Neuroscience 147, 277-285, 2007

Differential regulation of the expression of Period2 protein in the limbic forebrain and dorsomedial hypothalamus by daily limited access to highly palatable food in food-deprived and free-fed rats

Michael Verwey, Zehra Khoja, Jane Stewart and Shimon Amir

Center for Studies in Behavioral Neurobiology, Department of Psychology, Concordia University, Montréal, Quebéc H4B 1R6, Canada

Corresponding Author: Shimon Amir

Center for Studies in Behavioral Neurobiology

Concordia University, SP-244

7141 Sherbrooke St. West

Montreal, QC, Canada H4B 1R6

Tel: 514 848 2424 (EXT 2188)

Fax: 514 848 2817

e-mail: shimon.amir@concordia.ca

Acknowledgements

Supported by the Canadian Institutes of Health Research, the Natural Science and Engineering Council of Canada, and the Concordia Research Chairs Program.

Abbreviations: AL, ad libitum; ANOVA, Analysis of Variance; BLA, basolateral amygdala; BNSTov, oval nucleus of the bed nucleus of the stria terminalis; CEA, central nucleus of the amygdala; DG, dentate gyrus; DMH, dorsomedial hypothalamus; Ensure, Complete Meal Replacement, Chocolate Ensure Plus; LD, light dark;PER2, Period2; RF, restricted feeding; RT, Restricted treat; SCN, suprachiasmatic nucleus; ZT, zeitgeber time

Abstract

Circadian clock genes are rhythmically expressed in many areas of the brain and body and are thought to underlie most endogenous circadian behaviors and physiological processes. Daily rhythms of clock gene expression throughout the brain and body are normally coordinated by the suprachiasmatic nucleus (SCN), but they are also strongly influenced by daily temporal restrictions of food availability. Here, we studied the effects of a daily restricted presentation of highly palatable complete meal replacement, chocolate Ensure Plus (Ensure) in food-deprived (restricted feeding, RF) and free-fed (restricted treat, RT) rats, on the expression of the clock protein, Period2 (PER2) in regions of the brain involved in motivational and emotional regulation; these include the oval nucleus of the bed nucleus of the stria terminalis (BNSTov), the central nucleus of the amygdala (CEA), the basolateral amygdala (BLA), the dentate gyrus (DG) and the dorsomedial hypothalamus (DMH). RF and RT rats consumed similar amounts of Ensure, but changes in the pattern of PER2 expression were seen only in the RF condition, suggesting that changes in PER2 expression in these regions are triggered by the daily alleviation of a negative metabolic state associated with RF and are independent of the positive incentive properties of the consumed substance, per se. In contrast, the expression of the immediate early gene, Fos, was increased in these regions by both RF and RT schedules, showing that signals concerning the incentive value of the consumed food reach these regions. No changes in either PER2 or Fos expression were observed in the SCN of RF or RT rats. These findings demonstrate that mechanisms leading

to changes in the expression of PER2 and those affecting the induction of Fos under RF and RT are, at least in part, dissociable.

Key words: Restricted feeding, Clock genes, Bed nucleus of the stria terminalis, amygdala, hippocampus, food-anticipatory behavior

Introduction

Circadian rhythms in the expression of clock genes in peripheral tissues and brain are orchestrated by a master pacemaker located in the suprachiasmatic nucleus (SCN) (Lowrey and Takahashi, 2004, Yoo et al., 2004, Guo et al., 2005, Guo et al., 2006). These extra-SCN rhythms are also strongly influenced by feeding schedules that restrict food access to the same time each day (Damiola et al., 2000, Hara et al., 2001, Stokkan et al., 2001, Wakamatsu et al., 2001, Challet et al., 2003, Mieda et al., 2006, Zvonic et al., 2006, Angeles-Castellanos et al., 2007, Waddington Lamont et al., 2007). Such schedules lead to novel daily fluctuations in energy balance as well as changes in the patterns of arousal, circulating corticosterone and daily activity rhythms (Mistlberger and Marchant, 1995, Stephan, 2002, Davidson et al., 2005, Mendoza, 2007), suggesting that expression of clock genes in the brain and periphery is not only sensitive to signals from the SCN but also responds to changes in nutritional and/or motivational state independently of the SCN (Hara et al., 2001).

We have shown recently that scheduled restricted feeding in rats strongly affects the rhythm of expression of the clock protein PERIOD2 (PER2) in limbic forebrain areas involved in the control of motivation and emotion, 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) (Lamont et al., 2005a, Waddington Lamont et al., 2007). Interestingly we also found that in the absence of food deprivation, daily limited access to highly palatable substances such as sucrose or saccharine, had no effect on the rhythm

of PER2 expression in these areas in spite of the fact that these substances were consumed in large quantities. Based on these findings, we hypothesized that the expression of PER2 in the limbic forebrain is sensitive to homeostatic signals arising from the daily alleviation of a negative metabolic state associated with scheduled feeding and is independent of the positive incentive properties of the consumed substance, per se (Waddington Lamont et al., 2007).

To study this issue further, we assessed the effect of daily limited access to a highly palatable complete meal replacement, Chocolate Ensure Plus (Ensure), on PER2 expression in the BNSTov, CEA, BLA, and DG in both food-deprived and free-fed rats. In addition, we assessed the effect of daily limited access to Ensure on neuronal activation in these brain regions in food-deprived and free-fed rats using the transcription factor, Fos, as a marker (Angeles-Castellanos et al., 2004, Angeles-Castellanos et al., 2007). Also, we assessed the effect of limited access to Ensure in food-deprived and free-fed rats on PER2 and Fos expression in the dorsomedial hypothalamic nucleus (DMH), an area shown recently to play a role in the interface between restricted feeding, PER2 expression and certain food anticipatory rhythms (Gooley et al., 2006, Landry et al., 2006, Mieda et al., 2006). Finally, in order to differentiate the circadian effects of serial food presentations from the potential acute effects of a single food presentation, we also studied rats on the first day of our restricted Ensure schedules. Preliminary results have been presented in an abstract form (Verwey et al., 2005).

Materials and Methods

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. A total of 96 male Wistar rats were used (225-250g; Charles River Laboratories, St. Constant, QC, Canada). The rats were housed individually in cages equipped with running wheels, under a 12h:12h light-dark (LD) schedule (300 lux at cage level) and had free access to Purina rat chow and water for at least 10 days before the start of each experiment. Running wheel activity was recorded by computer (Vitalview, Minimitter, OR, USA) and analyzed with Circadia software.

