Circadian oscillators in the central extended amygdala are selectively sensitive to restricted feeding but not similarly restricted treats

Michael A. Verwey

A Thesis

in

The Department

of

Psychology

Presented in Partial Fulfillment of the Requirements for

the Degree of Master of Arts (Psychology) at

Concordia University

Montreal, Quebec, Canada

January, 2006

Your file Votre référence
ISBN: 0-494-14204-9
Our file Notre référence
ISBN: 0-494-14204-9

ABSTRACT

Circadian oscillators in the central extended
amygdala are selectively sensitive to restricted feeding
but not similarly restricted treats

Michael A. Verwey

When food-access is restricted to a consistent time of day (restricted feeding), rodents reorganize many circadian behaviors around the mealtime. The suprachiasmatic nucleus (SCN) is the master circadian pacemaker, while the oval nucleus of the bed nucleus of the stria terminalis (BNST-OV) and the central nucleus of the amygdala (CEA) are important in stress, motivation and feeding. All these regions express the 'clock protein' Period2 (PER2) with a circadian rhythm. Restricted feeding changes PER2 expression patterns in the BNST-OV and CEA, but not in the SCN. To test the relative importance of motivational and homeostatic variables on PER2 expression in the BNST-OV and CEA, we contrasted daily restricted feeding (containing homeostatic and motivational components) with daily restricted treats (no weight loss, a motivational challenge only). Restricted feeding was comprised of fasted animals receiving repeated daily access to chocolate Ensure, whereas restricted treats consisted of sated animals receiving similar Ensure-access. In restricted feeding groups, the daily pattern of running wheel activity was consistently reorganized. However, in restricted treat groups, behavioral reorganization was more variable. Daily oscillations of PER2 expression in the BNST-OV and CEA were altered only under restricted-feeding, but not restricted-treat conditions. In contrast, cFOS expression in both the BNST-OV and CEA was

enhanced in response to Ensure-access regardless of whether it was presented to fasted or sated rats. These findings suggest that some aspect of the homeostatic challenge associated with restricted feeding is important for shifting circadian PER2 expression in these areas of the central extended amygdala.

Acknowledgements

First and foremost, I would like to thank my thesis supervisor, Dr. Shimon Amir for his guidance and support throughout this work.

I would also like to thank Dr. Uri Shalev and Dr. Jane Stewart for all their valuable feedback on the writing of this thesis.

Thanks to Dr. Martin Ralph and his graduate student (now Dr.) Sean Cain for encouraging my interest in circadian rhythms.

To Barry Robinson and all members of the Amir-Lab (past and present) who made this work enjoyable and possible, thank you. Also, financial support from the Canadian Institutes of Health Research was greatly appreciated.

Finally, to my family and Sabine thank you for your unconditional love, friendship and "cheering-on" throughout this experience.
This would have been impossible without you.

Table of Contents

List of Figures

Introduction

The patterns of behavior and metabolic activity in cyanobacteria, plants, flies, rodents and humans are often circadian in nature (Bell-Pedersen et al. 2005). In mammals, the suprachiasmatic nucleus (SCN) is the master circadian pacemaker (Abe et al. 1979; Ralph et al. 1990). Biological clocks require a mechanism to generate circadian rhythms; cells within the SCN rely on protein autoregulatory feedback loops. These feedback loops are comprised of several 'clock genes' and their protein products, including *period* (*1,2,3*), *cryptochrome* (*1, 2*), *bmal1*, *clock*, and *reverb-α* (Reppert and Weaver, 2002). In addition, experiments that monitor the expression of clock genes and their protein products (e.g. *period2*) have revealed circadian oscillations in non-SCN tissues of the brain (Wakamatsu et al. 2001; Amir et al. 2004; Fukuhara et al. 2005; Lamont et al. 2005a) and periphery (Damiola et al. 2000; Challet et al. 2003; Yoo et al. 2004; Ishida et al. 2005). Moreover, many aspects of behavior and metabolism regulated by these non-SCN structures also display circadian changes. Daily clock protein oscillations within these structures raise the possibility that circadian regulation of these behaviors could be generated locally.

The oval nucleus of the bed nucleus of the stria terminalis (BNST-OV) and the central nucleus of the amygdala (CEA) perform many important functions, including roles in feeding and other motivated behaviors. The BNST-OV has been implicated in endocrine and behavioral responses to drugs of abuse (Erb et al. 1999, 2001; Leri et al. 2002; Colusi-Mas et al. 2005), stress (Day et al. 1999, 2005; Kozicz, 2002) and feeding (Ciccocioppo et al. 2003). Furthermore, binge eating can be induced in sated rats by injection of opioids into the amygdala (Stanley et al. 1988), and the CEA is important in

mediating this behavior (Will et al. 2004). Recently, the BNST-OV and CEA have also been shown to exhibit circadian oscillations of the clock protein *period2* (PER2; Amir et al. 2004; Lamont et al. 2005a). These circadian PER2 oscillations depend on the SCN (Amir et al. 2004) as well as adrenal hormones (Amir et al. 2004; Lamont et al. 2005a; Segall et al. 2005). As such, local clock protein expression in these areas seems to be regulated by both SCN-driven circadian rhythms and by peripheral signals that are responsive to environmental influence.

Restricted feeding is a regimen where animals receive access to food for a limited period of time each day. After several days, rats develop a food 'anticipatory' wheel running bout in addition to normal nocturnal wheel running (Richter, 1922). Many peripheral tissues (Damiola et al. 2000; Le Minh et al. 2001; Stokkan et al. 2001; Davidson et al. 2003), and some brain regions (Wakmatsu et al. 2001) have been shown to exhibit altered clock protein oscillations under restricted feeding. Daily PER2 expression profiles in the BNST-OV and CEA are also modified under restricted feeding (Lamont et al. 2004, 2005b). Restricted feeding involves both motivational and homeostatic changes that contribute (perhaps not equally) to the reorganization of behavior and clock protein expression. If rats have continued access to *ad libitum* rat chow and are provided daily restricted treats, 'anticipatory' wheel running develops that is similar to that observed under restricted feeding (Mistlberger and Rusak, 1987; Mendoza et al. 2005a). Here, restricted feeding was comprised of fasted animals receiving repeated daily access to a highly palatable meal (chocolate Ensure). Restricted treats consisted of sated animals receiving similar repeated daily access to Ensure. These feeding challenges were used to test whether changes in motivational state (in the

absence of homeostatic challenge) would be sufficient to affect PER2 expression rhythms in BNST-OV and CEA.

In Experiment 1, the effects of daytime restricted feeding and daytime restricted treats on PER2 immunoreactivity in the SCN, BNST-OV and CEA were assessed. This experiment was designed to test if shifts in PER2 expression could be observed in rats given restricted treats, without the associated weight loss or homeostatic challenge of daytime restricted feeding. In Experiment 2, the effects of nighttime (instead of daytime) restricted feeding and nighttime restricted treats on PER2 expression were studied. Nocturnal rodents such as the rat normally confine their feeding to the dark period of the circadian cycle. Nighttime restricted feeding can be argued to be a more natural, or less disruptive, feeding restriction. Consistent with previous work, PER2 expression in the SCN was unaffected by restricted feeding or restricted treats (Lamont et al. 2004; Mendoza et al 2005b). In contrast, PER2 expression patterns in the BNST-OV and CEA were selectively sensitive to restricted feeding and unaffected by restricted treats.

In addition to PER2 expression, Experiments 1 and 2 also assessed the effects of restricted feeding and restricted treats on cFOS expression in the SCN, BNST-OV and CEA. This tested for differences in the cFOS response to food (or feeding) that might depend on homeostatic factors. cFOS expression is a valuable marker of neuronal activity that has helped researchers describe the functional connectivity of the SCN with other regions of the brain (Rea 1989; Mead et al. 1992; Beaule et al. 2001). Furthermore, cFOS expression has been used extensively to describe local activations of the BNST-OV and CEA in response to various drug treatments and stressors (Day et al. 1999, 2001, 2005; Kozicz, 2002; Thompson and Rosen, 2006). In accord with previous findings, we

found cFOS expression in the SCN was unaffected by restricted feeding or restricted treats (Angeles-Castellanos, 2004). In contrast, cFOS expression in the BNST-OV and CEA was similarly affected (after Ensure presentation) regardless of whether it was presented under conditions of restricted feeding or restricted treat.

Materials and Methods

Animals and housing - A total of 168 male, Wistar rats (~ 225-250 g; Charles River Laboratories, St. Constant, Quebec, Canada) were used. Rats were individually housed in sound-attenuated, light tight, ventilated boxes with free access to running wheels. Running wheel activity was continuously recorded by computer (Vitalview – Minimitter, OR, USA) and analyzed with Circadia software. Rats had free access to Purina rat chow in all pre-experimental stages; food availability during each experiment will be described below. Water was freely available at all stages.

Animals were housed in a 12:12 light-dark schedules for at least 2 weeks prior to all experimental stages. Lights-on was defined as Zeitgeber time (ZT) 0 and animals were perfused at one of six timepoints across the day (ZT 1, 5, 9, 13, 17, 21). All experiments respected the guidelines set out by the Canadian Council on Animal Care and were approved by the Animal Care Committee at Concordia University.

Restricted feeding and restricted treats – Animals were assigned to restricted feeding, restricted treat or *ad libitum* rat chow groups. Restricted feeding groups had no access to rat chow for 10 days and during this period were fed exclusively with unlimited Chocolate Ensure Plus for 2 hours each day. Restricted treat groups had continued *ad libitum* access to rat chow and received a similarly restricted access to chocolate Ensure for 13 days. Finally, *ad libitum* control groups received no Ensure access and were fed exclusively on rat chow. An anti-phase entrainment method was used to test whether time of feeding (day or night) was an important factor in these feeding challenges. Daytime groups received unlimited Ensure access from ZT 4 – 6. Nighttime groups received unlimited Ensure access from ZT 16 – 18 (twelve hours anti-phase to daytime

presentations). Experiment 1 assessed PER2 and cFOS expression profiles across the day in control, daytime restricted feeding (Ensure: ZT 4-6) and daytime restricted treat (Ensure: ZT 4-6) groups. Experiment 2 assessed PER2 and cFOS expression profiles across the day in control, nighttime restricted feeding (Ensure: ZT 16-18) and nighttime restricted treat (Ensure: ZT 16-18) groups. Experiment 3 assessed PER2 and cFOS expression profiles before (ZT 1), during (ZT 5) and after (ZT 9) the first daytime Ensure-presentation (ZT4-6). In experiment 3 'restricted feeding'-like rats were fasted beginning at ZT 6 the previous day, while 'restricted treat'-like rats had continued access to *ad libitum* rat chow. Experiment 4 assessed PER2 and cFOS expression profiles before (ZT 13), during (ZT 17) and after (ZT 21) the first nighttime Ensure-presentation (ZT 16-18). In experiment 4 'restricted feeding'-like rats were fasted beginning at ZT 18 the previous day, while 'restricted treat'-like rats had continued access to *ad libitum* rat chow.

Tissue preparation – Animals were deeply anaesthetized with an intraperitoneal dose (~ 100 mg/kg) of sodium pentabarbitol (Somnotol). The descending aorta was clamped and the upper body was perfused transcardially with cold physiological saline (9g/L NaCl in distilled water, ~300 ml) followed by cold 4% paraformaldehyde solution (40g paraformaldehyde/1L 0.1 M Phosphate Buffer, ~300 ml). Brains were post-fixed in 4% paraformaldehyde solution for 24 hours before slicing. Serial coronal brain sections (50 μm) were taken on a Vibratome (Leica) in 50 mM trizma buffered saline (TBS ~ pH 7.6). Alternate sections were collected for either cFOS or PER2 staining. Before staining, all slices were washed in cold TBS and stored at –20°C in Watsons

cryoprotectant solution (30% sucrose, 0.1% Polyvinylpyrrolidone, 30% Ethylene Glycol, in 0.1M sodium phosphate buffer pH ~7.2; Watson et al. 1986).

