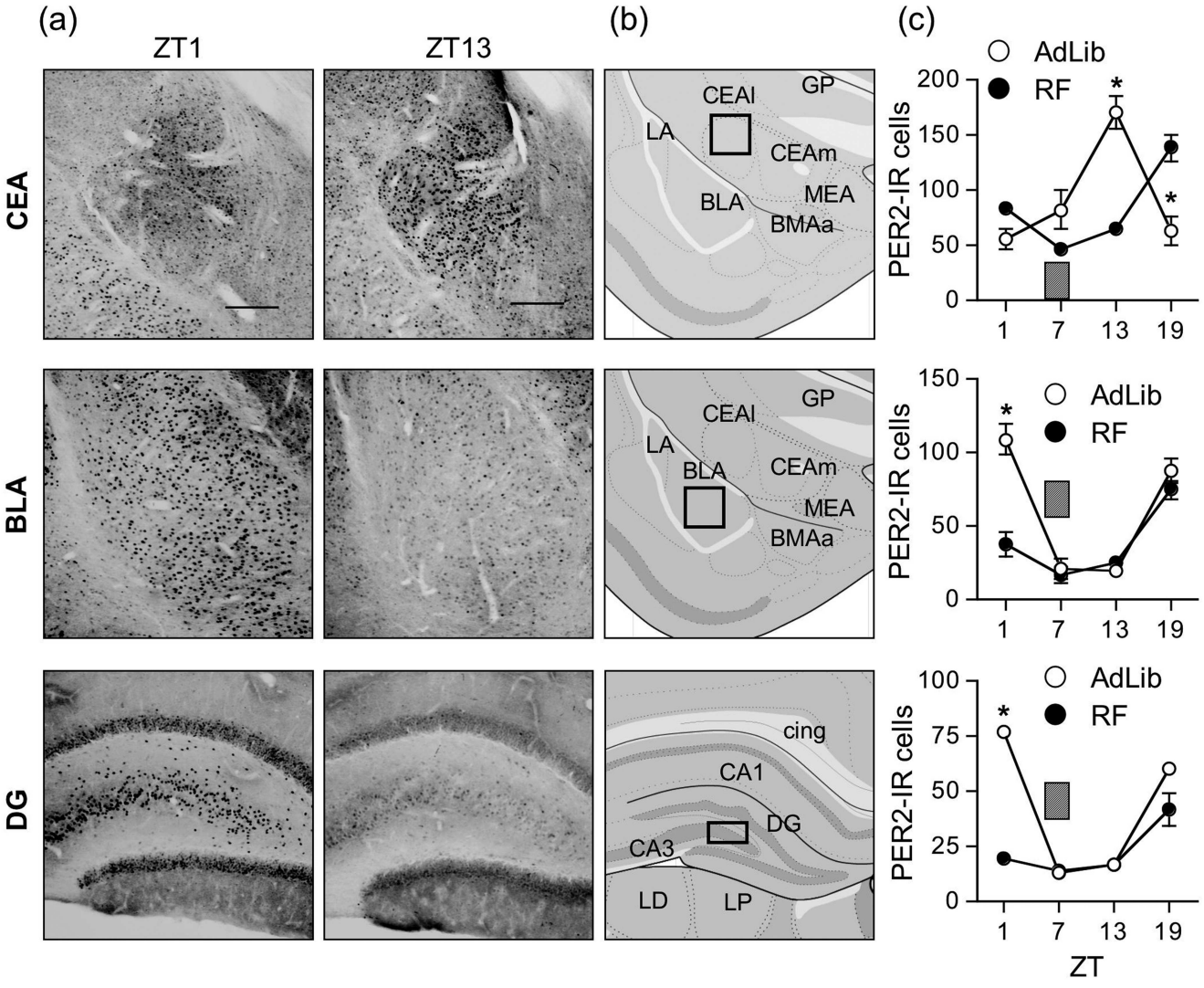
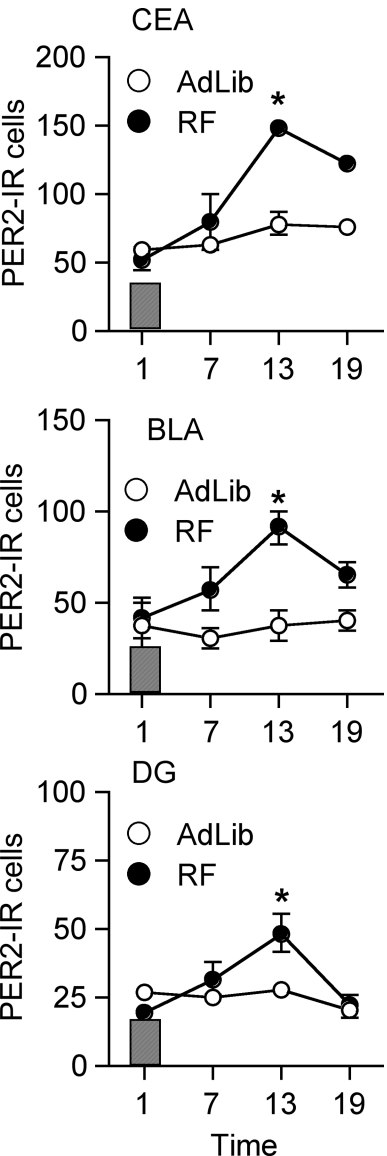