Restricted feeding and restricted treats

Rats were assigned randomly to one of three groups: restricted feeding (RF) group, restricted treat (RT) group or ad libitum (AL) group. During experimental stages, rats in the RF group were fed exclusively with unlimited Ensure for 2 h each day, during the middle of the day, from zeitgeber time (ZT) 4–6 (ZT0 denotes time of lights on in a 12:12 light-dark schedule). Rats in the RT group had continued access to Purina rat chow and in addition received an identical restricted access to Ensure during the day (ZT4-6). Rats in the AL control group had free access to Purina rat chow only. These schedules lasted for 10-13 days. This relatively short RT schedule was deliberately chosen so the RT group could be compared to the RF group. Other experiments have

generally used longer (4-6 week) palatable meal entrainment protocols and have focused more on treat-anticipatory behavior (Mistlberger and Rusak, 1987, Mendoza et al., 2005c, a). Our experiments did not set out to study the anticipation of a daily treat, per se, instead we focused on the metabolic and motivational consequences of the daily consumption of Ensure between fasted and free-fed rats.

Tissue preparation and immunocytochemistry

On the last day of the scheduled feeding, rats were deeply anaesthetized with sodium pentobarbital (Somnotol, ~ 100 mg/kg) at one of six zeitgeber times (ZT1, 5, 9, 13, 17, 21) and 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) containing regions of interest were collected from each animal using a vibratome and stored in Watson's cryoprotectant solution until use (Watson et al., 1986). Immunocytochemistry for PER2 was performed on one set of brain sections as previously described (Amir et al., 2004) using an affinity purified rabbit polyclonal antibody raised against PER2 (1:800, Alpha Diagnostics International, San Antonio, TX). Immunocytochemistry for Fos was performed on a second set of brain sections collected from each rat as previously described (Beaule et al., 2001) using a polyclonal *cFos* antibody, raised in rabbit (1:100,000, Oncogene Sciences, Boston, MA).

Image analysis and statistics

PER2- and Fos-stained brain sections were mounted on gelatin-coated glass slides, coverslipped and examined under a light microscope. Images of brain areas containing the SCN, BNSTov, CEA, BLA, DG and DMH were captured using a Sony XC-77 video camera, a Scion LG-3 frame grabber, and Image SXM software (v1.6, S D Barrett, http://www.ImageSXM.org.uk). Cells immunopositive for PER2 or Fos were counted on captured images using a 400 x 400 μm (SCN, BNSTov, CEA, BLA, DMH) or a 200 x400μm (DG) frame. The mean number of PER2 or Fos immunoreactive cells per region 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). Alpha level was set at 0.05 for all analyses.

Results

Ensure consumption and locomotor activity rhythms under restricted feeding and restricted treat schedules

Daily rhythms of wheel running activity were assessed in AL rats housed under a 12h:12h LD schedule and in similarly housed rats that were placed on either a RF or RT schedule in which Ensure was given for 2 h each day, from ZT4-6. Fig. 1 shows the amounts of Ensure consumed each day during the 2-h access period by rats from the RF and RT groups. It can be seen that with the exception of the first day of limited access, rats from the two groups consumed

similar amounts of Ensure throughout the experiment. Notwithstanding, RT rats continued to eat regular chow and thus consumed more calories each day than RF rats (data not shown). As expected, rats from the RF group showed consistent changes in running wheel patterns and developed an anticipatory running wheel bout which began 2-3 h before daily food presentation (Fig. 2). In contrast, only nine of the 24 rats from the RT group showed anticipatory running wheel activity (Fig. 2). The remaining rats from the RT group did not develop anticipatory running and their circadian running patterns resembled those of AL rats that had free access to normal rat chow but not to Ensure. When the consumption of Ensure was reexamined, treat-anticipating RT rats tended to eat moderately more Ensure than non-anticipating RT rats, but this effect was not significant ($p=0.075$).

Restricted feeding but not restricted treat modifies the daily pattern of PER2 expression

Examples of PER2 expression in the SCN, BNSTov, CEA, BLA, DG and DMH of AL rats killed at ZT1 or ZT13 and graphs showing daily patterns of PER2 expression in AL, RF and RT groups are shown in Fig. 3. and results from two-way analyses of variance (ANOVA) carried out for each brain region to assess group differences and changes across time are shown in Table 1. In AL rats the expression of PER2 in the SCN, BNSTov, CEA, BLA and DG was rhythmic, whereas expression in the DMH was arrhythmic, as previously reported (Amir et al., 2004, Lamont et al., 2005b, Mieda et al., 2006). Specifically, in the SCN,

BNSTov and CEA maximal nuclear staining for PER2 was seen in the evening, at ZT 13, whereas in BLA and DG PER2 expression was maximal in the morning, at ZT1 (see Fig. 3).

In food deprived rats, restricted access to Ensure for 10 days had no effect on PER2 expression in the SCN. The rhythm of PER2 expression in the SCN in RF rats was similar to that seen in the SCN of AL rats, peaking at ZT13. In contrast, peak PER2 expression in the BNSTov, CEA , BLA and DG of rats from the RF group shifted to ZT17, 12 hours after the daily Ensure presentation (Fig. 3). Restricted feeding induced a strong PER2 rhythm in the DMH which peaked around the time of food presentation, consistent with a previous report in mice (Mieda et al., 2006).

Finally, robust rhythms of PER2 expression were seen in the SCN, BNSTov, CEA, BLA and DG of rats from the RT group (Fig. 3). However, contrary to the effect of daily restricted feeding, daily restricted treat had no effect on the rhythm of PER2 expression in these areas. In these rats, PER2 expression in all regions resembled that of AL rats, consistent with our previous observation that consumption of a highly palatable substance in the absence of food deprivation is insufficient to bring about a change in the rhythm of PER2 expression (Waddington Lamont et al., 2007).

Fos expression under scheduled daily limited access to Ensure

Daily restricted feeding, but not restricted treat, shifted the phase of PER2 expression in the limbic forebrain and induced rhythms in PER2 expression in the

DMH, suggesting that the two feeding schedules might exert quantitatively or qualitatively different effects on neural activity within these brain regions. To investigate this possibility we assessed the expression of the cellular activity marker, Fos before, during and after Ensure presentation in a second set of brain sections obtained from AL, RF and RT rats.

