# NOTE TO USERS

# Subordinate Circadian Oscillators of the Limbic Forebrain: Synchronization by Motivational and Metabolic State

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# ABSTRACT

Subordinate Circadian Oscillators of the Limbic Forebrain: Synchronization by Motivational and Metabolic State

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The master circadian clock in the suprachiasmatic nucleus (SCN) is responsible for circadian rhythms in physiology and behavior. However, putative clock cells are found in nearly every mammalian tissue, suggesting that circadian rhythms are regulated both globally by the SCN and locally at the tissue level, downstream from the master clock. Immunocytochemistry for the clock gene protein Period2 (PER2), reveals two synchronized, opposite patterns of PER2 expression in the limbic forebrain of Wistar rats: the oval nucleus of the bed nucleus of the stria terminalis (BNST-OV) and central nucleus of the amygdala (CEA) are synchronized and in phase with the SCN; the basolateral amygdala (BLA) and dentate gyrus (DG) of the hippocampus are synchronized but 180° out of phase with the SCN. SCN lesions abolish rhythmic expression of PER2 in the limbic forebrain. The BNST-OV and CEA also require adrenal hormones for the rhythmic expression of PER2, as adrenalectomy causes a loss of PER2 rhythm in these two regions, leaving the SCN, BLA, and DG unaffected. In addition, a schedule of restricted feeding (RF) that limits food access to three hours during the day causes behavioral anticipation prior to food availability, and shifts the time of peak PER2 expression. Thus, compared to *ad libitum* (AL) fed animals, in the RF group the BNST-OV, CEA, BLA and DG all show peak PER2 expression about 12 hours

after food access, but the SCN is unaffected. In constant light (LL), AL fed rats show no circadian rhythm of behavior or PER2 expression in the SCN or limbic forebrain. In contrast, RF in LL results in behavioral anticipation of the food access time, and restoration of rhythmic PER2 expression, peaking 12 hours after feeding time, in the BNST-OV, CEA, DG, and surprisingly, the SCN. Limited daily access to nutritive or non-nutritive treats does not produce significant behavioral anticipation or changes in the phase of PER2 expression in the limbic forebrain, whereas sodium deprived animals given limited daily access to saline showed a reduction in PER2 expression in the BLA around the time of saline access, but there was no behavioral effect, suggesting both reward and negative energy balance are necessary to alter the phase of PER2 expression in the limbic forebrain. These results indicate the presence of subordinate circadian oscillators in the limbic forebrain responsible for the integration of emotional, motivational, and behavioral state with circadian rhythms.

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## CONTRIBUTION OF AUTHORS

Dr. Shimon Amir acted as Ph.D. supervisor and principle investigator, and therefore is senior author on Chapters 2, 3, and 4. He is also responsible for the demonstration of rhythmic expression of PER2 in the oval nucleus of the bed nucleus of the stria terminalis, and as such, is first author on Chapter 1. Dr. Amir also performed some data analysis, and the trans orbital enucleation surgeries for an experiment presented in Chapter 1.

Dr. Jane Stewart provided extensive editorial input on each of the manuscripts.

Mr. Barry Robinson is the laboratory coordinator and is responsible for all of the immunocytochemistry protocols used for these experiments. He also performed the SCN lesion surgeries and contributed to the data collection of the experiments presented in Chapter 1.

Ms. Jane Barry-Shaw was a research assistant and contributed to the data collection of the experiments presented in Chapters 2 and 4.

Ms. Laura Renteria Diaz was an undergraduate honors student. She contributed to the data collection of the experiments presented in Chapters 2 and 4.

Ms. Valerie Harbour was a research assistant and contributed to the data collection of the experiments presented in Chapter 4.

## NOTES ON NOMENCLATURE

References to genes, their transcripts, and protein products have been made throughout this thesis. To discriminate between the message (mRNA) and the signal (protein product), the following nomenclature was used as consistently as possible.

Genes, mRNA, and any signal other than the final protein product are written in *Italics* with the first letter capitalized. For example: *cFos, Per1, Bmal1*.

Protein products of these genes and transcripts were written in the regular type face using all CAPITAL letter. For example: FOS, PER1, BMAL1.

**Notes on clock genes.**

Circadian clock genes have been cloned and described in a number of species. The species is often indicated by using the lower case first letter of the species name before the name of the gene. For example, if the *Period1* gene had been cloned from a mouse, the gene would be written *mPer1*. The experiments presented here used rats except as indicated in Chapter 1, therefore the r before the name of the gene has been dropped. Whenever genes from other species are discussed, the type of species is named explicitly.

## LIST OF ABBREVIATIONS

ACTH: Adrenocorticotropic hormone

ADX: Adrenalectomy, adrenalectomized

AgRP: Agouti-related gene product

AL: *ad libitum*

ANOVA: Analysis of variance

AVP: Arginine vasopressin

bHLH: Basic helix loop helix

BLA: Basolateral amygdala

BMAa: Basomedial nucleus of the amygdala: anterior part

*Bmal1*, BMAL1: Brain and muscle ARNT-like *gene*, protein 1

BNST: Bed nucleus of the stria terminalis

BNST-OV: Oval nucleus of the bed nucleus of the stria terminalis

CCK: Cholecystokinin

CEA: Central nucleus of the amygdala

CEAl: Central nucleus of the amygdala, lateral part

CEAm: Central nucleus of the amygdala, medial part

CKIε, CKIδ: Casein Kinase 1 epsilon, delta

CORT: Corticosterone

CP: Caudoputamen

CRF: Corticotrophin releasing factor; also corticotrophin releasing hormone

CRH: Corticotrophin releasing hormone; also corticotrophin releasing factor

*Cry,* CRY: Cryptochrome *gene*, protein

CS: Conditioned stimulus

CS+: Conditioned stimulus paired with food delivery

CS-: Conditioned stimulus not paired with food delivery

CT: Circadian time

DD: Constant darkness

DG: Dentate gyrus of the hippocampus

DMH: Dorsomedial nucleus of the hypothalamus

ENK: Enkephalin

GR: Glucocorticoid receptor

GRP: Gastrin-releasing peptide

HPA: hypothalamic pituitary adrenocortical (axis)