Immunocytochemistry – Sections were removed from Watsons cryoprotectant and were rinsed in cold TBS. A 30 minute quench phase in 3% hydrogen peroxide (in TBS) was followed by another set of rinses in cold TBS. Then a 1 hour pre-block of 5% normal goat serum (NGS) in a 0.3% triton trizma buffered saline (TTBS) solution at 4°C followed. Tissue was incubated for 48 hours with the primary antibody in TTBS at 4°C. Sections stained for cFOS were incubated with a polyclonal cFOS antibody (1° Oncogene Sciences, Boston, MA) at a concentration of 1:100 000. Sections stained for PER2 were incubated with a polyclonal PER primary antibody (ADI – San Antonio, TX) at a concentration of 1:800. Following incubation with the primary antibody, sections were rinsed in cold TBS and were incubated for 1 hour at 4°C in a solution of biotinylated anti-rabbit made in goat (Vector Labs, Burlingame, California) in TTBS (1:200). Tissue was rinsed in cold TBS and was incubated for 2 hours at 4°C in a tertiary avidin-biotin complex solution (Vectastain Elite ABC Kit; Vector Labs) in TBS. Next, tissue was rinsed in TBS and then rinsed in trizma buffer (50mM HCl ~ pH 7.6, 10 minutes). Tissue was incubated on an orbital shaker, in a 0.05% 3, 3'diaminobenzidine (DAB) trizma buffered solution for 10 minutes at room temperature. This was followed immediately by another 10 minute incubation in 0.05% DAB trizma buffer solution with 8% $NiCl_2$, and 0.01% H_2O_2. Sections were rinsed and wet-mounted (in TBS) onto gelatin-coated slides, dehydrated through serial alcohol treatments, soaked in Citrosolv (Fisher Scientific, Houston, TX) and coverslipped with Permount (Fisher Scientific, Houston, TX).

Data Analysis – Slides were inspected under a light microscope with the 20X objective and digital images (400μm by 400μm) were captured using a Sony XC-77 camera, and NIH image (v1.63) software. Between 9 and 13 pictures were captured bilaterally for each BNST-OV, CEA and SCN. Immunopositive cells were counted using NIH image; data from both right and left hemispheres were pooled. The 5 images with the highest counts were averaged to represent the data point for each subject.

Statistical Analysis – Measures of Ensure consumption were taken within subjects. Each rat only experienced a single feeding regimen; therefore, group differences were all tested between subjects. Least significant difference pairwise comparisons were used to contrast Ensure consumptions between groups within a single day.

Measures of PER2 and cFOS immunoreactivity were analyzed using standard analyses of variance (ANOVA - SPSS 11). Analyses for each structure (SCN, BNST-OV and CEA) were performed separately. Two main factors were considered in each analysis: Feeding group and time. Feeding group in all experiments had 3 main levels: Control, restricted feeding and restricted treat. In experiments 1 and 2, time had 6 levels, evenly distributed across the day: ZT 1, 5, 9, 13, 17, and 21. In experiment 3, time had 3 levels: ZT 1, 5 and 9. In experiment 4, time had 3 levels: ZT 13, 17, and 21. All immunocytochemistry counts were analyzed between subjects. If there was a significant interaction between feeding condition and time, variables were tested individually with simple effects ANOVAs. Within a timepoint, least significant difference pairwise comparison tests were used to compare PER2 or cFOS levels between groups.

Results

Experiment 1 – Daytime Restricted Feeding and Treats

Daily presentations of Ensure (ZT 4-6) to restricted-feeding and restricted-treat groups led all rats to engorge themselves during a discrete and consistent time each day. On the tenth presentation of Ensure, rats in both groups ate approximately 35ml of Ensure per 2-hour session. This experiment studied the effects of daily Ensure presentations on running wheel activity rhythms, and rhythms of PER2 and cFOS expression in SCN, BNST-OV and CEA.

1.1 Ensure consumption and Running wheel activity

Rats in restricted-feeding groups showed consistent changes of running wheel patterns and developed an 'anticipatory' running wheel bout which began 2-3 hours prior to food presentation (Figure 1a). Some rats receiving daytime restricted treats showed similar reorganization of running wheel activity (Figure 1b) whereas others failed to develop treat-'anticipatory' running (Figure 1c) and circadian running patterns resembled *ad libitum* control animals (Figure 1d). With the exception of day 1 when the restricted feeding group ate more Ensure than the restricted treat group ($p=0.001$), all rats ate similar amounts of Ensure during the access period (Figure 2a).

1.2 PER2 expression profiles

Representative photomicrographs showing circadian PER2 expression in the SCN, BNST-OV, and CEA of *ad libitum* controls can be seen in Figure 3. PER2 expression profiles for all feeding groups across the 24 hour period are shown in Figure

4a. In the SCN, profiles were similar in all feeding conditions (Figure 4ai), but as expected, levels changed significantly with time of day ($ANOVA_{TIME}$: $F_{5, 51}$= 103.851, $p<0.001$; Appendix A). Consistent with previous results (Lamont et al. 2004) we found that daytime restricted feeding shifted peak PER2 expression in the BNST-OV (Figure 4aii) and CEA (Figure 4aiii) later than that seen in *ad libitum* controls. PER2 expression normally peaked around ZT 13, but under daytime restricted feeding this peak was shifted to ZT 17. Ensure-consumption was similar in restricted-feeding and restricted-treat groups, but rats receiving restricted treats showed no differences in PER2 expression at any time when compared to controls. In the BNST-OV (Figure 4aii) and CEA (Figure 4aiii), PER2 expression in daytime restricted treat groups continued to peak at ZT 13 as it did in the control groups. Overall, there were significant interactions between feeding group and time in the BNST-OV (Figure 4aii; $ANOVA_{TIMExGROUP}$: $F_{10,53}$= 7.555; $p<0.001$; Appendix A) and CEA (Figure 4aiii; $ANOVA_{TIMExGROUP}$: $F_{10,53}$=11.377; $p<0.001$; Appendix A). This experiment suggests an important role for the homeostatic challenge of restricted feeding in the modification of PER2 oscillations in the BNST-OV and CEA.

1.3 cFOS expression profiles

Figure 5 shows representative photomicrographs of cFOS immunoreactivity one hour after Ensure-access began (ZT 5; a: Control, b: Restricted feeding and c: Restricted treat). It can be seen that cFOS expression in the CEA increased in all groups that were fed between ZT 4-6. No differences between groups were seen (at ZT 5) in either the SCN or BNST-OV.

cFOS expression over the day is graphed in Figure 4b for the SCN, BNST-OV and CEA. In the SCN, similar cFOS expression profiles were observed in all groups; as expected, cFOS expression changed with time (Figure 4bi; $ANOVA_{TIME}$: $F_{5,53} = 121.112$; $p < 0.001$; Appendix B). In the BNST-OV (Figure 4bii) however, each feeding group had a unique profile of cFOS expression. Specifically, each feeding condition showed significant variation in cFOS expression across the day, but the daily trends were different ($ANOVA_{TIMExGROUP}$: $F_{10,53} = 2.669$; $p = 0.01$; Appendix B). In the CEA, cFOS staining (Figure 4biii) also showed a significant interaction between feeding group and time ($ANOVA_{TIMExGROUP}$; $F_{10,53} = 8.738$; $p < 0.001$; Appendix B). In the CEA, higher cFOS expression was observed at the time of Ensure presentation (ZT 5) in both restricted feeding and restricted treat groups ($p<0.001$) as compared to rats that received no Ensure. CEA cFOS expression remained higher than controls for at least 3 hours post-Ensure (ZT 9) in restricted-feeding ($p<0.001$) and restricted-treat ($p=0.002$) groups. During the night (ZT 17), rats in the daytime restricted-feeding group (fasted rats) had lower cFOS levels in the CEA than those seen in either restricted treat ($p=0.016$) or *ad libitum* control ($p=0.037$) groups.

Experiment 2 – Nighttime restricted feeding and treats

Nighttime restricted feeding and nighttime restricted treats (Ensure: ZT 16-18) were used to test whether the Ensure-associated effects seen in Experiment 1 were independent of time of day. Experiment 1.2 showed that circadian PER2 expression patterns in the BNST-OV and CEA were altered by daytime restricted feeding. Here, nighttime restricted treat and nighttime restricted feeding paradigms were used to test if

these effects were dependent on Ensure presentation time. In Experiment 1.3, the effects of daytime-Ensure on cFOS levels in the BNST-OV were unclear because of high daytime levels observed in *ad libitum* control animals (Figure 4bii). A nighttime meal would help to better describe the cFOS response in the BNST-OV as cFOS expression in *ad libitum* control animals is comparatively low during the night.

2.1 Ensure consumption and Running wheel activity

Running wheel recordings show that restricted feeding rats consistently stopped using the running wheel while Ensure was available (ZT 16-18), and remained off the running wheels for 1-2 hours post-meal (Figure 6a). Most rats receiving restricted treats showed similar reorganization of running wheel use (Figure 6b). Some rats receiving restricted treats did not alter running wheel use (Figure 6c) and resembled *ad libitum* controls (Figure 6d). Restricted-feeding and restricted-treat groups ate similar amounts of Ensure on the first day, but on days 2 through 10 the restricted treat group ate more Ensure than the restricted feeding group (Figure 2b; $p<0.001$). Comparing these values to Experiment 1, all rats under restricted feeding ate similar amounts of Ensure on days 2 through 10 regardless of whether the Ensure was presented during daytime (ZT 4-6) or nighttime (ZT 16-18) hours. However, nighttime restricted treat animals ate more Ensure than daytime restricted treat animals (and day or night restricted feeding groups).

2.2 PER2 expression profiles

In the SCN, feeding group had no effect on daily rhythms of PER2 expression (Figure 7ai; $ANOVA_{TIME}$: $F_{5,52}=85.358$, $p<0.001$; Appendix C). In the BNST-OV

(Figure 7aii) and CEA (Figure 7aiii), peaks in PER2 expression were observed 12 hours after Ensure presentation in the restricted feeding group only. This is consistent with the observed phase relationship in Experiment 1.2 and supports a hypothesis of PER2 entrainment by mealtimes in the BNST-OV and CEA. Significant interactions were found between feeding group and time in the BNST-OV (Figure 7bii; $ANOVA_{TIMExGROUP}$: $F_{10,54}$= 23.547; p<0.001; Appendix C) and CEA (Figure 7biii; $ANOVA_{TIMExGROUP}$: $F_{10,54}$= 23.747 ; p<0.001;Appendix C). At most timepoints, restricted treat PER2 expression in the BNST-OV and CEA was similar to *ad libitum* (no Ensure access) control groups.

2.3 cFOS expression profiles

All feeding groups exhibited similar circadian cFOS expression patterns in the SCN (Figure 7bi; $ANOVA_{TIME}$: $F_{5,54}$ = 111.968; p < 0.001; Appendix D). In contrast, the BNST-OV (Figure 7bii) exhibited a large increase in cFOS expression at the time of Ensure presentation (ZT 17) in restricted-treat (p=0.002) and restricted-feeding (p<0.001) groups as compared to controls. At ZT 1 and ZT 5, restricted-feeding and restricted-treat groups showed significantly lower cFOS levels than control animals in the BNST-OV (ZT 1: p<0.01 ; ZT 5: p<0.01). cFOS expression in the BNST-OV demonstrated a significant interaction between feeding group and time (Figure 7bii; $ANOVA_{TIMExGROUP}$: $F_{10,54}$ = 5.308; p < 0.001; Appendix D). The CEA (Figure 7biii) also demonstrated a significant interaction between feeding group and time ($ANOVA_{TIMExGROUP}$: $F_{10,54}$ = 6.960; p < 0.001; Appendix D). Similar to the BNST-OV, cFOS was significantly increased in the CEA at the time of feeding (ZT17) in restricted-treat (p<0.003) and

restricted-feeding ($p<0.003$) groups. Furthermore, this increase in cFOS expression over controls was sustained until ZT21 ($p<0.014$). At ZT 5, restricted-feeding and restricted-treat groups showed lower cFOS levels in the CEA when compared to controls ($p<0.02$).