Representative photomicrographs of Fos immunoreactivity from AL, RF and RT rats killed 1 h after Ensure presentation (ZT5) and graphs showing the levels of Fos expression from these groups 3 h before (ZT1) and 1 and 5 h after (ZT5, ZT9) Ensure presentation (ZT4-6) are shown in Fig 4. In the SCN, the expression of Fos varied as a function of Time peaking at ZT1, 1 h after lights on in all three groups ($F[2,27]=6.86$; $p<.001$). Likewise, Fos expression in the BNSTov varied as a function of Time ($F[2,27]=10.90$; $p<.0003$). Furthermore, in the BNSTov there was also a significant Time x Group interaction ($F[4,27]=5.31$; $p<.003$); expression of Fos at ZT5, 1 h after Ensure presentation, was significantly higher in the RF group than in the AL group (Fig. 4), but there was no difference between the AL the RT group.

In the CEA, Fos expression varied as a function of Time ($F[2,27]=6.29$; $p<.006$), and there was a significant Time x Group interaction ($F[4,27]=8.25$; $p<.002$). In this case, however, the level of Fos expression at ZT5 and ZT9, 1 and 5 h after Ensure presentation was greater in both the RF and RT groups compared with the AL group (Fig. 4). In the BLA, the RF and RT groups showed a similar patter of Fos expression at ZT1 and ZT5 and in each case levels were significantly different from those seen in the AL group (Time x Group:

F(4,27)=11.18; p<.0001). In the DG only the RF group showed elevated Fos expression at ZT5 (Time x Group: F(4,27)=23.36; p<.0001). Finally, in the DMH Fos expression in both RF and RT groups was significantly higher than in AL rats at ZT5, 1 h after ensure presentation (Time x Group: F(4, 27)=19.03; p<.0001).

Taken together, the results show that after 10 days of repeated exposure, presentation of Ensure to RF rats increases Fos expression in all the regions studied, except the SCN. In contrast, in RT rats, exposure to Ensure increases Fos expression only in the CEA, BLA and DMH relative to AL rats. Furthermore, at the time of food presentation the increases in Fos expression were usually greater in RF than in RT rats. Thus, although the presentation of Ensure activates neural elements in most regions in both RF and RT groups after repeated Ensure presentation, the magnitude of this activation appears to be modulated by the metabolic and motivational consequences of food deprivation.

PER2 and Fos expression following acute Ensure feeding

The results from the scheduled feeding experiment suggest that the mechanism regulating the expression of PER2 in the limbic forebrain and DMH is sensitive to signals associated with the repeated mitigation of a negative metabolic state. Daily limited access to Ensure in the absence of food deprivation did not alter PER2 expression, suggesting that incentive signals associated with daily presentation of Ensure are not effective. However, both restricted feeding and restricted treat induced Fos expression in these same regions, albeit somewhat differentially, suggesting that Fos expression is related

to the incentive or nutritive aspects of Ensure. To further test this idea, in a final experiment, we assessed Fos and PER2 expression before, during and after an acute presentation of Ensure in 24-h food-deprived and free-fed rats. Groups of free-fed and fasted (24 h) rats were killed 3 h before (ZT 1), and 1 (ZT5) and 5 h (ZT 9) after a single presentation of Ensure (ZT4-6).

As shown in Fig. 5, the first presentation of Ensure induced equally strong Fos expression in both groups in all regions, with the exception of the SCN where Fos expression was similar in all groups. PER2 expression was unaffected in all regions.

Discussion

The results of the present experiments show that in food-deprived rats daily restricted access to a highly palatable food, chocolate Ensure, synchronizes the rhythms of PER2 expression in limbic forebrain regions involved in motivational and emotional regulation and uncouples them from the rhythm in the SCN. These results are consistent with previous findings on the synchronizing effect of daily restricted feeding on the expression of PER2 and other clock genes in the brain and periphery in rodents (Damiola et al., 2000, Hara et al., 2001, Stokkan et al., 2001, Wakamatsu et al., 2001, Challet et al., 2003, Mieda et al., 2006, Zvonic et al., 2006, Angeles-Castellanos et al., 2007, Waddington Lamont et al., 2007). We also found that in the absence of food deprivation daily restricted access to Ensure had no effect on PER2 rhythms in the limbic forebrain in spite of the fact that consumption equaled that in food-deprived rats.

These results confirm and extend our previous observations in free-fed rats given restricted daily access to sucrose or saccharine solution (Waddington Lamont et al., 2007) by showing that even a highly palatable complete meal replacement has no effect on PER2 expression, suggesting that it is not the intake of nutrients, per se, that results in altered PER2 expression patterns. Furthermore, this supports the hypothesis that the effect on PER2 rhythms in these brain areas arises from signals associated with the daily alleviation of a negative metabolic state, and not from those associated with the incentive properties of the food. To the best of our knowledge there is one other study of the effect of daily restricted treat on the expression of a clock gene in the brain in rats (Mendoza et al., 2005a). In this study it was found that presentation of a daily palatable meal changed the pattern of PER1 expression in the SCN and the paraventricular thalamic nucleus, an area known to be highly sensitive to a range of arousing and rewarding stimuli. These results are at odds with the present finding of a lack of effect of restricted treat on PER2 expression in the SCN. These differences could be attributed to methodological differences, such as the number of days of limited access (6 weeks in the Mendoza et al. study compared to <2 weeks in the present study), the nature of the treat (chocolate bar in the Mendoza et al. study), type of limitation (limiting the amount of chocolate in the Mendoza et al. study instead of limiting the time treat was available in the current study), lighting conditions (constant darkness in the Mendoza et al. study instead of a light-dark cycle in the present study), and the type of clock protein measured (PER1 in the Mendoza et al. study instead of PER2 in the present study). Furthermore,

differences in patterns of expression of PER1 and PER2 have also been observed in rats under restricted feeding schedules (Lamont et al., 2005a, Angeles-Castellanos et al., 2007, Waddington Lamont et al., 2007) suggesting that the mechanism(s) that control the expression of different clock genes in the brain could be differentially sensitive to signals associated with feeding.