LA: Lateral nucleus of the amygdala

LD: Light-dark cycle

LH: Luteinizing hormone

LHA: Lateral hypothalamic area

LL: Constant light

MPA: Medial preoptic area

MSG: Monosodium glutamate

NPAS2: Neuronal PAS domain protein 2

NPY: Neuropeptide Y

PAS: PER-ARNT-SIM (domain)

PB: Parabrachial nucleus

*Per1*, PER1: Period1 clock *gene*, protein

*Per2,* PER2: Period2 clock *gene*, protein

PK2: Prokineticin 2 protein

PVN: Paraventricular nucleus of the hypothalamus

PVT: Paraventricular nucleus of the thalamus

RHT: Retinohypothalamic tract

RF: Restricted feeding

SCN: Suprachiasmatic nucleus

SI: Substantia innominata

SPVZ: Subparaventricular zone

SS: Somatostatin

VIP: Vasoactive intestinal polypeptide

ZT: Zeitgeber time

# GENERAL INTRODUCTION

Life on earth evolved under the rising and setting sun. As a result, all organisms have an internal clock that cycles about once a day and can entrain to the daily light-dark cycle. One example of such a phenomenon is the unicellular marine alga *Gonyaulax polyedra,* which travels up to the higher levels of the ocean to absorb sunlight and $CO_2$ for photosynthesis during the day, but descends to the nutrient rich lower layers during the night to absorb nitrogen and phosphorous (Roenneberg & Merrow, 2002). What is so interesting about this organism is that both light and nitrogen can reset its circadian clock. This suggests that either the clock can be entrained by more than one stimulus, or that multiple oscillators are altering the timing of its rise and descent to obtain an optimal balance of energy and nutrients (Roenneberg & Merrow, 2002).

In mammals, the master circadian clock is located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Klein et al., 1991). The SCN can receive photic information directly from the eye via the retinohypothalamic tract (RHT; Rusak & Zucker, 1979); but like algae, mammals must also be able to respond to non-photic stimuli in order to maximize the use of resources and increase their chances of survival. These non-photic cues have relatively little impact on the SCN (Hara et al., 2001; Wakamatsu et al., 2001), but nevertheless, profoundly influence circadian behavior (Amir & Stewart, 1998; Stephen, 2002). The non-photic stimuli known to affect circadian rhythms include olfactory cues (Amir et al., 1999; Funk & Amir, 2000; Goel, et al., 1998; Governale & Lee, 2001) food (Berridge, 2004; Stephan et al., 1979a, b; Stephan, 2002), health, (Hastings et al., 2003), social interactions (Mistlberger & Skene, 2004; Mrosovsky, 1996), and emotional state (Cardinal et al., 2002; Cardinali, 2000).

Oscillations in clock gene expression have been discovered in the mammalian brain (Abe et al., 2002, Shieh, 2003) and most tissues of the body (Balsalobre et al., 1998, 2000a,b). The presence of circadian gene expression outside of the SCN suggests that a single master clock entrained by the light dark cycle may not be sufficient for the regulation of all circadian controlled behaviors and physiological processes. Non-photic cues may impact circadian rhythms by altering clock gene expression throughout the cortex and limbic forebrain, in the very cells where processing of non-photic sensory, metabolic, emotional, and motivational information takes place, rather than acting on a single "non-photic oscillator system" (Mrosovsky, 1996). The goal of this thesis is to use evidence of rhythmic clock gene expression to reveal brain regions that may be involved in the integration of non-photic stimuli with photic input from the master clock to control circadian rhythms.

## The Suprachiasmatic Nucleus: The Circadian Master Clock

### Anatomy of the SCN.

The SCN is a bilateral structure named for its anatomical location directly above the optic chiasm, on either side of the third ventricle. It is made up of approximately 20,000 neurons, and is divided into distinct ventrolateral core and dorsomedial shell regions (Van den Pol, 1980; Moore & Speh, 1993). The core, immediately adjacent to the optic chiasm, receives direct input from the eye and projects primarily to the shell (Leak et al., 1999). The SCN shell, located directly dorsal and medial to the core, sends sparse projections to the core, but dense projections to the adjoining hypothalamus (Leak et al., 1999). Both the core and shell neurons are GABA-ergic, but the distribution of the

neuropeptides in each subregion differs (Moore & Speh, 1993). The majority of core neurons produce either vasoactive intestinal polypeptide (VIP) or gastrin-releasing peptide (GRP), with a smaller number producing calbindin-$D_{28K}$, cholecystokinin (CCK), enkephalin (ENK), or somatostatin (SS; LeSauter et al., 2002; Moore et al., 2002). The calbindin-containing neurons in particular seem to be essential for the circadian control of locomotor activity (LeSauter & Silver, 1999). Most neurons in the shell produce arginine vasopressin (AVP), but a smaller population produce calretinin, neurotensin, and substance P (Moore et al., 2002).