Experiment 3 – Acute effects of Daytime Ensure-presentation

This experiment tested if acute fasting or refeeding might effect PER2 and cFOS expression in the SCN, BNST-OV and CEA. Fasted ("restricted feeding") or sated ("restricted treat") rats were tested before (ZT 1), during (ZT 5) and after (ZT 9) the first presentation of daytime Ensure (ZT 4-6). Running wheel use in this experiment was similar to control animals since there was not sufficient time for food entrained activity rhythms to develop. Furthermore, Ensure consumptions (data not shown) were consistent with levels observed on day 1 in Figure 2.

3.1 PER2 expression

PER2 immunocytochemistry showed similar variation with time in the SCN among all groups (Figure 8ai; $ANOVA_{TIME}$: $F_{2,27}= 155.953$; $p<0.001$; Appendix E). In contrast, PER2 expression in the BNST-OV and CEA tended to be lower in restricted feeding groups (Figure 8aii and Figure 8aiii) as compared to animals that had access to *ad libitum* rat chow (Control and Treat groups). For instance, at ZT 5, PER2 expression in the BNST-OV was significantly lower in the acute restricted feeding (fasted + Ensure) group when compared to *ad libitum* rat chow controls ($p=0.01$). At ZT9, BNST-OV PER2 expression was significantly lower in acute restricted feeding (fasted + Ensure) as compared to acute restricted treat animals ($p=0.006$). In the CEA, restricted feeding rats

displayed significantly lower PER2 expression in the CEA at ZT 5 than restricted treat rats ($p=0.013$). A significant main effect of group was found in the BNST-OV (Figure 8aii; $ANOVA_{GROUP}$: $F_{2,27}= 4.825$, $p=0.016$; Appendix E) and CEA (Figure 8aiii; $ANOVA_{GROUP}$: $F_{2,27}=4.920$, $p=0.015$; Appendix E). An exact role for fasting or refeeding in regulating PER2 expression in the BNST-OV and CEA is difficult to determine from this study.

3.2 cFOS expression

In the SCN, cFOS staining demonstrated similar changes with time among all feeding groups (Figure 8bi; $ANOVA_{TIME}$: $F_{2,27}= 30.192$; $p<0.001$; Appendix F). The BNST-OV displayed large increases in cFOS expression in restricted feeding and restricted treat groups over control animals at the time of Ensure presentation (ZT 5, $p<0.001$, Figure 8bii). Ensure-associated increases were sustained for several hours after Ensure (ZT 9, $p<0.001$, 3 hours post-Ensure). In the BNST-OV, a significant interaction was found in cFOS expression between feeding group and time (Figure 8bii; $ANOVA_{TIMExGROUP}$: $F_{4,27}= 3.339$; $p=0.024$; Appendix F). cFOS staining in the CEA also showed a large increase at the time of Ensure feeding (ZT 5, $p<0.001$, Figure 8biii), this increase was also sustained for several hours after Ensure (ZT 9, $p<0.001$, 3 hours post-Ensure). A significant interaction was found in the CEA between feeding group and time (Figure 8biii; $ANOVA_{TIMExGROUP}$: $F_{4,27}= 8.752$; $p<0.001$; Appendix F). There were no significant differences between groups at ZT 1 (Figure 8b) demonstrating that fasting alone does not have a significant effect on cFOS immunoreactivity in the BNST-OV or

CEA. Furthermore, Ensure-associated cFOS increases in the BNST-OV and CEA were similar regardless of whether the rats were fasted or sated.

Experiment 4 – Acute effects of Nighttime Ensure-presentation

This experiment was designed to test if acute fasting or refeeding might effect PER2 and cFOS expression in the SCN, BNST-OV and CEA. Fasted ("restricted feeding") or sated ("restricted treat") rats were tested before (ZT 13), during (ZT 17) and after (ZT 21) the first presentation of nighttime Ensure (ZT 16-18). Running wheel use in these acute experiments was similar to control animals since there was not sufficient time for food entrained activity rhythms to develop. Furthermore, Ensure consumptions (data not shown) were consistent with levels observed on day 1 in Figure 2.

4.1 PER2 expression

Similar changes in PER2 expression over time were observed in the SCN among all nighttime feeding groups (Figure 9ai; $ANOVA_{TIME}$: $F_{2,27}=137.900$; $p<0.001$; Appendix G). Fasting (19 hour fast ending ZT13) in the restricted feeding group caused a significant decrease of PER2 in the BNST-OV (Figure 9aii: $p<0.01$) and CEA (Figure 9aiii: $p<0.01$) as compared to restricted-treat and *ad libitum* control groups. Post-Ensure (ZT 21, 3 hours after Ensure) there was a significant increase in PER2 expression in both the BNST-OV (Figure 9aii: $p<0.01$) and CEA (Figure 9aiii: $p<0.01$) as compared to restricted-treat and *ad libitum* control groups. This resulted in a significant interaction between nighttime Ensure groups in both the BNST-OV (Figure 9aii; $ANOVA_{TIMExGROUP}$: $F_{4,27}= 7.599$; $p<0.001$; Appendix G) and CEA (Figure 9aiii;

$ANOVA_{TIMExGROUP}$: $F_{4,27}$= 18.321; p<0.001; Appendix G). These data show that feeding manipulations have effects on PER2 expression in the BNST-OV and CEA.

4.2 cFOS expression

cFOS staining in the SCN showed similar effects of time among all feeding groups (Figure 9bi; $ANOVA_{TIME}$: $F_{2,27}$= 73.190; p<0.001; Appendix H). Staining in the BNST-OV showed increased cFOS expression in restricted-feeding and restricted-treat groups over control animals at the time (ZT 17, p<0.001) of Ensure presentation (Figure 9bii). Ensure-associated increases were sustained for several hours after Ensure (ZT 21, p<0.001, 3 hours post-Ensure). A significant interaction was found in the BNST-OV between feeding group and time (Figure 9bii; $ANOVA_{TIMExGROUP}$: $F_{4,27}$=8.475; p<0.001; Appendix H). The CEA also showed a large increase in cFOS expression at the time of nighttime Ensure presentation (ZT 17, p<0.001; Figure 9biii) and this increase was sustained for several hours after Ensure removal (ZT21, p<0.001, 3 hours post-Ensure). A significant interaction was found in the CEA between feeding group and time (Figure 9biii; $ANOVA_{TIMExGROUP}$: $F_{4,27}$= 4.104; p=0.01; Appendix H). There were no significant differences between groups at ZT 13 (Figure 9b) demonstrating that fasting alone (as shown by the acute restricted feeding group) does not have a significant effect on cFOS immunoreactivity in the BNST-OV or CEA. Furthermore, Ensure-associated cFOS increases in the BNST-OV and CEA were similar regardless of whether the animal was fasted or sated.

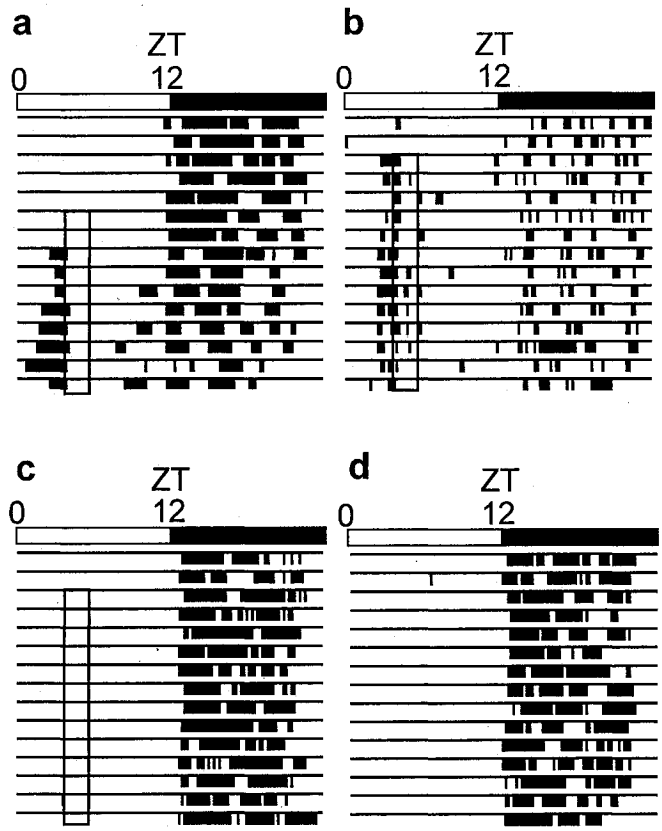

Figure 1: Characteristic running wheel records (Experiment 1). 12 hr light-dark cycles are marked above each record (ZT0 lights on; ZT12 lights off). Sequential days are arranged vertically and each horizontal line represents a single 24 hour time-period. (a) 'Anticipating' daytime restricted feeding (Ensure: ZT 4-6). (b) 'Anticipating' daytime restricted treats (Ensure: ZT 4-6) with continued access to *ad libitum* rat chow. (c) No 'anticipation' of daytime restricted treats (Ensure: ZT 4-6) with continued access to *ad libitum* rat chow. (d) *Ad libitum* rat chow only.

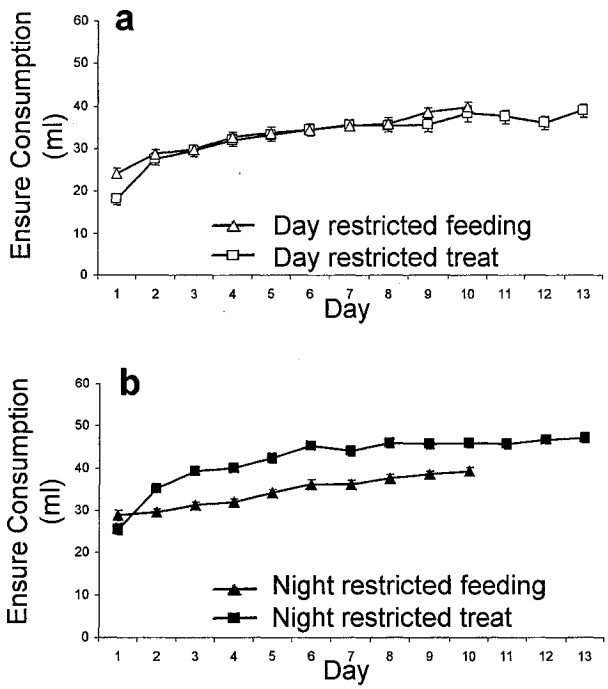

Figure 2: Daily consumption of Ensure (ml) in restricted feeding (no rat chow access) and restricted treat (*ad libitum* rat chow access) groups. Rats were housed in a 12 hour light-dark cycle (ZT0 lights on; ZT12 lights off). (a) Daytime Ensure presentations (ZT 4-6) (b) Nighttime Ensure presentations (ZT16-18)