Our findings indicate that the rhythms of PER2 expression in the limbic forebrain are modulated by nutritional status and are insensitive to the incentive properties of food, per se. To study whether the signals associated with the eating of Ensure in free-fed and food deprived rats gained access to the regions under study we measured Fos induction before, during and after chronic Ensure presentation. In addition, we assessed Fos expression in food-deprived or free-fed rats receiving an acute presentation of Ensure. In rats that received daily Ensure presentations, we found that with the exception of the BNSTov and DG, restricted daily access to Ensure induced a significant increase in the expression of Fos in the limbic forebrain in both food deprived and free-fed rats, indicating that the expression of Fos in these regions is modulated primarily by signals associated with the incentive or metabolic properties of the consumed food. This conclusion is further supported by the results from rats that received Ensure for the first time, where Fos expression increased equally in all regions (except the DG and SCN) regardless of nutritional state. However, a role for nutritional status in the modulation of Fos expression is suggested by the finding that, in rats given daily restricted access to Ensure, the increase in Fos expression in the different regions were greater in food deprived than free-fed rats, (except the

BLA and SCN). Together, the results from the rats given daily restricted access to Ensure are consistent with previous findings on the expression of Fos in multiple limbic areas in rats under restricted feeding and restricted treat (Angeles-Castellanos et al., 2004, Angeles-Castellanos et al., 2005, Mendoza et al., 2005c, b, a, Gooley et al., 2006, Angeles-Castellanos et al., 2007). Furthermore, they suggest that the neural signals and cellular events leading to Fos induction in the BNSTov, CEA, BLA and DG under scheduled restricted feeding and restricted treat and those responsible for the changes in PER2 expression these limbic forebrain regions are, at least in part, functionally dissociable.

In addition to the differences in PER2 and Fos expression discussed above, we observed differences in the prevalence of food anticipatory wheel-running. Previous studies have shown that daily limited access to a palatable meal can entrain food anticipatory rhythms in free-fed rats (Mistlberger and Rusak, 1987, Mendoza et al., 2005a); however, the aim of our study was to compare the metabolic and motivational consequences of the daily consumption of Ensure in food-deprived and free-fed rats and we used a protocol too short to reliably observe treat-anticipation. In the present study all food-deprived rats showed robust anticipatory wheel-running, whereas only 37% of free-fed rats that received daily limited access to Ensure did. To assess whether these behavioral differences, rather than differences in nutritional state, could account for the effects on PER2 expression, we compared rhythms of PER2 between free-fed rats that showed anticipatory wheel-running (n=9) and those that did not (n=15).

We found that the circadian patterns of PER2 expression in these two sub-groups were indistinguishable and not different from those in *ad libitum* control rats (data not shown). This finding suggests that anticipatory wheel-running, as such, does not account for the differences in the rhythms of PER2 expression.

We also studied PER2 and Fos expression in the DMH, a region of the hypothalamus that receives a major innervation from the SCN (Thompson and Swanson, 1998) and that has been implicated both in SCN-driven circadian rhythms (Chou et al., 2003) and in the control of circadian food anticipatory wheel-running activity and temperature rhythms (Gooley et al., 2006). Consistent with a previous study in mice (Mieda et al., 2006), we found that in free-fed rats the expression of PER2 in the DMH is arrhythmic and that in food deprived rats, daily limited access to Ensure induces a robust rhythm that peaks around the time of food presentation. Importantly, we found that as in the case of the limbic forebrain, daily limited access to Ensure in free-fed rats had no effect on PER2 expression in the DMH whereas Fos was expressed equally in the DMH in both food-deprived and free-fed rats. These findings further demonstrate the dissociation between the effects of Ensure presentation on neuronal activation and on changes in rhythms of PER2 expression.

The findings concerning PER2 expression in the DMH are at odds with the hypothesis that the induction of a rhythm of PER2 in this region is critical for the expression of food anticipatory behavior (Mieda et al., 2006). At least 33% of free-fed rats given daily access to Ensure showed anticipatory wheel-running yet no changes in PER2 expression were observed in the DMH. In fact, it is possible

that an even greater proportion of RT rats may have shown other forms of anticipatory behavior such as increased activity around the food receptacle before mealtime (Landry et al., 2006), but these alternate anticipatory behaviors were not tested. Thus, although our study confirms the previous finding that daily restricted access to food induces a robust rhythm of PER2 expression in the DMH, we do not have evidence that this rhythm plays a critical role in the expression of food-anticipatory behaviors.

Taken together the present experiments show that daily restricted access to a highly palatable meal produces marked changes in PER2 rhythms in BNSTov, CEA, BLA, DG, and DMH in food-deprived rats, but does not affect the rhythms in these structures in free-fed rats. The evidence from the studies of Fos induction, shows that this lack of effect is not due to differences in the ability of food stimuli to gain access to these various brain regions. Thus, we conclude on the basis of these data and those from our previous studies that the effects on PER2 rhythms are due to the daily alleviation of a negative metabolic state and not to signals arising from the incentive or metabolic properties of the food, per se. We cannot, however, at this time relate these changes to any particular behavioral outcome of scheduled feeding. Although there is evidence from studies in mutant mice that global functional disruption of PER2 does affect the expression food anticipatory locomotor activity and temperature rhythms (Feillet et al., 2006), the particular brain regions important for these changes remain to be identified.

References

Amir S, Lamont EW, Robinson B, 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.

Angeles-Castellanos M, Aguilar-Roblero R, Escobar C (2004) c-Fos expression in hypothalamic nuclei of food-entrained rats. Am J Physiol Regul Integr Comp Physiol 286:R158-165.

Angeles-Castellanos M, Mendoza J, Diaz-Munoz M, Escobar C (2005) Food entrainment modifies the c-Fos expression pattern in brain stem nuclei of rats. Am J Physiol Regul Integr Comp Physiol 288:R678-684.

Angeles-Castellanos M, Mendoza J, Escobar C (2007) Restricted feeding schedules phase shift daily rhythms of c-Fos and protein Per1 immunoreactivity in corticolimbic regions in rats. Neuroscience 144:344-355.

Beaule C, Arvanitogiannis A, Amir S (2001) Light suppresses Fos expression in the shell region of the suprachiasmatic nucleus at dusk and dawn: implications for photic entrainment of circadian rhythms. Neuroscience 106:249-254.

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

Chou TC, Scammell TE, Gooley JJ, Gaus SE, Saper CB, Lu J (2003) Critical role of dorsomedial hypothalamic nucleus in a wide range of behavioral circadian rhythms. J Neurosci 23:10691-10702.

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

Davidson AJ, Tataroglu O, Menaker M (2005) Circadian Effects of Timed Meals (and Other Rewards). Methods Enzymol 393:509-523.