(a)
ZT1
ZT13
CEA
BLA
DG
(b)
CEAl
GP
LA
CEAm
BLA
MEA
BMAa
cing
CA1
DG
CA3
LD
LP
(c)
AdLib
RF
PER2-IR cells
ZT

Figure 2


CEA
AdLib
RF
PER2-IR cells
200
150
100
50
0
1
7
13
19
*
BLA
AdLib
RF
PER2-IR cells
150
100
50
0
1
7
13
19
*
DG
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Time

Figure-3

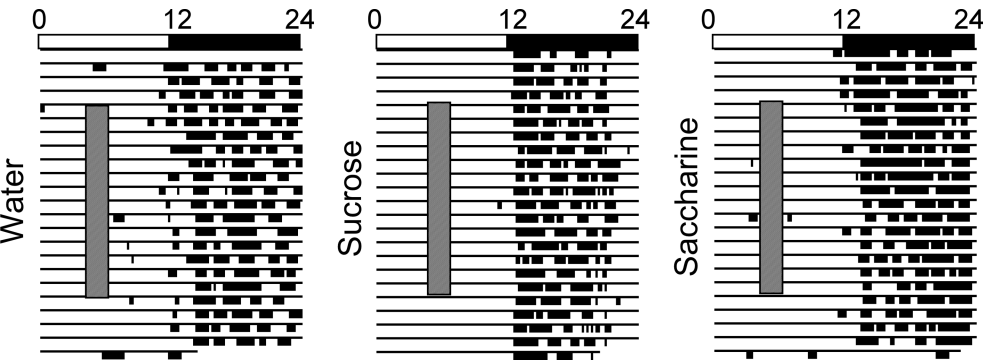


Figure-4

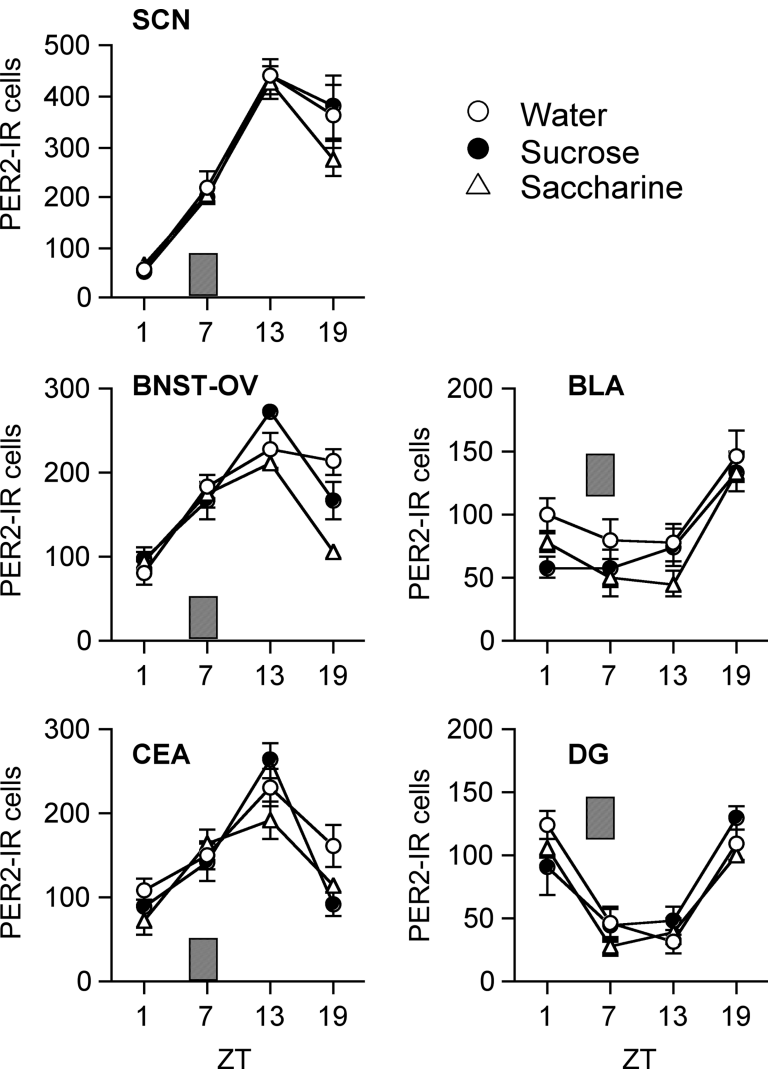
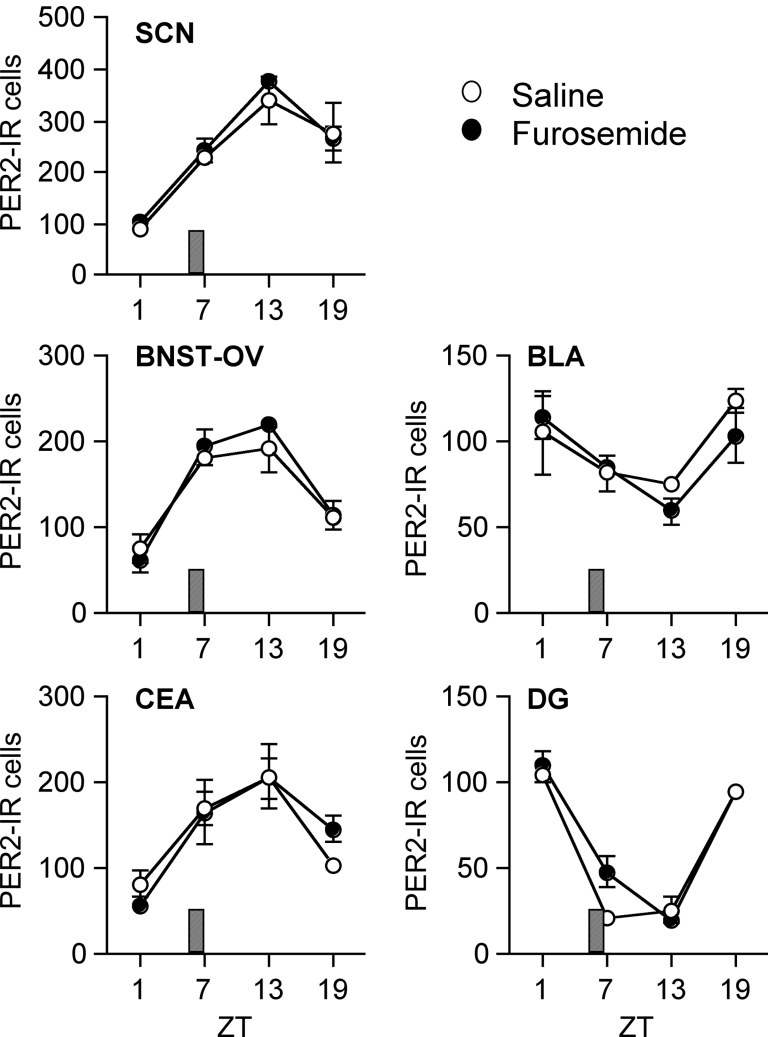