**Photic entrainment.**

The SCN is synchronized to the external light-dark (LD) cycle by a process called photic entrainment. Light is conveyed from the retina to the core region of the SCN via the RHT (Moore & Lenn, 1972; Rusak & Zucker, 1979). The primary circadian photoreceptor melanopsin is found in the soma region of most retinal ganglion cells that innervate the SCN (Berson et al., 2002; Gooley et al., 2001; Hannibal et al., 2002; Provencio et al., 2000). Unlike the rapid activation and habituation of the opsin-based rods and cones that are the photoreceptors of the image-forming retinal pathway, melanopsin containing retinal ganglion cells are relatively insensitive to brief light exposure, but gradually become more activated in response to constant illumination of an intensity similar to that of the dawn sky that persists for an extended time, from 2 seconds to about 2 minutes. This is consistent with the amount and duration of light exposure necessary for entrainment (Berson et al., 2002).

Other photoreceptors, including rods and cones, as well as the photopigment cryptochrome, also play a role in photic entrainment. Animals can become entrained to a

LD cycle if any one of these photoreceptors is spared, and only manipulations that completely abolish retinal input prevent photic entrainment (reviewed in Beaulé et al., 2003b).

Photic activation of the SCN core causes the release of glutamate, pituitary adenylate cyclase-activating polypeptide, and substance P (Harmar, 2003; Kim et al., 2001), which can cause either phase delays or phase advances depending on the time of day (Kim et al., 2001). SCN cells become synchronized with each other, via both electrical synapses and neurochemical signaling using GABA and VIP dependent mechanisms (Aton et al., 2005; Harmar, 2003; Liu & Reppert, 2000; Long et al., 2005; Yamaguchi et al., 2003), ultimately leading to the establishment of circadian rhythms and the synchronization of behavioral and physiological processes with the LD cycle.

**The SCN is the master circadian clock.**

The SCN has all of the defining attributes of a functional circadian pacemaker (Tosini & Menaker, 1996): intrinsic rhythmicity (Abe et al., 2002; Green & Gillette, 1982; Groos & Hendriks, 1982; Inouye & Kawamura, 1979; Shibata et al., 1982; Yamazaki et al., 2000), a free running period under constant conditions (Inouye & Kawamura, 1979; Yamazaki et al., 1998), and accurate time-keeping over a wide range of physiological temperatures (Herzog & Huckfeldt, 2003; Ruby et al., 1999). In addition, substantial evidence indicates that the SCN is the master circadian clock (Klein et al., 1991), as SCN lesions abolish circadian rhythmicity of locomotor activity, feeding, drinking, and hormone release (Moore & Eichler, 1972; Stephan & Zucker, 1972; Abe et al., 1979). Interestingly, knife cuts that create an isolated SCN "island" also cause arrhythmicity of locomotor activity, sleep wakefulness, and electrical activity in the brain

outside the island (Inouye & Kawamura, 1979), but the circadian rhythm of the hormone corticosterone (CORT) is spared (Honma et al., 1984), suggesting that the master clock uses multiple signals to confer rhythmicity.

Further evidence for the essential role of the SCN in circadian rhythmicity was provided by the discovery that a surgically transplanted graft of SCN cells could restore a circadian rhythm of locomotor activity to recipient animals with SCN ablation. Hamsters with the *tau* mutation have an endogenous circadian rhythm with a period shorter than 24 hours. Animals homozygous or heterozygous for the mutation have periods of 20 and 22 hours respectively, whereas wild-type animals have an endogenous period of about 24 hours (Ralph et al., 1990). SCN grafts were taken from a homozygous *tau* mutant donor and placed in a wild-type recipient, and vice versa. Animals that demonstrated restoration of locomotor activity rhythms after the graft had a new period that reflected the genotype of the donor animal, rather than the recipient, thus demonstrating that the circadian period was conferred by the donor SCN (Ralph et al., 1990). As a replication and extension of this study, Silver and colleagues (1996) showed that SCN grafts were able to restore a rhythm of locomotor activity to the recipient even when the graft was contained within a capsule that allowed only the diffusion of humoral signals between the graft and host, but prevented the formation of any neural connections, demonstrating that a diffusible signal was sufficient to confer a circadian rhythm to the host animal. Recent evidence indicates that prokineticin 2 (PK2), the protein product of clock controlled gene *PK2*, and transforming growth factor $\alpha$ may be the SCN-secreted factors responsible for the control of locomotor activity and sleep (Cheng et al., 2002, 2005; Kramer et al., 2001).

The results from SCN lesion, island, and graft experiments are not entirely consistent with each other, because SCN grafts can restore the circadian rhythm of locomotor activity, presumably via a diffusible signal from the SCN itself, yet knife cuts that sever only neural connections lead to arrhythmic locomotor activity. However, all of these results provide support for the hypothesis that the SCN is the structure responsible for circadian rhythms in mammals.

**Molecular clockwork.**

The molecular mechanisms that underlie circadian rhythmicity are fairly well understood in mammals and show remarkable homology with other species. The oscillatory mechanism is contained within each cell of the SCN master clock (Herzog et al., 1998; Welsh et al., 1995), and is composed of positive and negative interconnected transcriptional and translational feedback loops. In mammals, the essential clock genes are *Clock, Bmal1, Period* (*Per*), and *Cryptochrome* (*Cry*), along with *Rora,* and *Rev-Erbα* (Reppert & Weaver, 2001, 2002) (see Figure 1).

Evidence that these are the essential components for circadian rhythmicity comes from knock out studies showing that mice with mutations of *Clock, Bmal1, Per1,* and *Per2* (but not *Per3*) become arrhythmic under constant conditions, as do *Cry1/Cry2* double mutant mice (Albrecht et al., 2001; Bae et al., 2001; Bunger et al., 2000; Herzog et al., 1998; van der Horst et al., 1999; Zheng et al., 1999). In contrast *Rev-Erbα* and *Rora* are not essential, but are responsible for fine-tuning the oscillation (Preitner et al., 2002; Sato et al., 2004).