Feillet CA, Ripperger JA, Magnone MC, Dulloo A, Albrecht U, Challet E (2006) Lack of Food Anticipation in Per2 Mutant Mice. Curr Biol 16:2016-2022.

Gooley JJ, Schomer A, Saper CB (2006) The dorsomedial hypothalamic nucleus is critical for the expression of food-entrainable circadian rhythms. Nat Neurosci 9:398-407.

Guo H, Brewer JM, Champhekar A, Harris RB, Bittman EL (2005) Differential control of peripheral circadian rhythms by suprachiasmatic-dependent neural signals. Proc Natl Acad Sci U S A 102:3111-3116.

Guo H, Brewer JM, Lehman MN, Bittman EL (2006) Suprachiasmatic regulation of circadian rhythms of gene expression in hamster peripheral organs: effects of transplanting the pacemaker. J Neurosci 26:6406-6412.

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

Lamont EW, Diaz LR, Barry-Shaw J, Stewart J, Amir S (2005a) Daily restricted feeding rescues a rhythm of period2 expression in the arrhythmic suprachiasmatic nucleus. Neuroscience 132:245-248.

Lamont EW, Robinson B, Stewart J, Amir S (2005b) The central and basolateral nuclei of the amygdala exhibit opposite diurnal rhythms of expression of the clock protein Period2. Proc Natl Acad Sci U S A 102:4180-4184.

Landry GJ, Simon MM, Webb IC, Mistlberger RE (2006) Persistence of a behavioral food-anticipatory circadian rhythm following dorsomedial hypothalamic ablation in rats. Am J Physiol Regul Integr Comp Physiol 290:R1527-1534.

Lowrey PL, Takahashi JS (2004) Mammalian circadian biology: elucidating genome-wide levels of temporal organization. Annu Rev Genomics Hum Genet 5:407-441.

Mendoza J (2007) Circadian clocks: setting time by food. J Neuroendocrinol 19:127-137.

Mendoza J, Angeles-Castellanos M, Escobar C (2005a) A daily palatable meal without food deprivation entrains the suprachiasmatic nucleus of rats. Eur J Neurosci 22:2855-2862.

Mendoza J, Angeles-Castellanos M, Escobar C (2005b) Differential role of the accumbens Shell and Core subterritories in food-entrained rhythms of rats. Behav Brain Res 158:133-142.

Mendoza J, Angeles-Castellanos M, Escobar C (2005c) Entrainment by a palatable meal induces food-anticipatory activity and c-Fos expression in reward-related areas of the brain. Neuroscience 133:293-303.

Mieda M, Williams SC, Richardson JA, Tanaka K, Yanagisawa M (2006) The dorsomedial hypothalamic nucleus as a putative food-entrainable circadian pacemaker. Proc Natl Acad Sci U S A 103:12150-12155.

Mistlberger R, Rusak B (1987) Palatable daily meals entrain anticipatory activity rhythms in free-feeding rats: dependence on meal size and nutrient content. Physiol Behav 41:219-226.

Mistlberger RE, Marchant EG (1995) Computational and entrainment models of circadian food-anticipatory activity: evidence from non-24-hr feeding schedules. Behav Neurosci 109:790-798.

Stephan FK (2002) The "other" circadian system: food as a Zeitgeber. J Biol Rhythms 17:284-292.

Stokkan KA, Yamazaki S, Tei H, Sakaki Y, Menaker M (2001) Entrainment of the circadian clock in the liver by feeding. Science 291:490-493.

Thompson RH, Swanson LW (1998) Organization of inputs to the dorsomedial nucleus of the hypothalamus: a reexamination with Fluorogold and PHAL in the rat. Brain Res Brain Res Rev 27:89-118.

Verwey M, Khoja Z, Amir S (2005) Feeding-induced c-Fos activation is insufficient for reentrainment of PER2 rhythms in the limbic forebrain. Program No 606 2005 Abstract Viewer/Itinerary Planner Washington, DC: Society for Neuroscience Online

Waddington Lamont E, Harbour VL, Barry-Shaw J, Renteria Diaz L, Robinson B, Stewart J, Amir S (2007) Restricted access to food, but not sucrose, saccharine, or salt, synchronizes the expression of Period2 protein in the limbic forebrain. Neuroscience 144:402-411.

Wakamatsu H, Yoshinobu Y, Aida R, Moriya T, Akiyama M, 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.

Watson RE, Jr., Wiegand SJ, Clough RW, Hoffman GE (1986) Use of cryoprotectant to maintain long-term peptide immunoreactivity and tissue morphology. Peptides 7:155-159.

Yoo SH, Yamazaki S, Lowrey PL, Shimomura K, Ko CH, Buhr ED, Siepka SM, Hong HK, Oh WJ, Yoo OJ, Menaker M, Takahashi JS (2004) PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. Proc Natl Acad Sci U S A 101:5339-5346.

Zvonic S, Ptitsyn AA, Conrad SA, Scott LK, Floyd ZE, Kilroy G, Wu X, Goh BC, Mynatt RL, Gimble JM (2006) Characterization of peripheral circadian clocks in adipose tissues. Diabetes 55:962-970.

Figure captions

Fig. 1

Mean (±sem) daily intake (ml) of chocolate Ensure in food deprived (RF, n=24) and free-fed (RT, n=24) rats. Ensure was presented for 2 hours each day from ZT4-6 (4-6 hours after lights-on).

Fig. 2

Representative single-plotted actograms of wheel-running activity in *ad libitum* (AL) control rats, as well as food deprived (Restricted feeding; RF) and free-fed (Restricted treat; RT) rats that received a daily presentation of chocolate Ensure from ZT4-6 (4-6 hours after lights on; illustrated by grey rectangles). All RF rats showed 'anticipatory' wheel running preceding the daily meal, while only 33% of RT rats showed a similar behavioral pattern (bottom panel, on left). All rats were housed under a 12h:12h LD cycle. The vertical marks indicate periods of activity of at least 10 wheel-revolutions/10 min. Successive days are plotted from top to bottom.

Fig. 3

Restricted feeding (RF), but not restricted treat (RT) synchronizes PER2 expression. (a) Brain maps showing location of regions under study. The dotted square in each map indicates the area scanned for quantification of PER2 immunoreactivity. (b) Examples of PER2 expression in the SCN, BNSTov, CEA,

BLA, DG and DMH in control (AL) rats killed at ZT1 or 13 (Scale bar = 200μm). (c) Graphs showing mean (±SEM) number of PER2-immunoreactive (PER2-IR) nuclei in the SCN, BNSTov, CEA, BLA, DG and DMH as a function of ZT in AL, RF and RT rats (n=3-4/group). Vertical dotted rectangles inside the graphs indicate the time of Ensure presentation. Asterisks indicate significant difference from corresponding AL group (Student-Newman-Keuls, $p<.05$).