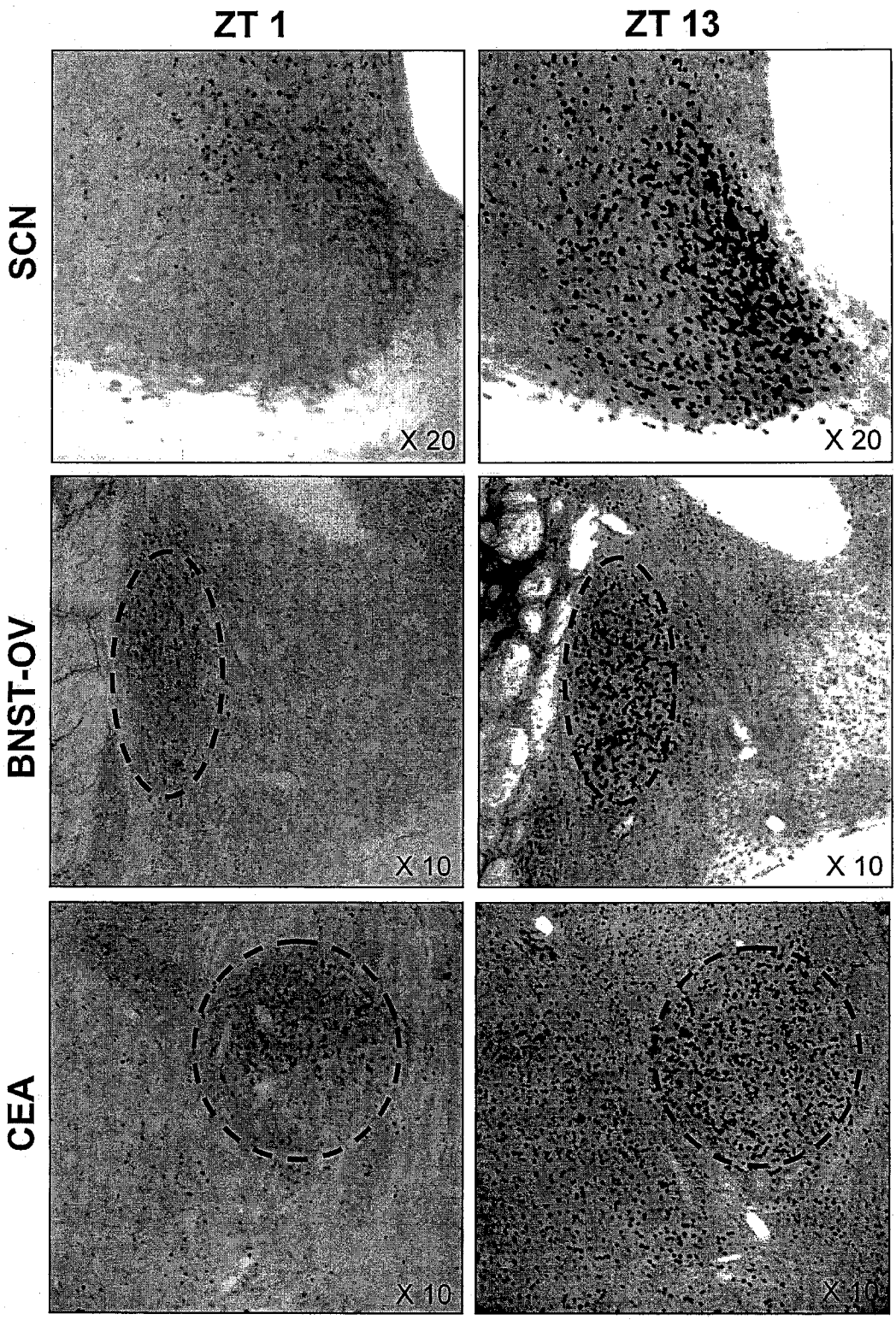

Figure 3: Characteristic photomicrographs of daily PER2 oscillations in the SCN, BNST-OV and CEA of freely fed rats housed in a 12:12 light-dark cycle (ZT 0 = Lights on, ZT 12 = Lights off). Minimum (ZT 1) and maximum (ZT 13) PER2 expression times are shown.

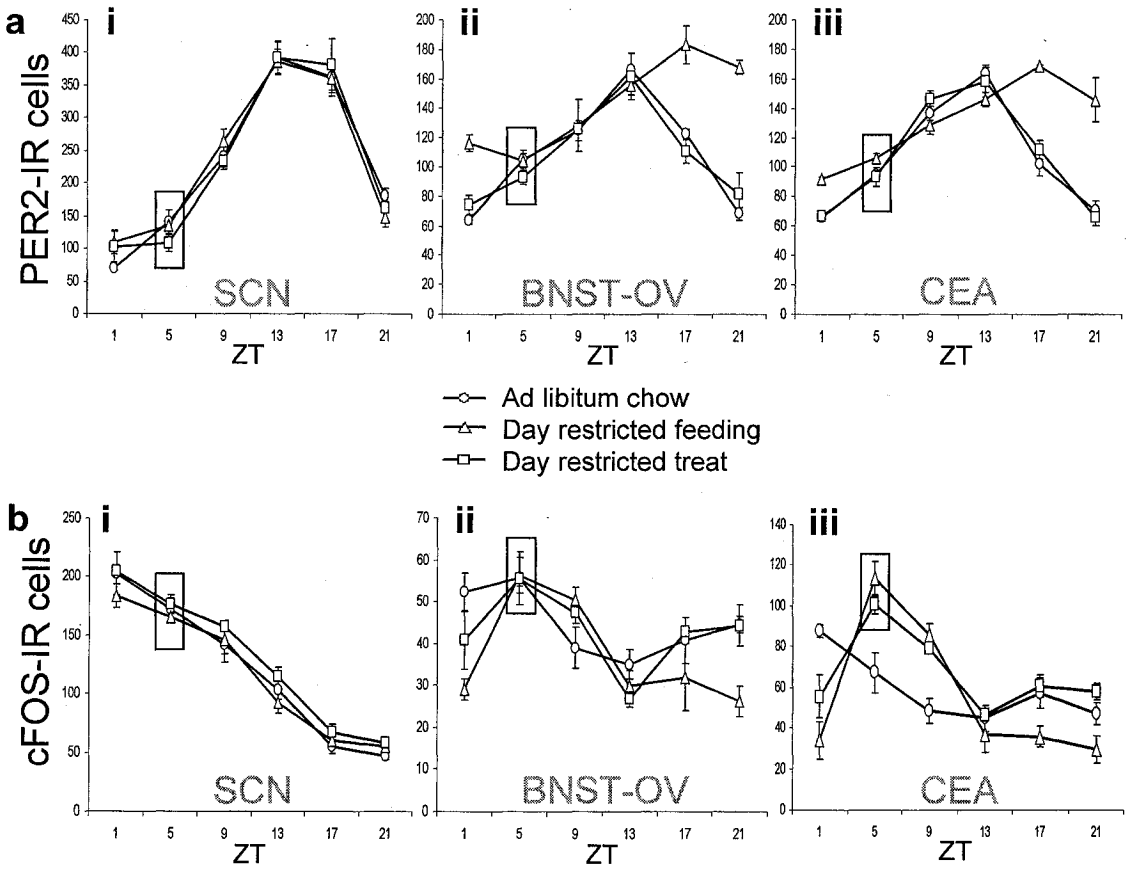

Figure 4: Profiles of cFOS and PER2 expression (Experiment 1) in the SCN, BNST-OV and CEA under control, daytime restricted feeding or daytime restricted treat conditions (Daytime Ensure access marked with open rectangles). (A) Mean ± SEM number of PER2 immunoreactive (PER2-IR) cells across the day (B) Mean ± SEM number of cFOS immunoreactive (cFOS-IR) cells across the day. Rats were perfused (n=4) at each zeitgeber time (ZT 1, 5, 9, 13, 17, or 21) under a 12:12 light-dark cycle (ZT0 lights on; ZT12 lights off).

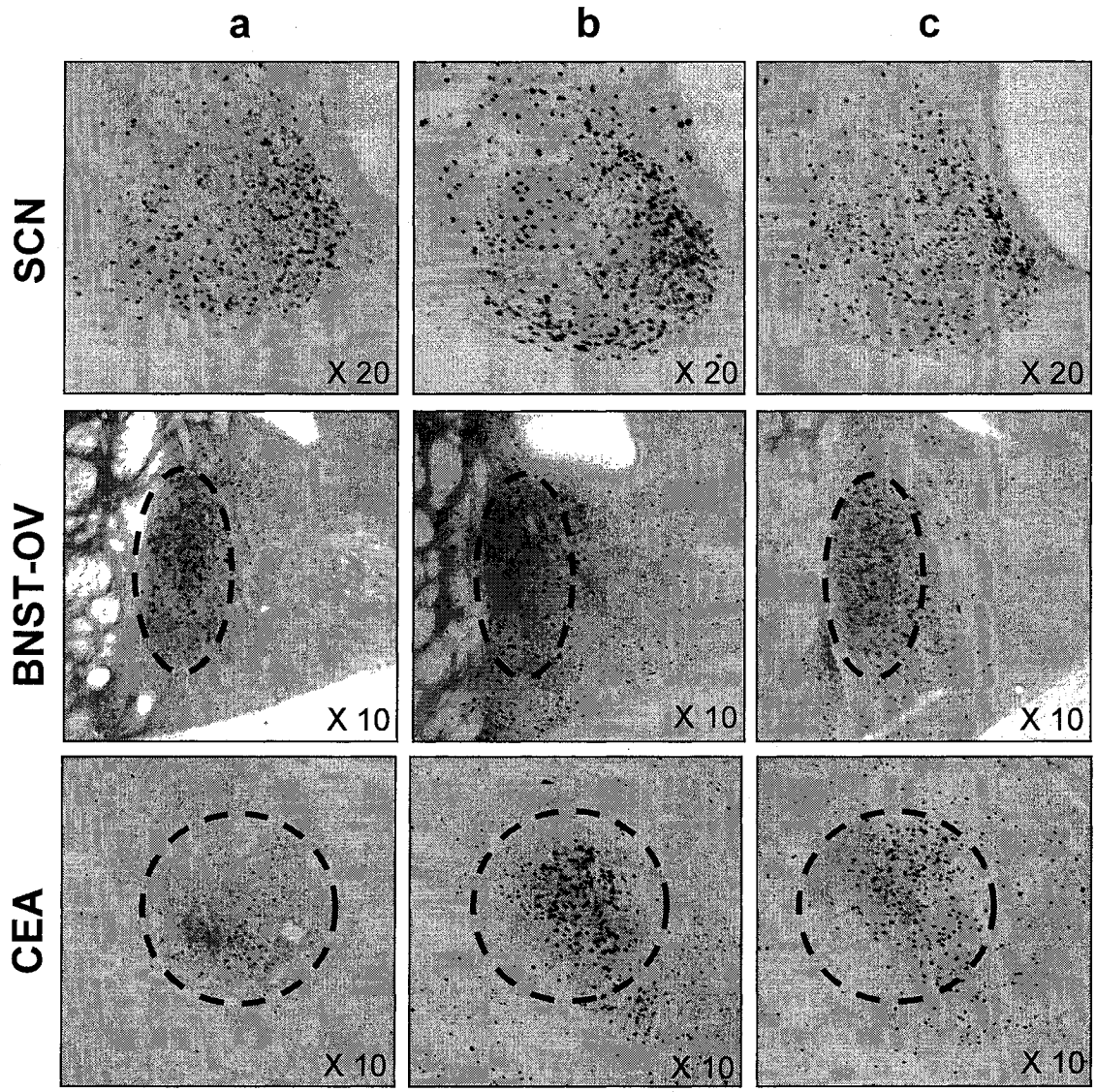

Figure 5: Characteristic photomicrographs of cFOS expression in the SCN, BNST-OV and CEA at ZT 5. (a) *Ad libitum* rat chow controls (b) Daytime restricted feeding group (1 hour after Ensure-presentation began) (c) Daytime restricted treat group (1 hour after Ensure-presentation began).