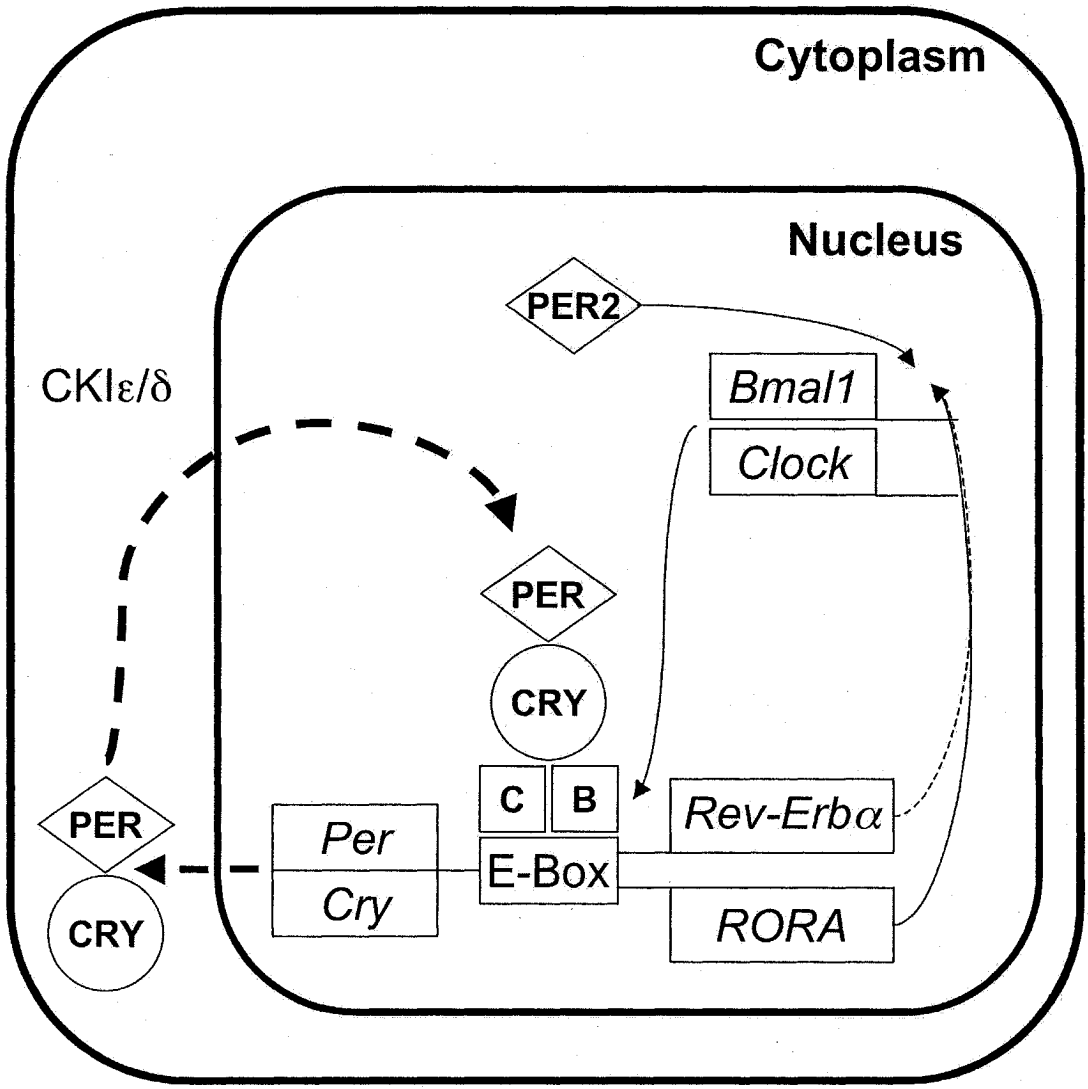


Figure 1. Simplified schematic diagram showing the positive (solid arrows) and negative (dashed arrows) transcription/translation feedback loops of the molecular clock. Clock gene mRNA is indicated by italics, proteins are in bold. **C** = CLOCK protein, **B** = BMAL1 protein. See text for a detailed explanation. Adapted from Reppert & Weaver, 2002.

*Clock* and *Bmal1* encode two basic helix-loop-helix (bHLH)-PER-ARNT-SIM (PAS) domain-containing transcription factors called CLOCK and BMAL1 (brain and muscle ARNT-like protein 1; also known as MOP3), respectively (Hogenesch et al., 1997, 1998; King et al., 1997; Yu et al., 2002). CLOCK and BMAL1 form heterodimers that activate the transcription of three *Period* and two *Cryptochrome* genes through E-box enhancers, and are highly selective for those with the nucleotide sequence CACGTG (Gekakis, et al., 1998; Hogenesch et al., 1997, 1998; King et al., 1997; Kume et al., 1999). The products of the *Per* genes are PAS domain proteins called PERIOD (PER) 1, 2, and 3 (Albrecht et al., 1997, 2001; Shearman et al., 2000; Sun et al., 1997; Zheng et al., 1999). The *Cry* gene encodes the pterin/flavin proteins CRYPTOCHROME (CRY) 1 and 2 (van der Horst et al., 1999).