Fig. 4

After 10 days of repeated exposure, presentation of Ensure increases Fos expression in all regions (except the SCN) in restricted feeding (RF) rats, but only the CEA, BLA and DMH in restricted treat (RT) rats. (a) Brain maps showing location of regions under study. The dotted square in each map indicates the area scanned for quantification of Fos immunoreactivity. (b) Examples of Fos expression in the SCN, BNSTov, CEA, BLA, DG and DMH in control (AL), RF and RT rats killed at ZT5 (Scale bar = 200μm). (c) Graphs showing mean (±SEM) number of Fos-immunoreactive (Fos-IR) nuclei in the SCN, BNSTov, CEA, BLA, DG and DMH as a function of ZT in AL, RF and RT rats (n=3-4/group). Vertical dotted rectangles inside the graphs indicate the time of Ensure presentation (ZT4-6). Asterisks indicate significant difference from corresponding AL group (Student-Newman-Keuls, $p<.05$).

Fig. 5

Ensure presentation for the first time does not affect PER2 expression but it increases Fos expression in all areas (except the SCN) in food-deprived (24hr; RF) and free-fed (RT) rats. (a) Brain maps showing location of regions under study. The dotted square in each map indicates the area scanned for quantification of Fos and PER2 immunoreactivity. (b) Graphs showing mean (±SEM) number of PER2-immunoreactive (PER2-IR) nuclei in the SCN, BNSTov, CEA, BLA, DG and DMH as a function of ZT in control (AL), RF and RT rats (n=4/group). (c) Graphs showing mean (±SEM) number of Fos-immunoreactive (Fos-IR) nuclei in the SCN, BNSTov, CEA, BLA, DG and DMH as a function of ZT in AL, RF and RT rats (n=4/group). Dotted rectangles inside the graphs indicate the time of Ensure presentation (ZT4-6). Asterisks indicate significant difference from corresponding AL group (Student-Newman-Keuls, $p<.05$).

Table 1

Results from ANOVAs carried out to assess the effect of feeding schedule and time of day on PER2 expression in each brain area under study

Brain area	Group	Time of day	Group x Time
SCN	F(2, 54)=0.68 p=.5	F(5, 54)= 91.40 p<0001	F(10, 54)= 1.18 p=.3
BNSTov	F(2, 54)= 3.65 p=.03	F(5, 54)= 30.68 p<0001	F(10, 54)= 5.85 p<.0001
CEA	F(2, 54)= 17.31 p<0001	F(5, 54)= 27.70 p<0001	F(10, 54)= 7.59 p<.0001
BLA	F(2, 54)= 2.02 p=.14	F(5, 54)= 8.24 p<0001	F(10, 54)= 5.00 p<.0001
DG	F(2, 54)= 34.71 p<0001	F(5, 54)= 34.52 p<0001	F(10, 54)= 11.53 p<.0001
DMH	F(2, 54)= 248.61 p<0001	F(5, 54)= 4.18 p=.002	F(10, 54)= 5.36 p<.0001