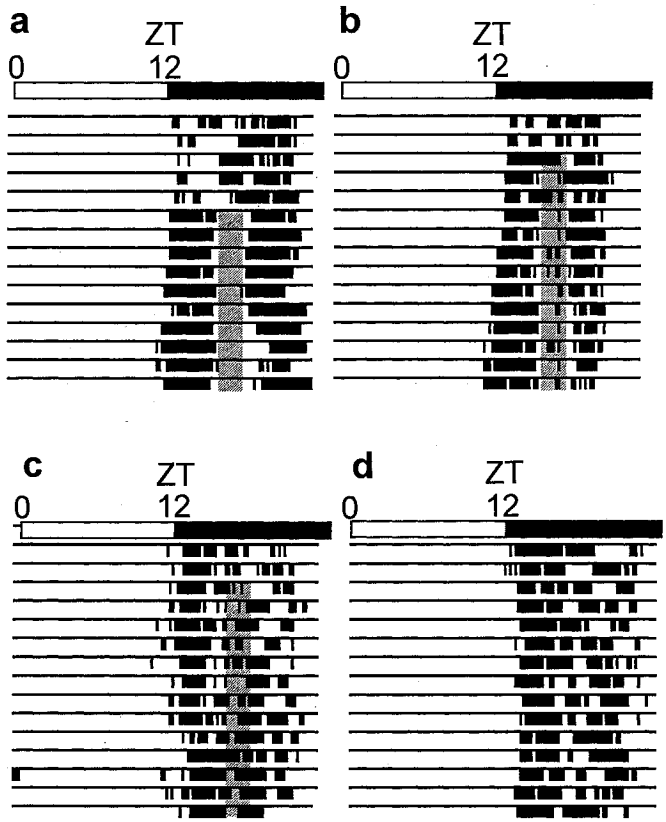

Figure 6: Characteristic running wheel records (Experiment 2).
12 hr light-dark cycles are marked above each record (ZT0 lights on; ZT12 lights off). Sequential days are arranged vertically and each horizontal line represents a single 24 hour time period. (a) 'Reorganization' under nighttime restricted feeding (Ensure: ZT 16-18). (b) 'Reorganization' under nighttime restricted treats (Ensure: ZT 16-18) with continued access to *ad libitum* rat chow. (c) No 'reorganization' under nighttime restricted treats (Ensure: ZT 16-18) with continued access to *ad libitum* rat chow. (d) *Ad libitum* rat chow only.

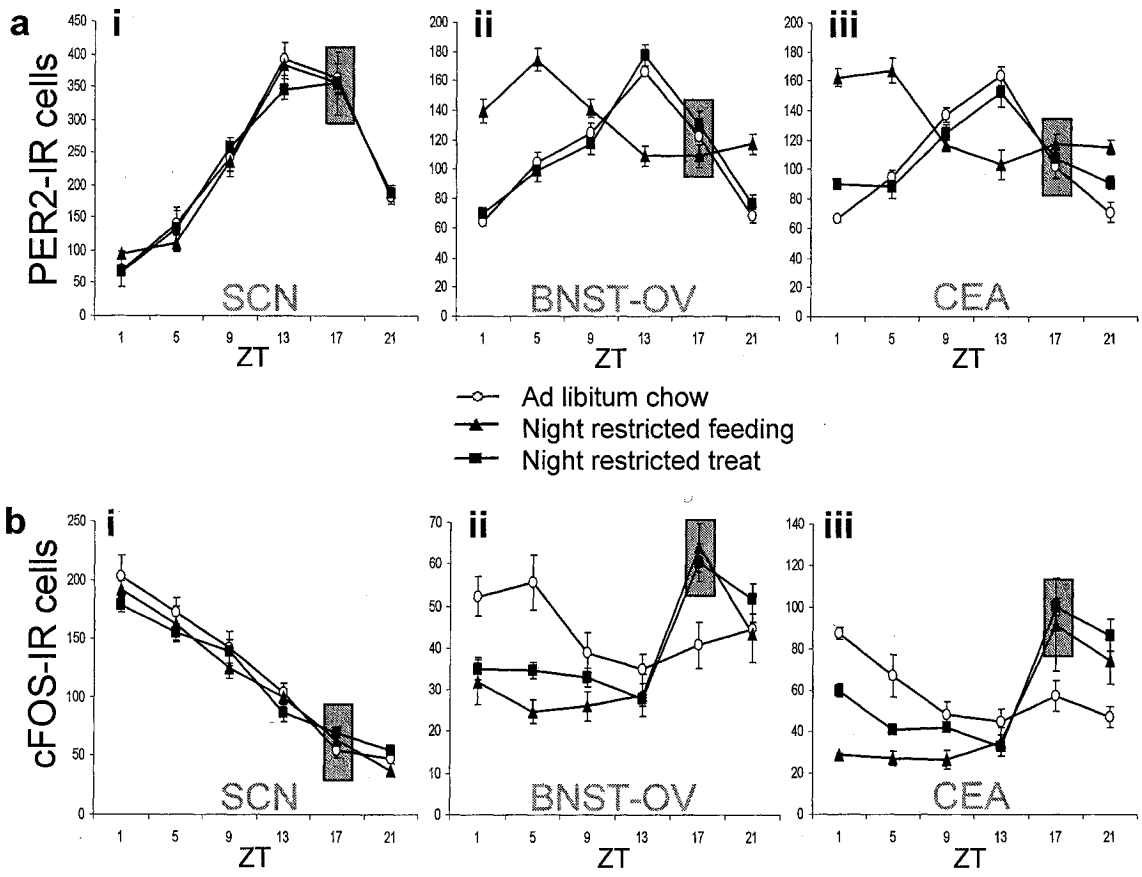

Figure 7: Profiles of cFOS and PER2 expression (Experiment 2) in the SCN, BNST-OV and CEA under control, nighttime restricted feeding or nighttime restricted treat conditions (Nighttime Ensure access marked with shaded rectangles). (A) Mean ± SEM number of PER2 immunoreactive (PER2-IR) cells across the day. (B) Mean ± SEM number of cFOS immunoreactive (cFOS-IR) cells across the day. Animals were perfused (n=4) at each zeitgeber time (ZT 1, 5, 9, 13, 17, 21) under a 12 hr light-dark cycle (ZT0 lights on; ZT12 lights off).

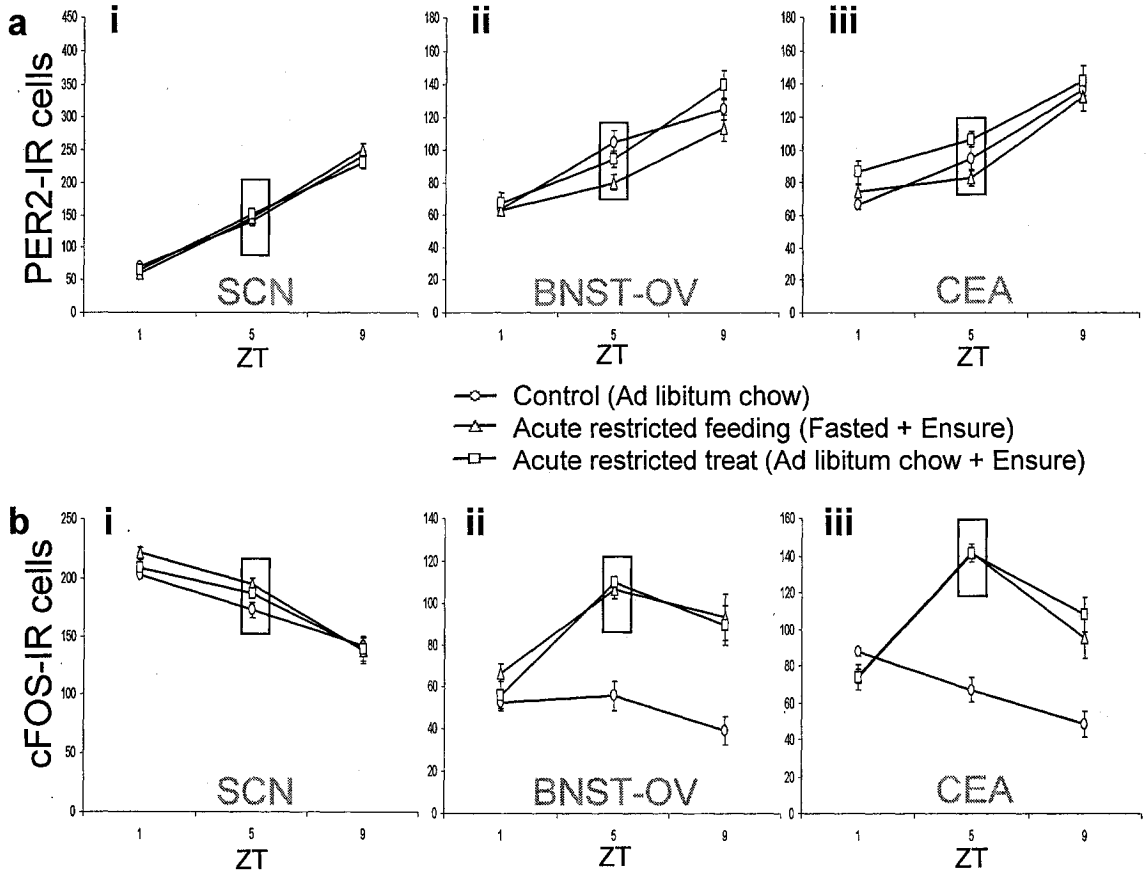

Figure 8: PER2 and cFOS expression (Experiment 3) in the SCN, BNST-OV and CEA under control, acute restricted feeding (fasted rats) or acute restricted treat (sated rats). (a) Mean ± SEM number of PER2 immunoreactive (PER2-IR) cells in animals presented a single daytime Ensure access (open rectangles). (b) Mean ± SEM number of cFOS immunoreactive (cFOS-IR) cells in animals presented a single daytime Ensure access (open rectangles). Animals were perfused (n=4) at each zeitgeber time (ZT 1, 5, 9) under a 12 hr light-dark cycle (ZT0 lights on; ZT12 lights off).