SCN
BNST-OV
BLA
CEA
DG
PER2-IR cells
ZT
Water
Sucrose
Saccharine

Figure-5


SCN
BNST-OV
BLA
CEA
DG
PER2-IR cells
ZT
Saline
Furosemide
500
400
300
200
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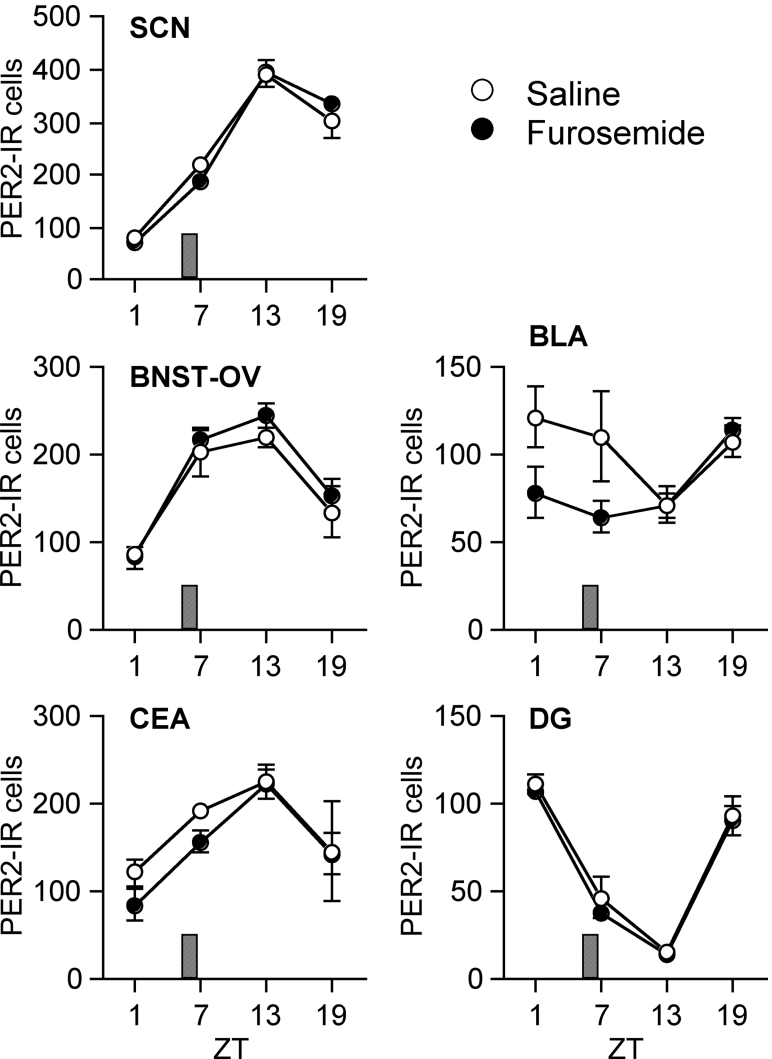
50
1
7
13
19

Figure-6


SCN
BNST-OV
BLA
CEA
DG
PER2-IR cells
ZT
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Furosemide
500
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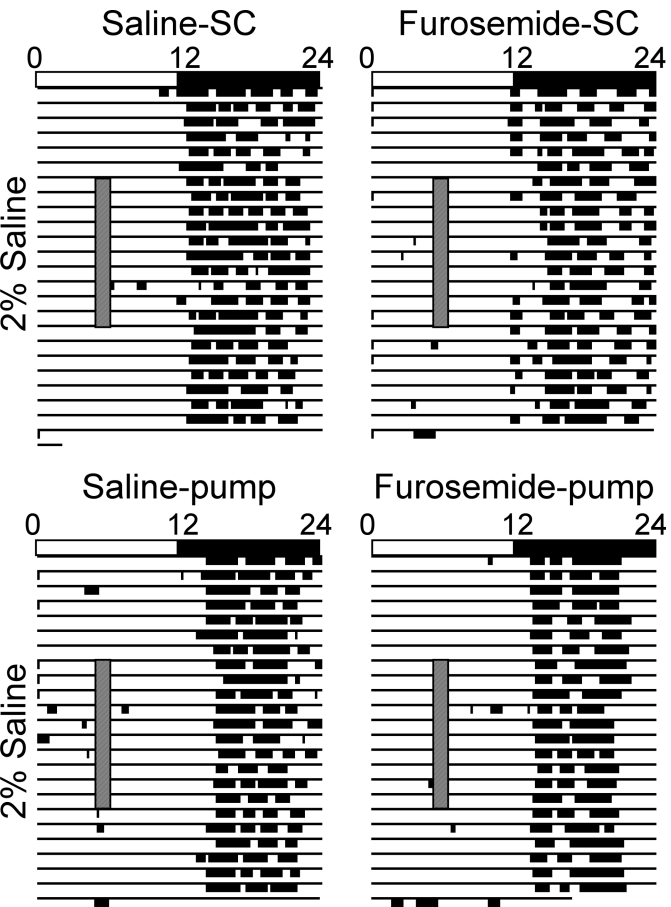
7
13
19

Figure-7


Saline-SC
Furosemide-SC
Saline-pump
Furosemide-pump
0
12
24
2% Saline