Figure 1

Click here to download high resolution image

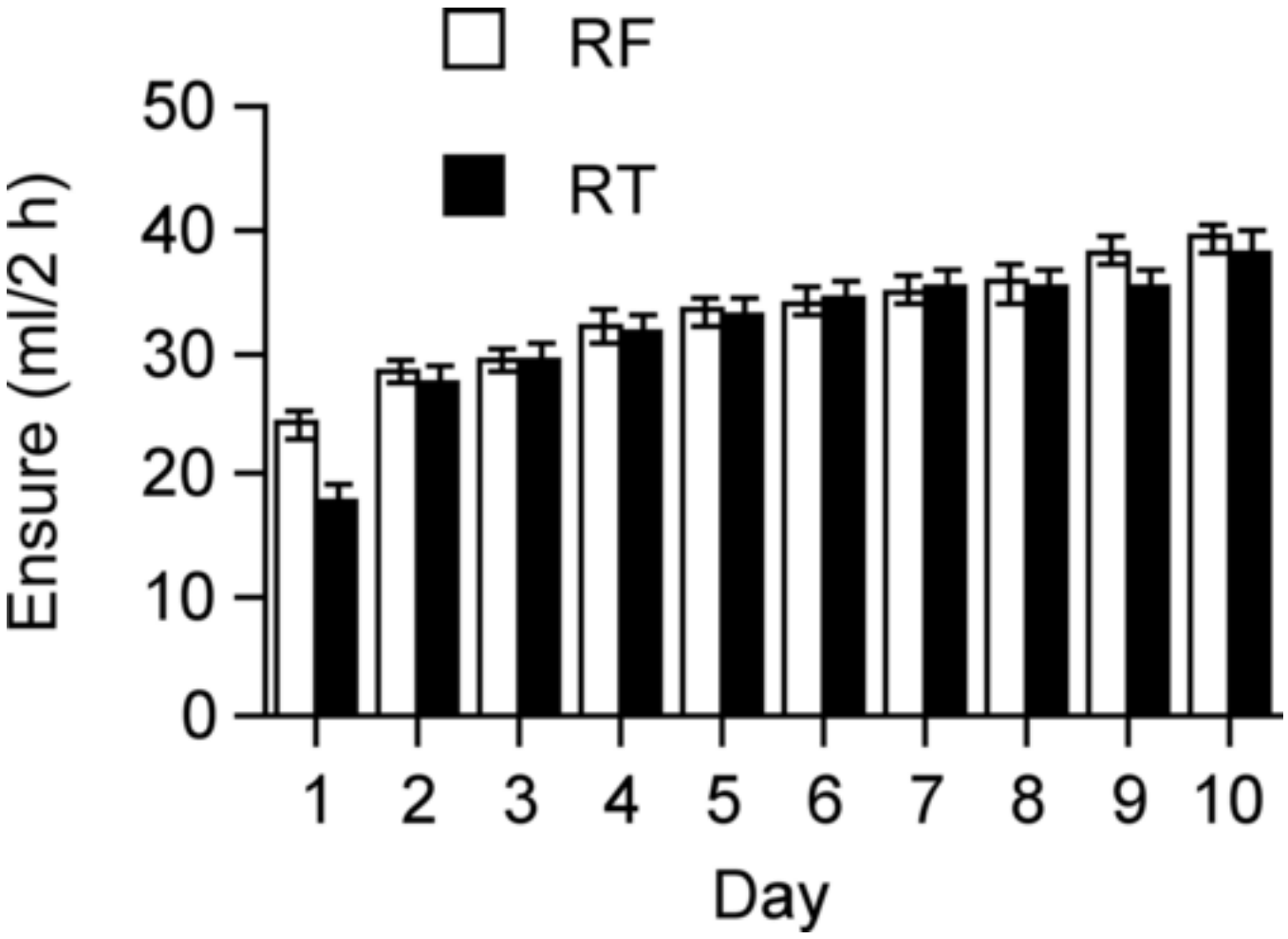

Figure 2

Click here to download high resolution image

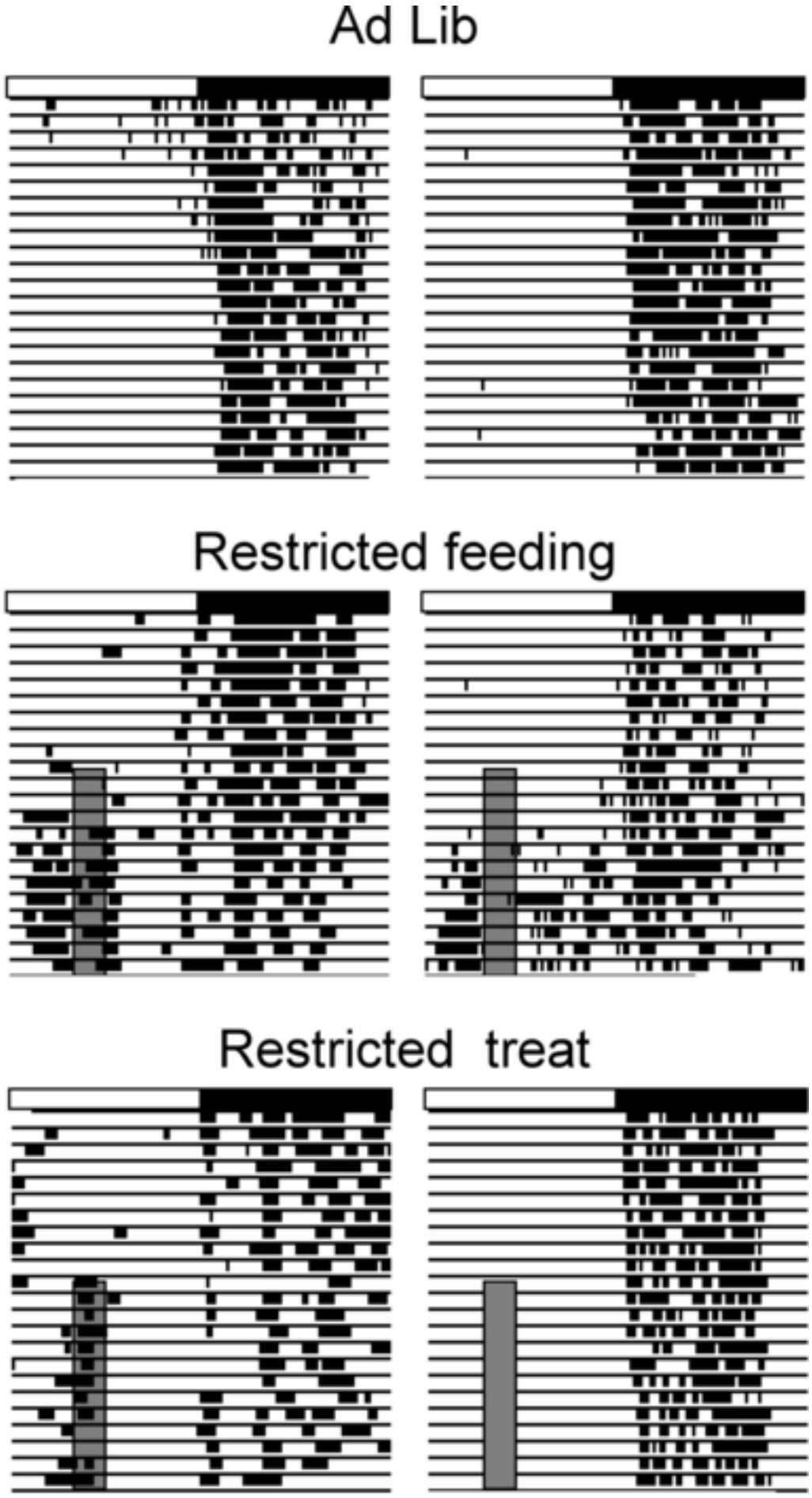

Figure 3