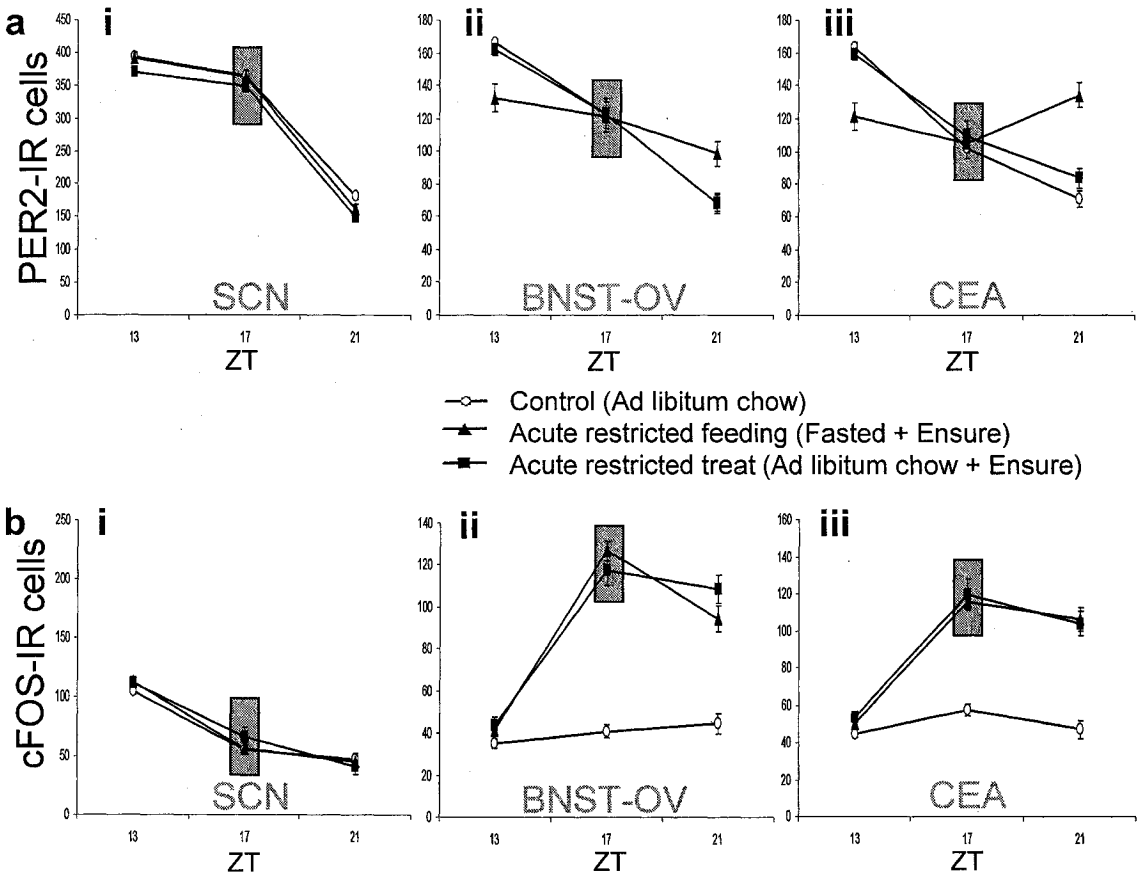

Figure 9: PER2 and cFOS expression (Experiment 4) in the SCN, BNST-OV and CEA under control, acute restricted feeding (rats were fasted) or acute restricted treat (rats were sated). (a) Mean ± SEM number of PER2 immunoreactive (PER2-IR) cells in animals presented a single daytime Ensure access (open rectangles). (b) Mean ± SEM number of cFOS immunoreactive (cFOS-IR) cells in animals presented a single daytime Ensure access (open rectangles). Animals were perfused (n=4) at each zeitgeber time (ZT 1, 5, 9) under a 12 hr LD cycle (ZT0 lights on; ZT12 lights off).

Discussion

Restricted feeding schedules entrain circadian rhythms of behavior and physiology (Richter, 1922; Davidson et al. 2003). Similar effects on behavioral rhythms have been observed in non food deprived animals given limited daily access to a 'treat' (Mistlberger and Rusak, 1987; Mendoza et al. 2005a), suggesting that motivational aspects of eating are also effective in modulating circadian rhythms of behavior. At the cellular level, restricted feeding entrains clock gene expression profiles in both the brain (Wakmatsu et al. 2001) and periphery (Damiola et al. 2000; Le Minh et al. 2001; Stokkan et al. 2001; Davidson et al. 2003). Work from our laboratory has shown that restricted feeding can modify expression of the clock protein PER2 in the BNST-OV and CEA (Lamont et al. 2004; Lamont et al. 2005b). The BNST-OV and CEA are limbic forebrain structures that have been implicated in the regulation of several homeostatic and motivational states, including feeding (Ciccocioppo et al. 2003; Will et al. 2004). The impact of restricted treats on clock gene expression in the BNST-OV and CEA is unknown. In the present thesis we used presentations of a highly palatable food, chocolate Ensure, in food deprived and sated rats to compare the effects of restricted feeding (homeostatic and metabolic challenge) and restricted treats (motivational challenge) on PER2 rhythms in the BNST-OV and CEA. In addition, we assessed the effect of restricted feeding and restricted treats on cFOS expression within the BNST-OV and CEA in order to compare the impact of the two feeding paradigms on these regions. We found that the presentation of Ensure caused similar increases in cFOS expression within the BNST-OV and CEA whether it was presented under restricted feeding or restricted treat conditions. However only restricted feeding, but not restricted treat, was

effective in shifting the phase of PER2 expression patterns within these regions. These findings point to the existence of a functional dissociation between the vulnerability of cells in the BNST-OV and CEA and the responsiveness of circadian oscillators within these structures to the homeostatic and motivational inputs associated with restricted of feeding.

Experiment 1 showed that daytime restricted feeding caused a phase delay of peak PER2 expression in the BNST-OV and CEA. Experiment 2 showed that nighttime restricted feeding caused a phase advance of peak PER2 expression in the BNST-OV and CEA. Individually, either experiment suggests that restricted feeding (and the associated homeostatic challenge) changes PER2 expression profiles in the BNST-OV and CEA. This is consistent with many other experiments that show modified clock gene oscillations in the periphery under restricted feeding (Damiola et al. 2000; Le Minh et al. 2001; Stokkan et al. 2001; Davidson et al. 2003). It is important to note that both experiments 1 and 2 exhibited maximum PER2 expression 12 hours after daily Ensure-access (under restricted feeding conditions). This demonstrates that the time of the meal (under homeostatic challenge) is an important factor in determining PER2 expression profiles in the central extended amygdala. Collectively this supports the view that clock gene expression in these areas is food-entrained under conditions of restricted feeding. The consequence(s) of reentrained clock gene expression in these areas are unknown. One possibility is that circadian clock proteins could regulate local cellular activity rhythms.

Experiments 1 and 2 showed that control rats exhibit significant daily oscillations of cFOS expression in the BNST-OV and CEA. This is the first experiment to show that

cFOS expression in these regions exhibits a circadian rhythm. cFOS expression in the BNST-OV peaked midday, which is consistent with multi-unit activity recordings that showed peak BNST activity in phase with the SCN and peaking midday (Yamazaki et al. 1998). cFOS expression in the CEA peaked at ZT 1 in control animals. Daily oscillations in cFOS expression do not necessarily result from local circadian timing and could be explained by circadian behaviors (e.g. rest-activity cycles) or endocrine signals (e.g. corticosterone) feeding back to these areas. Experiments 3 and 4 demonstrated that cFOS expression in the BNST-OV and CEA is highly reactive to the presentation of Ensure. At the time of Ensure-presentation, observed cFOS expression in Experiments 1 and 2 (after several Ensure-presentations) was greatly reduced as compared to Experiments 3 and 4 (the first Ensure presentation). This suggests that some habituation-like process may attenuate cFOS inductions in the BNST-OV and CEA after repeated daily presentations. These data demonstrate that restricted feeding and restricted treats have similar effects on cFOS expression in the BNST-OV and CEA. Therefore, the unique effect of restricted feeding on PER2 expression profiles does not seem to result from differential immediate early gene expression. Consistent with results in the SCN (Rea et al. 1993), this also suggests that cFOS expression is not sufficient to phase shift circadian clocks in the BNST-OV or CEA. Conversely, these data (timepoints around Ensure presentations) suggest no clear role for local PER2 oscillations in regulating cFOS expression.

Phase-shifted PER2 expression profiles (under restricted feeding) provide a manipulation that could be used to test whether circadian PER2 expression might correlate with daily rhythms in cFOS expression. Experiments 1 and 2 tested cFOS

expression across the day immediately after the last meal. In order to properly observe 'basal' cFOS expression, Ensure should not be delivered on the day before perfusions. This proposed experiment would also test whether cFOS is expressed in the central extended amygdala in 'anticipation' of Ensure-presentation. In the present thesis, Ensure-presentation potentially masks basal cFOS expression in the BNST-OV and CEA around mealtimes. In order to study times when subjects were relatively undisturbed within these experiments, timepoints distant from mealtimes should be examined (e.g. nighttime in day-Ensure groups and daytime in night-ensure groups). These data clearly demonstrate that the effect of repeated Ensure-presentation on cFOS expression seem to extend across the circadian cycle. Experiment 1 (Ensure ZT 4-6) resulted in elevated cFOS expression during the night in the BNST-OV and CEA of restricted-treat and control groups as compared to the restricted feeding group (Figures 4bii and 4biii). This observation suggests that continued access to rat chow (in restricted-treat and control groups) and the associated nighttime feeding could feedback to maintain elevated cFOS expression during the night. Experiment 2 (Ensure ZT 16-18), resulted in decreased daytime cFOS expression in the BNST-OV and CEA of restricted feeding and restricted treat rats as compared to controls (Figure 7bii and 7biii). Nocturnal rats do not normally eat much during the day suggesting that feeding-induced cFOS expression is an inadequate hypothesis to account for this observation. Daily treats have been reported to change cFOS expression around treat-time in the CEA (Mendoza et al. 2005a), showing that some circadian changes in cFOS expression can occur in the absence of reentrainment of local clock proteins. cFOS expression is often discussed as being responsive to environmental stimuli, in the SCN however, there is a clear component that

is regulated by the circadian clock (Geusz et al. 1997; Guido et al. 1999; Allen and Earnest, 2002). Restricted feeding animals had reentrained PER2 expression profiles in the BNST-OV and CEA. Hence, changes in cFOS expression could be partially attributed to local changes in clock protein oscillations. To firmly establish this point in the BNST-OV or CEA, a cFOS-luciferase construct could be used to study these structures *in vitro* and monitor cFOS expression within a single preparation across the day (Geusz et al. 1997; Allen and Earnest, 2002). If cFOS were expressed with a circadian rhythm in the explanted BNST-OV and CEA, this would support the idea that local circadian timing modulates cellular activities within these areas. Our present data do not clearly disprove or support this hypothesis. These experiments could help to establish a local significance of cycling clock proteins in the central extended amygdala.

Under *ad libitum* feeding conditions there exists a clear role for the SCN in maintaining circadian PER2 expression in the BNST-OV (Amir et al. 2004). Unilateral SCN lesions dampen PER2 oscillations in the BNST-OV on the ipsilateral side, suggesting a neural connection between the SCN and ipsilateral BNST-OV is important in maintaining circadian PER2 expression. A similar experiment studying the CEA after unilateral SCN-lesions has not been performed. Furthermore, previous experiments have shown that PER2 oscillations in the BNST-OV and CEA also rely on rhythmic corticosterone from the adrenal gland (Amir et al. 2004, Lamont et al. 2005; Segall et al. 2005). Both rhythmic corticosterone and ipsilateral SCN integrity seem to be required for rhythmic PER2 expression in the BNST-OV and CEA in *ad libitum* fed animals. Such experiments should also be performed under restricted feeding paradigms since it is unclear if similar entrainment pathways apply to restricted feeding schedules.

Corticosterone profiles are changed dramatically under restricted feeding conditions (Wilkinson et al. 1979). Daily peaks shift from early night to early day ('anticipating' the daytime meal). Corticosterone has also been shown to have a role in stabilizing peripheral biological clocks (Le Minh et al. 2001). Furthermore, glucocorticoid receptors are present in large numbers in both the BNST-OV and CEA (Ruel and de Kloet, 1985; Ozawa et al. 1999). Therefore, corticosterone might be attractive as a possible mechanism for PER2 reentrainment in the BNST-OV and CEA under restricted feeding. However, daytime corticosterone injections (to mimic feeding-associated corticosterone peaks) are insufficient to phase shift clock protein oscillations in the liver and lungs (Stokkan et al. 2001). In nighttime restricted feeding, corticosterone release is part of the 'anticipation' of nighttime meals and this creates a profile similar to *ad libitum* control animals (Ahlers et al. 1980). In Experiment 2, there was a large phase shift in PER2 expression in nighttime restricted feeding animals and this is inconsistent with the presumed normality of corticosterone profiles in these animals. In order to test this, corticosterone levels should be monitored systematically across the day in restricted feeding, restricted treat and control groups. This is important because it would describe the daily rhythm of corticosterone levels in nighttime restricted-feeding, as well as in restricted-treat groups. Collectively, these data would help explain the role of corticosterone in regulating PER2 expression in the BNST-OV and CEA under restricted feeding or restricted treat diets.