CRY1, CRY2, PER1, and PER2 form multimeric complexes (Eide et al., 2002) that are translocated back into the nucleus of the cell where they interact with CLOCK and/or BMAL1 to inhibit CLOCK::BMAL1 mediated transcription of *Per* and *Cry* (Albrect et al., 1997; Brown et al., 2005; Kume et al., 1999; Shearman et al., 2000; Yamamoto et al., 2005; Yu et al., 2002). In addition, CRY1, CRY2, and especially PER2 provide positive drive on *Bmal1,* contributing to the positive arm of the transcription/translation system (Reppert & Weaver, 2001; Shearman et al., 2000; Yu et al., 2002; Zheng et al., 1999).

CLOCK::BMAL1 heterodimers also activate the transcription of the orphan nuclear receptor genes *Rora* and *Rev-Erbα*, members of the retinoic acid-related orphan receptor (ROR) family (Preitner et al., 2002; Sato et al., 2004; Ueda et al., 2002). The resulting REV-ERBα and RORA proteins actively compete for binding at the Rev-

Erb/ROR response element in the promoter region of *Bmal1* in a circadian manner (Sato et al., 2004). RORA activates *Bmal1* transcription (Sato et al., 2004), and REV-ERBα represses it (Preitner et al., 2002; Yu et al., 2002).

The time course of this process is as follows: At the beginning of the circadian day (defined as circadian time 0; CT0), CLOCK::BMAL1 heterodimers transcribe *Per*, *Cry, Rora,* and *Rev-Erbα*.. REV-ERBα inhibits *Bmal1* transcription causing BMAL1 levels to fall. By CT12, both PER and CRY proteins are at a high level in the nucleus. At this time CRY, and to a lesser extent PER2, inhibit CLOCK::BMAL1 mediated transcription. At the same time PER2, as well as CRY and RORA, enhance *Bmal1* transcription, causing peak *Bmal1* mRNA levels between CT15 and CT18. BMAL1 protein levels, which lag behind mRNA levels by approximately 4-6 hours, peak from about CT22 to CT24, which begins the next cycle of *Per*, *Cry, Rora,* and *Rev-Erbα* transcription (Reppert & Weaver, 2001; Shearman et al., 2000; Yu et al., 2002).

Post transcriptional phosphorylation and proteolysis are also critical for the function of the circadian clock and control the period and phase of the oscillation (Eide et al., 2002, 2005; Lee et al., 2004; Lowrey et al., 2000; Sherman et al. 2000). For example, casein kinase I epsilon (CKIε) and delta (CKIδ) enzymes phosphorylate PER1 and PER2, which affects the formation of dimers with CRY proteins, movement between cellular compartments, and causes degradation of the proteins. It is hypothesized that CKIs control the timing of nuclear entry by destabilizing PER1 and PER2 and preventing their accumulation until CRY levels are high enough to allow PER::CRY dimers to translocate into the nucleus (Reppert & Weaver 2001).

The locus of the mutation responsible for the short period of the *tau* mutant hamster encodes CKIε (Lowrey et al., 2000). In the *tau* mutant, CKIε binds PER1 and PER2 less efficiently, leading to earlier peak expression of PER1 and PER2 and, in turn to abnormalities in both the positive and negative limbs of the molecular clock oscillation.

Other evidence for the importance of clock protein phosphorylation comes from a recent study showing that PER2 protein expression remains rhythmic in cell cultures where *Per2* mRNA is constitutively expressed, and that the cyclical regulation of PER2 expression is required to maintain the oscillation of the clock gene feedback loop (Yamamoto et al., 2005). Therefore, posttranscriptional regulation of PER2 is essential for the oscillation of the circadian molecular clock in mammals.

In summary, the mammalian circadian clock is comprised of a small number of essential clock genes that together form interactivating feedback loops that produce a circadian rhythm, along with transcriptional and post transcriptional factors that increase the stability of the oscillation.

**A role for clock genes in pathology.**

The essential circadian clock genes are ultimately responsible for circadian rhythms of physiology and behavior (Albrecht et al., 2001; Bunger et al., 2000; Herzog et al., 1998; van der Horst et al., 1999; Zheng et al., 1999). However, there is also evidence implicating clock genes in human and animal pathology. For instance, familial advanced sleep phase syndrome, a dominant genetic trait that causes people to become "morning larks", is caused by a genetic anomaly on the human *Per2* gene (Jones et al., 1999; Toh et al., 2001). Sufferers have sleep episodes of normal duration, but have a short circadian

period and tend to fall asleep around 7:30 pm, a time when normal sleepers are awake and socializing, and wake at 4:30 am, the time of greatest sleepiness for most individuals. This disorder is caused by a mutation of a phosphorylation site within the CKI binding domain of the human *Per2* gene, resulting in reduced phosphorlyation of PER2. This shortens the circadian period and leads to early sleepiness and early wakefulness (Jones et al., 1999; Toh et al., 2001).

The *Per2* gene has also been implicated in the suppression of cancerous tumors. *Per2* knock-out mice fail to show induction of essential clock genes by gamma radiation, and are more likely to develop tumors than wild-type mice (Fu et al., 2002). This finding has also been extended to humans. In comparison with nearby non-cancerous cells, more than 95% of breast cancer cells had non-genetic abnormalities in the promoter regions of *Per1, Per2*, and *Per3* (Chen et al., 2005). This suggests a role for *Per* genes in the suppression of tumor growth or formation.

Circadian clock genes may be involved in the regulation of feeding. Support for this idea comes from research examining the Clock gene analogue neuronal PAS domain protein 2 (NPAS2, also known as MOP4; King et al., 1997; Zhou et al., 1997). Although not an essential circadian gene, NPAS2 is a functional CLOCK analogue that forms heterodimers with BMAL1 and can activate the transcription of *Per1, Per2,* and *Cry1*, and is found mainly in the mammalian forebrain (Kaasik & Lee, 2004; King et al., 1997; Reick et al., 2001; Zhou et al., 1997). NPAS2 mutant mice have difficulty adapting to a day-time feeding schedule, leading to sickness and death, and are impaired in both cued and contextual emotional long-term memory tasks (Dudley et al., 2003; Garcia et al., 2000).