Click here to download high resolution image

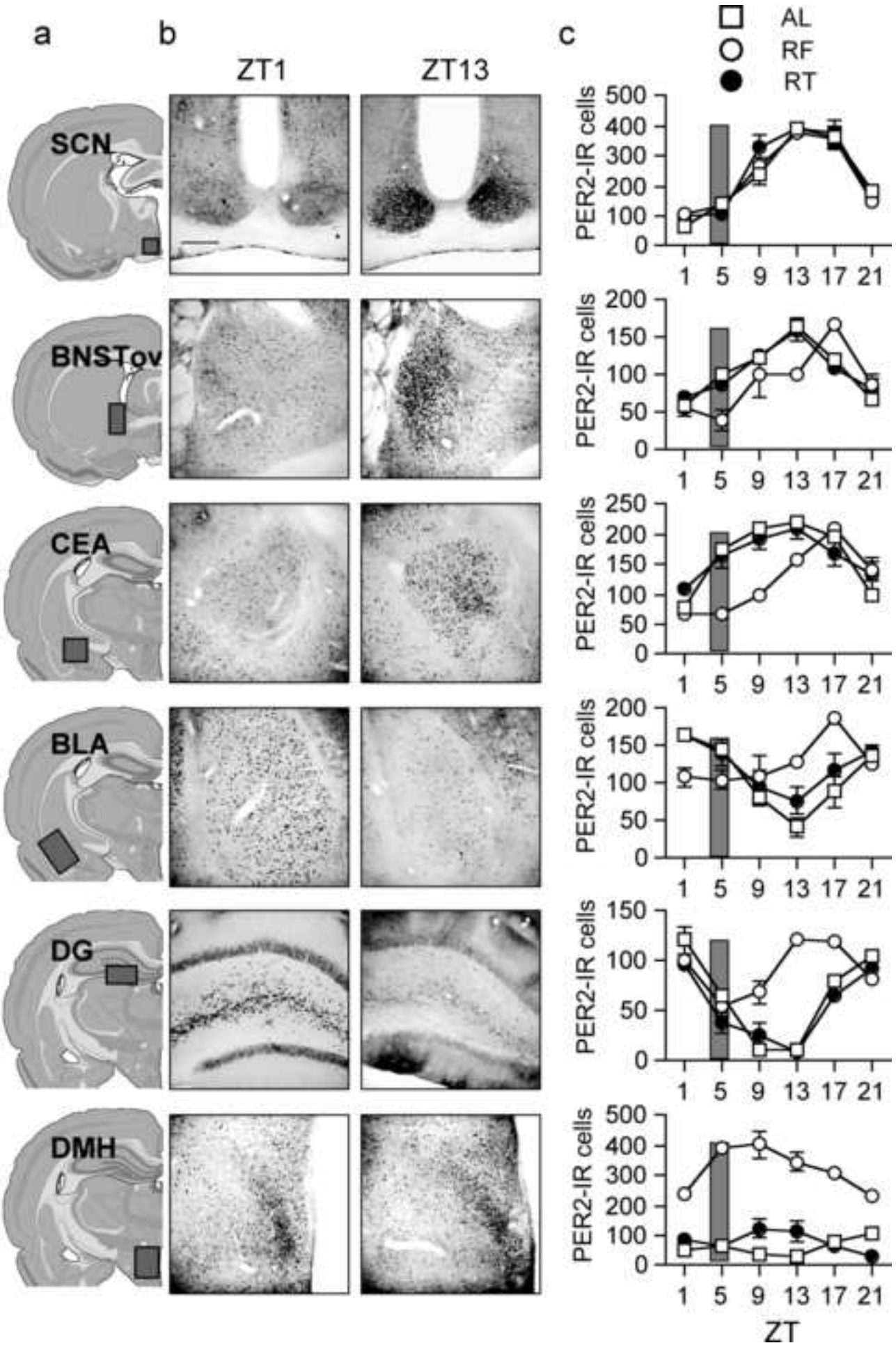

Figure 4

Click here to download high resolution image

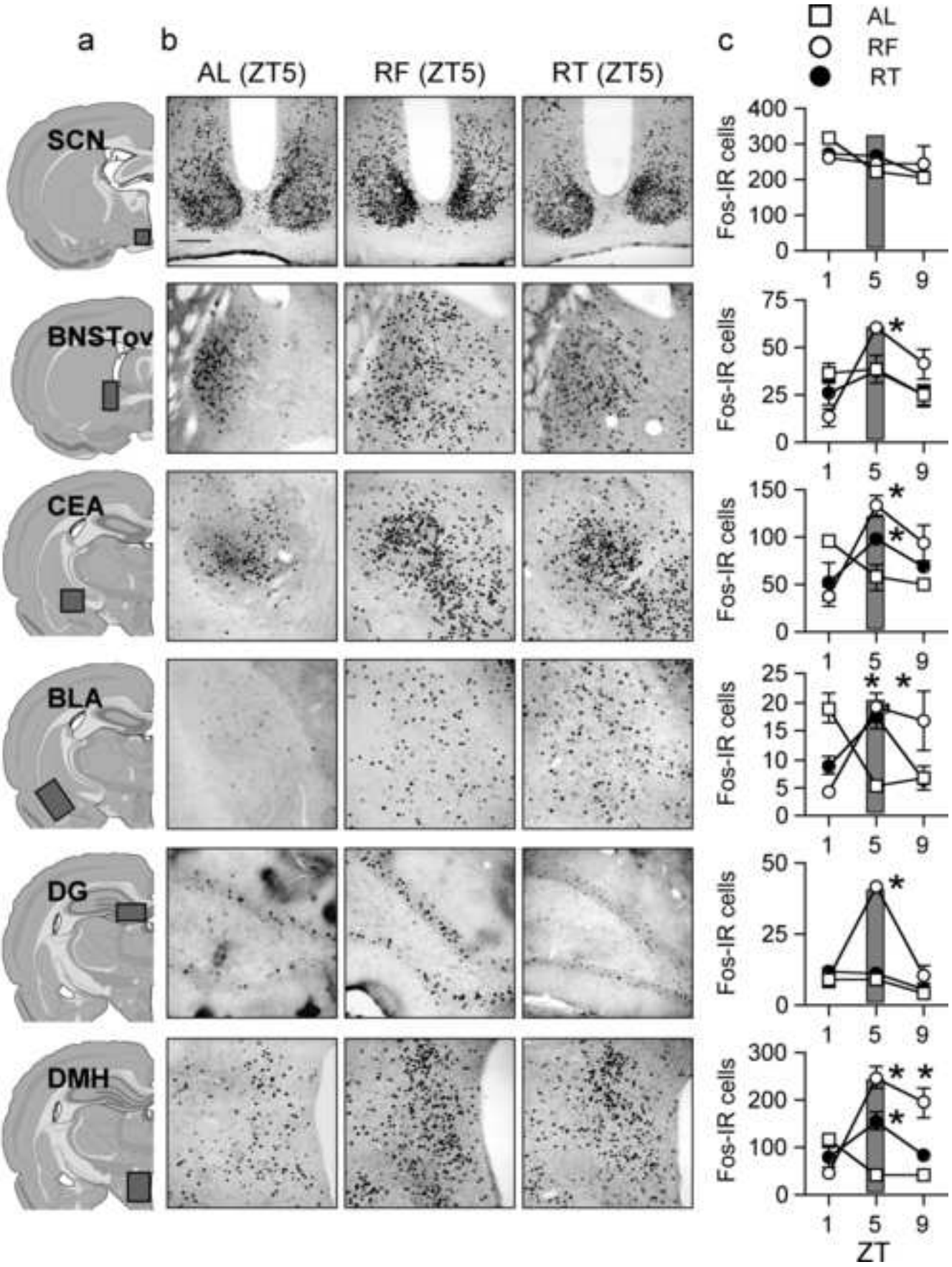

Figure 5

Click here to download high resolution image