Self-sustainability is a critical question when considering non-SCN circadian oscillators. Some non-SCN structures exhibiting clock protein oscillations are self-sustaining as the oscillations persist in SCN lesioned animals (Yoo et al. 2004). It is

important to discuss sustainability of non-SCN clock protein oscillations in the absence of entraining signals. In Experiment 4, the acute effects of fasting and nighttime refeeding in the BNST-OV and CEA led to intermediate PER2 immunoreactivity between control values and those values observed under entrained nighttime restricted feeding (Experiment 2). This suggests that it could take several days for PER2 expression to fully phase shift to the expression profiles observed under entrained conditions. In the BNST-OV, light-induced phase shifts also took several days for PER2 expression to re-entrain to new light-dark cycles (Amir et al. 2004). The autonomy of circadian oscillators in the BNST-OV and CEA could be further assessed by measuring PER2 expression soon after the removal of entraining signals (e.g. adrenalectomy or SCN lesion). Corticosterone delivery in the drinking water has been shown to rescue PER2 rhythms in the BNST-OV and CEA (Segall et al. 2005) in adrenalectomized rats. Invasive surgeries such as adrenalectomy or SCN lesions could have immediate effects on PER2 expression in the BNST-OV and CEA. In order to provide rats with recovery time, adrenalectomized rats with corticosterone-supplemented drinking water could be switched back to normal (no corticosterone) water and subsequent PER2 expression could be evaluated. If it takes several days for PER2 expression to become arrhythmic, this would show that PER2 expression is not simply a passive reaction to rhythmic corticosterone. This is an important experiment in establishing PER2 rhythms in the central extended amygdala as being valid circadian oscillators.

Daily injections of methamphetamine can also produce 'anticipatory' activity (Shibita et al. 1995) that are similar to meal 'anticipatory' activity. In addition, chronic methamphetamine treatments (in drinking water) have been shown to produce long

activity rhythms (Honma et al. 1988) that are independent of the SCN and have been studied in SCN lesioned animals (Honma et al. 1988, 1989). Remarkably, methamphetamine-induced activity rhythms can be entrained by restricted feeding (Honma et al. 1989). Amphetamines and motivated behaviors (such as restricted feeding) have effects on dopamine release. Dopamine has also been shown to have an important role in the stress induced cFOS increases in the enkephalinergic cells of the BNST-OV (Kozicz, 2002). This suggests that dopamine rhythms could be important in both methamphetamine and food entrained rhythms. Local unilateral injections of 6-hydroxydopamine (directly to the BNST-OV or CEA) would allow the comparison of PER2 expression in lesioned and intact sides within an individual. Local lesions would be preferential since they would avoid many of the behavioral consequences (including effects on feeding) of medial forebrain bundle or ventral tegmental area lesions. Such experiments should be performed under both free-feeding and restricted feeding paradigms as it is unclear if the relative importance of dopamine is similar in both feeding conditions.

Summary

Highly palatable meals under restricted feeding or restricted treat regimens, result in similar changes in cFOS expression in the BNST-OV and CEA around feeding time. Despite this similarity, PER2 expression is changed only under conditions of restricted feeding. This clearly shows that circadian oscillators in the central extended amygdala can be entrained by restricted feeding. Future research should attempt to determine the neurochemical inputs that entrain local clock proteins. Dopamine plays a key role in activating the BNST-OV during periods of stress (Kozicz, 2002) and could potentially

facilitate entrainment of local circadian oscillators. Under restricted feeding, changes in cFOS expression extend across the circadian cycle. In some cases feeding seems to cause an elevation in cFOS expression in the BNST-OV and CEA. An important possibility to consider is that changes in local clock protein rhythms could result in local changes in cFOS expression rhythms. Future research should attempt to firmly establish outputs of oscillating clock proteins. This could be addressed at the molecular level with immediate early genes such as cFOS. If daily cFOS rhythms persist in vitro, this would support a role for biological clockwork in modulating local cellular activity. Fundamentally, more work is needed to describe the generation, maintenance and consequences of the circadian oscillators in the BNST-OV and CEA.

References

Abe, K., Kroning, J., Greer, M.A., Critchlow, V. (1979) Effects of destruction of the suprachiasmatic nuclei on the circadian rhythms in plasma corticosterone, body temperature, feeding and plasma thyrotropin. *Neuroendocrinology, 29*, 119-131.

Ahlers, I., Smajda, B., Ahlersova, E. (1980) Circadian rhythm of plasma and adrenal corticosterone in rats: effect of restricted feeding schedules. *Endocrinologia experimentalis, 14*, 183-190.

Allen, G.C., & Earnest, D.J. (2002) Real-time analysis of rhythmic gene expression in immortalized suprachiasmatic nucleus cells. *Neuroreport, 13*, 2027-2030.

Amir, S., Lamont, E.W., 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. *Journal of Neuroscience, 24*, 781-790.

Angeles-Castellanos, M., Aguilar-Roblero, R., Escobar, C. (2004) c-Fos expression in hypothalamic nuclei of food-entrained rats. *American journal of physiology. Regulatory, integrative and comparative physiology, 286,* R158-165.

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*, 249-54.

Bell-Pedersen, D., Cassone, V.M., Earnest, D.J., Golden, S.S., Hardin, P.E., Thomas, T.L., Zoran, M.J. (2005) Circadian rhythms from multiple oscillators: lessons from diverse organisms. *Nature reviews. Genetics, 6,* 544-556.

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

Ciccocioppo, R., Fedeli, A., Economidou, D., Policani, F., Weiss, F., Massi, M. (2003) The bed nucleus is a neuroanatomical substrate for the anorectic effect of corticotropin-releasing factor and for its reversal by nociceptin/orphanin FQ. *Journal of Neuroscience*, *23,* 9445-9451.

Colussi-Mas, J., Panayi, F., Scarna, H., Renaud, B., Berod, A., Lambas-Senas, L. (2005) Blockade of beta-adrenergic receptors prevents amphetamine-induced behavioural sensitization in rats: a putative role of the bed nucleus of the stria terminalis. *The international journal of neuropsychopharmacology, 8*, 569-581.

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 & development,14*, 2950-2961.

Davidson, A.J., Poole, A.S., Yamazaki, S., Menaker, M. (2003) Is the food-entrainable circadian oscillator in the digestive system? *Genes, brain, and behavior, 2*, 32-39.

Day, H.E., Curran, E.J., Watson, S.J. Jr., Akil, H. (1999) Distinct neurochemical populations in the rat central nucleus of the amygdala and bed nucleus of the stria terminalis: evidence for their selective activation by interleukin-1beta. *The Journal of comparative neurology, 413,* 113-128.

Day, H.E., Badiani, A., Uslaner, J.M., Oates, M.M., Vittoz, N.M., Robinson, T.E., Watson, S.J. Jr., Akil, H. (2001) Environmental novelty differentially affects c-fos mRNA expression induced by amphetamine or cocaine in subregions of the bed nucleus of the stria terminalis and amygdala. *The Journal of neuroscience, 21*, 732-740.

Day, H.E., Nebel, S., Sasse, S., Campeau, S. (2005) Inhibition of the central extended amygdala by loud noise and restraint stress. *The European journal of neuroscience, 21,* 441-454.

Erb, S., Stewart, J. (1999) A role for the bed nucleus of the stria terminalis, but not the amygdala, in the effects of corticotropin-releasing factor on stress-induced reinstatement of cocaine seeking. *The Journal of neuroscience, 19*, RC35.

Erb, S., Salmaso, N., Rodaros, D., Stewart, J. (2001) A role for the CRF-containing pathway from central nucleus of the amygdala to bed nucleus of the stria terminalis in the stress-induced reinstatement of cocaine seeking in rats. *Psychopharmacology,* 158, 360-365.

Fukuhara, C., Yamazaki, S., Liang, J. (2005) Pineal circadian clocks gate arylalkylamine N-acetyltransferase gene expression in the mouse pineal gland. *Journal of neurochemistry, 93*, 156-162.

Geusz, M.E., Fletcher, C., Block, G.D., Straume, M., Copeland, N.G., Jenkins, N.A., Kay, S.A., Day, R.N. (1997) Long-term monitoring of circadian rhythms in c-fos gene expression from suprachiasmatic nucleus cultures. *Current Biology, 7*, 758-766.

Guido, M.E., de Guido, L.B., Goguen, D., Robertson, H.A., Rusak, B. (1999) Daily rhythm of spontaneous immediate-early gene expression in the rat suprachiasmatic nucleus. *Journal of biological rhythms, 14*, 275-280.

Honma, S., Honma, K., Shirakawa, T., Hiroshige, T. (1988) Rhythms in behaviors, body temperature and plasma corticosterone in SCN lesioned rats given methamphetamine. *Physiology & behavior, 44*, 247-255.

Honma, S., Honma, K., Hiroshige, T. (1989) Methamphetamine induced locomotor rhythm entrains to restricted daily feeding in SCN lesioned rats. *Physiology & behavior, 45*, 1057-1065.

Ishida, A., Mutoh, T., Ueyama, T., Bando, H., Masubuchi, S., Nakahara, D., Tsujimoto, G., Okamura, H. (2005) Light activates the adrenal gland: Timing of gene expression and glucocorticoid release. *Cell metabolism, 2*, 297-307.

Kozicz, T.(2002) Met-enkephalin immunoreactive neurons recruited by acute stress are innervated by axon terminals immunopositive for tyrosine hydroxylase and dopamine-alpha-hydroxylase in the anterolateral division of bed nuclei of the stria terminalis in the rat. *The European journal of neuroscience, 16*, 823-835.

Lamont, E.W., Barry-Shaw, J., Amir, S. (2004) Restricted feeding synchronizes Period 2 protein expression in the amygdala and hippocampus, but not the SCN. *Abstract Viewer/Itinerary Planner.* New Orleans, LA: Society for Neuroscience Program No. *428.2*

Lamont, E.W., Robinson, B., Stewart, J., Amir, S. (2005a) The central and basolateral nuclei of the amygdala exhibit opposite diurnal rhythms of expression of the clock protein Period2. *Proceedings of the National Academy of Sciences of the United States of America, 102*, 4180-4184.

Lamont, E.W., Diaz, L.R., Barry-Shaw, J., Stewart, J., Amir, S. (2005b) Daily restricted feeding rescues a rhythm of period2 expression in the arrhythmic suprachiasmatic nucleus. *Neuroscience, 132*, 245-248.

Le Minh, N., Damiola, F., Tronche, F., Schutz, G., Schibler, U. (2001) Glucocorticoid hormones inhibit food-induced phase-shifting of peripheral circadian oscillators. *The EMBO journal, 20,* 7128-7136.

Leri, F., Flores, J., Rodaros, D., Stewart, J. (2002) Blockade of stress-induced but not cocaine-induced reinstatement by infusion of noradrenergic antagonists into the bed nucleus of the stria terminalis or the central nucleus of the amygdala. *The Journal of neuroscience, 22*, 5713-5718.

Mead, S., Ebling, F.J., Maywood, E.S., Humby, T., Herbert, J., Hastings, M.H. (1992) A nonphotic stimulus causes instantaneous phase advances of the light-entrainable circadian oscillator of the Syrian hamster but does not induce the expression of c-fos in the suprachiasmatic nuclei. *The Journal of neuroscience, 12,* 2516-2522.

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

Mendoza J, Graff C, Dardente H, Pevet P, Challet E. (2005b) Feeding cues alter clock gene oscillations and photic responses in the suprachiasmatic nuclei of mice exposed to a light/dark cycle. *The Journal of neuroscience, 25*, 1514-1522.

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

Ozawa, H., Lion, J., Xie, C.X., Nishi, M., Steinbusch, H., Kawata, M. (1999) Down-regulation of ACTH and glucocorticoid receptor immunoreactivity in hypothalamic arcuate neurons after adrenalectomy in the rat. *Neuroreport, 10*, 1571-1575.

Ralph, M.R., Foster, R.G., Davis, F.C., Menaker, M. (1990) Transplanted suprachiasmatic nucleus determines circadian period. *Science, 247*, 975-978

Rea, M.A. (1989) Light increases Fos-related protein immunoreactivity in the rat suprachiasmatic nuclei. *Brain research bulletin, 23*, 577-581.