Another interesting example of the role of clock genes in both normal and pathological states is seen in *Clock* mutant mice. These mice are hyperphagic; eat a greater proportion of their daily food intake during the day than wild-type mice; are obese; and have elevated levels of cholesterol, leptin, lipids, glucose, and decreased levels of insulin (Turek et al., 2005). This increase in food intake may be related to an increase in the reward value of the food, based on evidence suggesting *Clock* is important for the regulation of dopamine transmission. *Clock* mutant mice show greater sensitivity to the rewarding effects of cocaine than wild-type animals, possibly due to increased dopamine transmission in the reward circuitry (McClung et al., 2005).

*Per1* and *Per2* have also been implicated in reward and motivational processes, as mice with mutations of either gene have dramatically different responses to chronic cocaine exposure than wild type animals (Abarca et al., 2002), and both alcohol (Chen et al., 2004) and opioids (Vansteensel et al., 2005) have been shown to alter the circadian rhythm of *Per* gene expression in the hypothalamus and SCN respectively. Together, these findings implicate clock genes in sleep disorders, cancers, feeding abnormalities, and the effects of drugs of abuse, suggesting the importance and wide ranging influence of clock genes in both normal and pathological states.

## Circadian Oscillators Outside of the SCN

The first mammalian extra-SCN clock was discovered in the retina of the golden hamster (Tosini & Menaker, 1996). In culture, the hamster retina had a circadian rhythm of melatonin synthesis that could be entrained to a LD cycle. In constant darkness, the rhythm was maintained with a period that reflected the genotype of the animal from

which the retinas were derived: approximately 24 hours in wild-type animals and approximately 21 hours in *tau* mutants. This showed that the mechanism that shortened the period of the master clock, which was unknown at that time, affected general circadian processes, not only the SCN (Tosini & Menaker, 1996). These experiments demonstrated that the eye has all of the properties of a circadian oscillator: intrinsic circadian rhythms, free-running under constant conditions, and temperature compensation (Tosini & Menaker, 1996).

Soon after, the identification of clock genes and the means to monitor their activity led to the discovery of circadian rhythms of clock gene expression in nearly every tissue studied (Abe et al., 2002; Balsalobre et al., 1998, 2000a; Sakamoto et al., 1998; Stokkan et al., 2001; Takata et al., 2002; Wakamatsu et al., 2001). This led to numerous experiments questioning whether the presence of clock genes in these extra-SCN brain regions and peripheral tissues indicated that that these cells were true circadian oscillators, and how circadian control of physiology and behavior might be orchestrated by multiple clocks.

A key piece of evidence that peripheral clock cells were functioning as circadian oscillators came from experiments using cultured hepatoma cells and fibroblasts (Balsalobre et al., 1998). Robust circadian rhythms of *Per1, Per2, Rev-Erbα*, and clock controlled genes *Dbp* and *Tef* lasting for several cycles could be induced in immortalized rat fibroblasts by incubating the cultures in high concentrations of serum. Amazingly, even though some of these cells had been maintained in culture for more than 25 years, they showed gene expression rhythms very similar to that of the SCN (Balsalobre et al., 1998).

Similar results have been found using a transgenic rat transfected with the firefly luciferase gene under the control of the *Per1* promoter to monitor circadian rhythms of tissues *in vitro* (*Per1::Luc*; Yamazaki et al., 2000). Using this technique, cultured SCN cells show a circadian rhythm of luminescence, peaking mid day, for the duration of the experiment (up to 32 days; Abe et al., 2002; Yamazaki et al., 2000). Liver, lung, and skeletal muscle also show circadian rhythms of *Per1::Luc* expression, peaking 7-11 hours later than the SCN. However, unlike the SCN, peripheral tissues remained rhythmic for only 2 to 7 cycles, gradually damping out. This was not due to cell death, as the rhythms of *Per1::Luc* could be reinstated by changing the medium. In addition, the SCN showed very rapid adjustment to a shift in the light schedule 1 or 6 days prior to culture, while the liver, lung and muscle all shifted at different rates, and more slowly than the SCN (Yamazaki et al., 2000). This suggests that the SCN may be driving the rhythm in these peripheral tissues.

Rhythmic expression of *Per1::Luc* has also been found in neural tissue (Abe et al., 2002). Tissue cultures of the pineal, pituitary, arcuate nucleus, and olfactory bulb show rhythmic expression of *Per1::Luc*, with a different phase in each tissue, but generally showing peak expression 9-12 hours after the SCN, during the expected dark phase of the light-dark cycle. Unlike the SCN, but like the organ and muscle tissues, rhythms of *Per1::Luc* in these areas gradually decreased in amplitude and damped out after several days in culture. Rhythmic *Per1::Luc* expression in brain tissues, with the exception of the olfactory bulb, was indifferent to a medium change, but could be restored by applying forskolin, a cAMP analog, to the culture medium (Abe et al., 2002). This suggests that the cultures were healthy and that the cells retained the capacity to

oscillate, but either *Per1::Luc* stopped cycling, or individual cells remained rhythmic but became desynchronized from each other. Subsequent studies in the mouse using a PER2/LUCIFERASE fusion protein (PER2::LUC) reporter indicate that it is the latter of these two explanations that is more likely because, unlike *Per1::Luc*, circadian rhythms of PER2:LUC in peripheral and extra-SCN neural tissues do not dampen out over time, but are self-sustaining (Yoo et al., 2004). This suggests that the observation of dampened rhythms in non-SCN tissues using the *Per1::Luc* reporter may have been an artifact of the methodology, rather than an indication of a gradual loss of circadian rhythmicity of the molecular machinery in those tissues (Yoo et al., 2004).

The hypothesis that peripheral and extra-SCN neural clock cells rely on the SCN for sustained oscillations of the molecular clock mechanism is further supported by examining tissues taken from animals that are behaviorally arrhythmic as a result of SCN lesion (Sakamoto et al., 1998; Yoo et al., 2004). Studies using the Northern blot technique to examine the expression of *Per2* mRNA in the eye, brain, muscle, heart, lung, spleen, liver, and kidney show that circadian rhythms in these tissues were abolished by SCN lesion (Oishi et al., 1998; Sakamoto et al., 1998; Yamazaki et al., 2000). The real time luminescence profile of the PER2::LUC fusion protein suggests that the individual clock cells in these tissues continued to show clock gene oscillation, but that they have become desynchronized from each other in SCN lesioned animals (Yoo et al., 2004). Whereas earlier findings indicated that the SCN was driving the rhythm of clock gene expression in peripheral and extra-SCN neural tissues, these data instead suggest peripheral clocks need continuous input from the SCN to maintain a 24-hour

synchronized phase relationship of clock gene expression between individual cells within tissues, and between tissues.

The exceptions to this rule are the eye and the olfactory bulb. As discussed earlier, retinal synthesis of melatonin does not require SCN input for synchronization, but is entrained by light directly (Tosini & Menaker, 1996). Similarly, the olfactory bulb contains cell autonomous clocks with rhythms of luminescence that match the firing pattern of the neurons (Granados-Fuentes et al., 2004b). Olfactory bulb oscillator is entrained by the SCN, but does not require SCN input for its rhythm, as it shows rhythmic *Per1::Luc* activity after SCN lesion or in constant light (LL), where the SCN *Per1::Luc* luminescence and running wheel behavior are arrhythmic (Granados-Fuentes et al., 2004a). Further, the olfactory bulb oscillator can be directly entrained by temperature and is temperature compensated (Granados-Fuentes et al., 2004b). The olfactory bulb oscillator is not necessary for locomotor activity rhythms but may play a role in olfactory entrainment of the SCN (Amir et al., 1999; Funk & Amir, 2000; Goel et al., 1998; Governale & Lee, 2001).

**Entrainment of peripheral and extra-SCN oscillators by the SCN.**

The SCN can synchronize peripheral and extra-SCN oscillators either by direct neural projections, or by a diffusible or blood borne signal. For example, the pineal gland receives indirect noradrenergic input from the SCN. In the pineal, *Per1* and *Cry2* mRNA are increased by daytime injections of the β-adrenergic agonist isoproterenol. In contrast the β-adrenergic antagonist propranolol, and light exposure given during the night, reduce clock gene mRNA expression. The similar effects of both light and propranolol

suggest that noradrenergic input from the SCN does control the phase of clock gene expression in the pineal (Simonneaux et al., 2004).

Similarly, the circadian modulation of the timing of preovulatory luteinizing hormone (LH) surges from the LH releasing hormone neurons located in the lateral septum, diagonal band, and preoptic nucleus are controlled by a direct ipsilateral projection from the SCN (de la Iglesia et al., 2000, 2003; Van der Beek et al.1997). Knife cuts dorsocaudal to the SCN, which have no effect on the rhythm of locomotor behavior, abolished LH surges (Watts et al. 1989), and once abolished by SCN lesion, LH surges were not restored by SCN transplantation (Meyer-Bernstein et al., 1999).

There are also numerous examples of diffusible or humoral signals mediating SCN control of the circadian rhythmicity of peripheral oscillators. As mentioned earlier, serum shock can induce rhythms of clock gene expression in culture (Balsalobre et al., 1998). The glucocorticoid analogue dexamethasone can also induce rhythmic expression of the *Per, Cry, Rev-Erbα,* and *Dbp* genes in cultured fibroblasts cells, and induce a shift in clock and clock controlled gene expression rhythms in the liver, kidney, and heart *in vivo*, but not the SCN, suggesting that glucocorticoids may act as a synchronizing signal for circadian phase of clock gene expression in peripheral oscillators (Balsalobre et al., 2000a).

The SCN itself seems capable of conferring rhythmicity directly via a diffusible signal, both *in vivo*, as demonstrated in the SCN transplant experiments described earlier (Ralph et al., 1990; Silver et al., 1996), and *in vitro*. Co-cultures of SCN cells separated from fibroblasts by a semi-permeable membrane results in analogous rhythms of 2-deoxyglucose and clock gene expression in both tissue types. SCN cultures remain

rhythmic for the duration of the experiments but fibroblasts show no circadian rhythm of clock gene expression or metabolism when removed from the SCN co-culture, when cultured alone, or when cultured with fibroblasts that have rhythmic gene expression induced by serum shock (Allen et al., 2001). Similarly, explants of adult SCN tissue were able to sustain the rhythmicity *Per1* and *Per2* gene expression of astrocytes in co-culture, whereas co-culture with cortical tissue was not able to do so (Prolo et al., 2005). In addition, nucleated blood cells, which have no neural connection to the SCN, also have rhythmic expression of clock genes in both rats (Oishi et al., 1998), and humans (Boivin et al., 2003; Kusanagi et al., 2004; Takata et al., 2002).