Rea, M.A., Michel, A.M., Lutton, L.M. (1993) Is Fos expression necessary and sufficient to mediate light-induced phase advances of the suprachiasmatic circadian oscillator? *Journal of biological rhythms, 8,* S59-64.

Reppert, S.M., Weaver, D.R. (2002) Coordination of circadian timing in mammals *Nature, 418*, 935-941.

Reul, J.M., & de Kloet, E.R. (1985) Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. *Endocrinology, 117*, 2505-2511.

Richter, C.P. (1922) A behavioristic study of the rat. *Comp Psychol Mono, 1*, 1-55

Segall, L.A., Perrin, J.S., Robinson, B., Rodaros, D., Amir, S. (2005) Exogenous corticosterone restores rhythmic expression of the clock gene PER2 in the central extended amygdala in adrenalectomized rats. *Abstract Viewer/Itinerary Planner.* Washington, DC: Society for Neuroscience. Program No. *60.3*

Stanley, B.G., Lanthier, D., Leibowitz, S.F. (1989) Multiple brain sites sensitive to feeding stimulation by opioid agonists: a cannula-mapping study. *Pharmacology, biochemistry, and behavior, 31,* 825-832.

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

Thompson, B.L., & Rosen, J.B. (2006) Immediate-early gene expression in the central nucleus of the amygdala is not specific for anxiolytic or anxiogenic drugs. *Neuropharmacology*, 50, 57-68.

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. *The European journal of neuroscience, 13,* 1190-1196.

Watson, R.E. Jr., Wiegand, S.J., Clough, R.W., Hoffman, G.E. (1986) Use of cryoprotectant to maintain long-term peptide immunoreactivity and tissue morphology. *Peptides, 7,* 155-159

Will, M.J., Franzblau, E.B., Kelley, A.E. (2004) The amygdala is critical for opioid-mediated binge eating of fat. *Neuroreport*, *15*, 1857-1860.

Yamazaki, S., Kerbeshian, M.C., Hocker, C.G., Block, G.D., Menaker, M. (1998) Rhythmic properties of the hamster suprachiasmatic nucleus in vivo. *The Journal of neuroscience*, *18*, 10709-10723.

Yoo, S.H., Yamazaki, S., Lowrey, P.L., Shimomura, K., Ko, C.H., Buhr, E.D., Siepka, S.M., Hong, H.K., Oh, W.J., Yoo, O.J., Menaker, M., Takahashi, J.S. (2004) PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. *Proceedings of the National Academy of Sciences of the United States of America, 101*, 5339-5346.

Yoshikawa, T., Yamazaki, S., Menaker, M. (2005) Effects of preparation time on phase of cultured tissues reveal complexity of circadian organization. *Journal of biological rhythms, 20*, 500-512.

Appendices

APPENDIX A

ANOVA source tables for Experiment 1.2 (PER2)

Groups (3): Daytime restricted feeding (Ensure ZT4-6), Daytime restricted treat (Ensure ZT 4-6) and control animals.

Times (6): ZT 1, 5, 9, 13, 17, 21

SCN

Source	SS	df	MS	F	P
Time	897756.883	5	179551.377	103.851	<0.001
Group	289.460	2	144.730	0.084	0.920
Time*Group	10210.870	10	1021.087	0.591	0.814
Error	88175.277	51	1728.927		
Total	4688277.720	69			

BNST-OV

Source	SS	df	MS	F	P
Time	44855.140	5	8971.028	30.300	<0.001
Group	18315.244	2	9157.622	30.930	<0.001
Time*Group	22367.600	10	2236.760	7.555	<0.001
Error	15691.907	53	296.074		
Total	1110226.560	71			

CEA

Source	SS	df	MS	F	P
Time	53784.574	5	10756.915	63.808	<0.001
Group	9432.033	2	4716.017	27.974	<0.001
Time*Group	19180.205	10	1918.021	11.377	<0.001
Error	8934.937	53	168.584		
Total	1017718.880	71			

Appendix B

ANOVA source tables for Experiment 1.3 (cFOS)

Groups (3): Daytime restricted feeding (Ensure ZT 4-6), Daytime restricted treat (Ensure ZT 4-6) and control animals.

Times (6): ZT 1, 5, 9, 13, 17, 21

SCN

Source	SS	df	MS	F	P
Time	206767.795	5	41353.559	121.112	<0.001
Group	1997.239	2	998.619	2.925	0.063
Time*Group	1299.832	10	129.983	.381	0.950
Error	18096.800	53	341.449		
Total	1304665.031	71			

BNST-OV

Source	SS	df	MS	F	P
Time	4286.552	5	857.310	11.728	<0.001
Group	665.722	2	332.861	4.554	0.015
Time*Group	1950.865	10	195.086	2.669	0.010
Error	3874.157	53	73.097		
Total	133843.400	71			

CEA

Source	SS	df	MS	F	P
Time	22295.936	5	4459.187	25.532	<0.001
Group	1493.232	2	746.616	4.275	0.019
Time*Group	15260.140	10	1526.014	8.738	<0.001
Error	9256.417	53	174.649		
Total	309025.640	71			

Appendix C

ANOVA source tables for Experiment 2.2 (PER2)

Groups (3): Nighttime restricted feeding (Ensure ZT 16-18), Nighttime restricted treats (Ensure ZT 16-18) and control animals.

Times (6): ZT 1, 5, 9, 13, 17, 21

SCN

Source	SS	df	MS	F	P
Time	867244.856	5	173448.971	85.358	<0.001
Group	510.988	2	255.494	0.126	0.882
Time*Group	9160.164	10	916.016	0.451	0.913
Error	105664.570	52	2032.011		
Total	4662063.480	70			

BNST-OV

Source	SS	df	MS	F	P
Time	35079.091	5	7015.818	42.567	<0.001
Group	7636.448	2	3818.224	23.166	<0.001
Time*Group	38809.112	10	3880.911	23.547	<0.001
Error	8900.120	54	164.817		
Total	1080061.680	72			

CEA

Source	SS	df	MS	F	P
Time	16425.196	5	3285.039	19.483	<0.001
Group	8534.781	2	4267.391	25.310	<0.001
Time*Group	40039.166	10	4003.917	23.747	<0.001
Error	9104.790	54	168.607		
Total	1025154.280	72			

Appendix D

ANOVA source tables for Experiment 2.3 (cFOS)

Groups (3): Nighttime restricted feeding (Ensure ZT 16-18), Nighttime restricted treats (Ensure ZT 16-18) and control animals.

Times (6): ZT 1, 5, 9, 13, 17, 21

SCN

Source	SS	df	MS	F	P
Time	195962.638	5	39192.528	111.968	<0.001
Group	702.939	2	351.469	1.004	0.373
Time*Group	3231.740	10	323.174	0.923	0.519
Error	18901.773	54	350.033		
Total	1186857.911	72			

BNST-OV

Source	SS	df	MS	F	P
Time	4998.338	5	999.668	13.135	<0.001
Group	779.274	2	389.637	5.120	0.009
Time*Group	4039.932	10	403.993	5.308	<0.001
Error	4109.700	54	76.106		
Total	131572.080	72			

CEA

Source	SS	df	MS	F	P
Time	19972.784	5	3994.557	16.771	<0.001
Group	2494.214	2	1247.107	5.236	0.008
Time*Group	16576.706	10	1657.671	6.960	<0.001
Error	12862.160	54	238.188		
Total	271867.200	72			

Appendix E

ANOVA source tables for Experiment 3.1 (PER2)

Groups (3): Nighttime restricted feeding (Ensure ZT 16-18), Nighttime restricted treats (Ensure ZT 16-18) and control animals.

Times (3): ZT 1, 5, 9

SCN

Source	SS	df	MS	F	P
Time	185896.296	2	92948.148	155.953	<0.001
Group	48.536	2	24.268	0.041	0.960
Time*Group	1259.778	4	314.944	0.528	0.716
Error	16092.040	27	596.001		
Total	1014977.520	36			

BNST-OV

Source	SS	Df	MS	F	P
Time	22379.002	2	11189.501	71.657	<0.001
Group	1506.816	2	753.408	4.825	0.016
Time*Group	1141.638	4	285.409	1.828	0.153
Error	4216.130	27	156.153		
Total	350997.240	36			

CEA

Source	SS	Df	MS	F	P
Time	23504.696	2	11752.348	73.384	<0.001
Group	1575.936	2	787.968	4.920	0.015
Time*Group	588.011	4	147.003	0.918	0.468
Error	4324.010	27	160.149		
Total	408012.680	36			

Appendix F

ANOVA source tables for Experiment 3.2 (cFOS)

Groups (3): Nighttime restricted feeding (Ensure ZT 16-18), Nighttime restricted treats (Ensure ZT 16-18) and control animals.

Times (3): ZT 1, 5, 9

SCN

Source	SS	df	MS	F	P
Time	31574.109	2	15787.054	30.192	<0.001
Group	1006.709	2	503.354	0.963	0.395
Time*Group	891.224	4	222.806	0.426	0.788
Error	14118.100	27	522.893		
Total	1193060.880	36			

BNST-OV

Source	SS	df	MS	F	P
Time	6372.842	2	3186.421	11.853	<0.001
Group	11685.396	2	5842.698	21.733	<0.001
Time*Group	3590.098	4	897.524	3.339	0.024
Error	7258.610	27	268.837		
Total	227436.600	36			

CEA

Source	SS	df	MS	F	P
Time	8927.336	2	4463.668	15.884	<0.001
Group	12898.382	2	6449.191	22.949	<0.001
Time*Group	9837.464	4	2459.366	8.752	<0.001
Error	7587.520	27	281.019		
Total	358551.040	36			

Appendix G

ANOVA source tables for Experiment 4.1 (PER2)

Groups (3): Nighttime restricted feeding (Ensure ZT 16-18), Nighttime restricted treats (Ensure ZT 16-18) and control animals.

Times (3): ZT 13, 17, 21

SCN

Source	SS	df	MS	F	P
Time	285158.547	2	142579.274	137.900	<0.001
Group	3318.518	2	1659.259	1.605	0.221
Time*Group	412.133	4	103.033	0.100	0.982
Error	25848.350	25	1033.934		
Total	3588750.040	34			

BNST-OV

Source	SS	df	MS	F	P
Time	34232.987	2	17116.493	100.933	<0.001
Group	19.980	2	9.990	0.059	0.943
Time*Group	5154.813	4	1288.703	7.599	<0.001
Error	4578.730	27	169.583		
Total	544542.760	36			

CEA

Source	SS	df	MS	F	P
Time	18253.069	2	9126.534	51.548	<0.001
Group	384.436	2	192.218	1.086	0.352
Time*Group	12974.631	4	3243.658	18.321	<0.001
Error	4780.340	27	177.050		
Total	524061.920	36			

Appendix H

ANOVA source tables for Experiment 4.2 (cFOS)

Groups (3): Nighttime restricted feeding (Ensure ZT 16-18), Nighttime restricted treats (Ensure ZT 16-18) and control animals.

Times (3): ZT 13, 17, 21

SCN

Source	SS	df	MS	F	P
Time	27232.390	2	13616.195	73.190	<0.001
Group	106.039	2	53.019	0.285	0.754
Time*Group	451.015	4	112.754	0.606	0.662
Error	5023.043	27	186.039		
Total	212993.871	36			

BNST-OV

Source	SS	df	MS	F	P
Time	21359.582	2	10679.791	52.453	<0.001
Group	17069.429	2	8534.714	41.918	<0.001
Time*Group	6901.944	4	1725.486	8.475	<0.001
Error	5497.400	27	203.607		
Total	244311.040	36			

CEA

Source	SS	df	MS	F	P
Time	15062.927	2	7531.463	25.095	<0.001
Group	14040.167	2	7020.083	23.391	<0.001
Time*Group	4926.787	4	1231.697	4.104	0.010
Error	8103.240	27	300.120		
Total	258172.160	36			