Taken together, these findings suggest that the SCN synchronizes clock gene expression in peripheral and extra-SCN oscillators by a number of different mechanisms. This point is particularly well illustrated by an experiment where parabiosis, the surgical union of two living animals (Finerty, 1952; Harris et al., 1997), was used to join a SCN lesioned mouse to an intact one. A blood-borne signal from the SCN-intact mouse was sufficient to restore the rhythms of *Per1, Per2,* and *Bmal1* expression in the liver and kidney but not the heart, spleen, or skeletal muscle of an SCN lesioned mouse (Guo et al., 2005).

## The Importance of Feeding as a Circadian Zeitgeber

In their research using the PER2::LUC fusion protein to examine circadian oscillations in peripheral tissues, Yoo et al., (2004) showed that the SCN and peripheral and extra-SCN neural tissues show sustained circadian rhythms of clock gene expression for extended periods of time, and even after SCN lesions, individual cells in peripheral

tissues continue to show rhythmic clock gene expression. However, they did reveal desynchronization of the phase of circadian gene expression between tissues, and between animals that had previously been entrained to the same LD cycle (Yoo et al., 2004). If individual cells within one tissue do remain synchronized with each other, it may be the result of a tissue specific signaling mechanism that can coordinate rhythms within organs (Yoo et al., 2004). Perhaps the most important stimulus known to affect peripheral organs is feeding.

Feeding is a potent synchronizer of behavior. Nocturnal rodents will consume the majority of their daily caloric requirement during the dark portion of the LD cycle, when they are active (Clifton, 2000; Rosenwasser et al., 1981; Stewart et al., 1985). When placed on a 24-hour schedule of restricted feeding (RF) that limits food access to a short period during the daytime, nocturnal rodents will show a reliable increase in activity several hours prior to food availability (Richter, 1922; see Stephan, 2002 for a review). This food anticipatory activity only occurs when the RF schedule is in the circadian range, as rats are unable to anticipate feeding schedules that are much shorter or longer than 24 hours (i.e. <18 hours or > 29 hours; Bolles & Stokes, 1965; Stephan et al., 1979a), but were able to anticipate a 23 hour feeding schedule (Stephan et al., 1979b).

Moreover, after entrainment to a 24-hour feeding schedule, a 12-hour phase shift in food availability produced a pattern of behavior essentially identical to that produced by a phase shift in the LD cycle, with rapid daily shifts in behavior occurring until a new stable pattern of entrainment to the feeding schedule was established (Stephan et al., 1979b; Stephan, 1984). In addition, all animals continued to show behavioral anticipation prior to feeding time for several days despite total food deprivation,

suggesting that the anticipatory behavior occurred in response to an internal circadian cue, rather than some external cue associated with the food itself or the experimental procedure (Stephan et al, 1979b). Feeding-related entrainment is not limited to behavioral activity, as the phase of the rhythms of temperature, CORT, insulin and glucagon secretion, and liver metabolism are all shifted by a RF schedule (Damiola et al., 2000; Davidson & Stephan, 1999b; Díaz-Muñoz et al., 2000; Honma et al., 1983; Krieger, 1974; Morimoto et al., 1977).

Interestingly, food anticipatory activity is not dependent on the SCN master clock as both intact and SCN lesioned animals are able to anticipate a 23-hour feeding schedule (Stephan et al., 1979b), but both fail to anticipate an 18-hour feeding schedule (Stephan et al., 1979a). Not only is the SCN not required for entrainment to RF, the SCN itself seems to be unaffected by changes in feeding schedules, as the rhythm of neural activity in the SCN is not entrained by day time feeding (Inouye, 1982; Shibata et al., 1983). This suggests that there are separate oscillators that are entrainable by light and feeding. Further evidence for the existence of multiple oscillators comes from studies showing that intact animals can simultaneously display behavioral anticipation of a 24-hour feeding schedule and an endogenous free-running circadian period of behavior of slightly more or less than 24-hours under constant lighting conditions (Castillo et al., 2004; Edmonds & Adler, 1977a; Mistlberger, 1993; Stephan, 1986). Entrainment to multiple feeding schedules in intact (Boulos & Logothetis, 1990; Edmonds & Adler, 1977b) and SCN-lesioned animals (Boulos & Logothetis, 1990; Stephan, 1989) has also been demonstrated.

These results clearly show that food is a potent Zeitgeber (time giver), capable of producing behavioral entrainment without SCN input. Lesions studies in which extensive damage to the olfactory bulbs (Davidson et al., 2001), the ventromedial, paraventricular, and lateral hypothalamic nuclei (Mistlberger & Rechtschaffen, 1984; Mistlberger & Rusak, 1988), the nucleus accumbens, bed nucleus of the stria terminalis (BNST), hippocampus, and amygdala (Mistlberger & Mumby, 1992) were produced, have failed to identify the neuroanatomical substrate of the food-entrainable oscillator. Vagotomy (Comperatore & Stephan, 1990) and even complete deafferentation of the visceral inputs to the brain also fail to block food anticipatory activity (Davidson & Stephan, 1998). Only the parabrachial nucleus (PB), an area that receives visceral and gustatory information, and projects to the thalamus, hypothalamus, BNST and amygdala (Saper & Loewy, 1980) has been identified as essential in the expression of food anticipatory activity (Davidson et al., 2000). This has led to the suggestion that restricted feeding does not entrain a single, anatomically discrete food entrainable oscillator, but likely affects multiple oscillators located in the brain and peripheral organs.

Evidence for this proposal comes from studies demonstrating that circadian oscillators identified in the periphery become uncoupled from the SCN master clock when animals are placed on a RF schedule. When feeding is restricted to the day in nocturnal rodents, clock gene mRNA expression in the liver and other peripheral tissues shifts up to 12 hours in anticipation of the scheduled feeding time, while gene expression in the SCN remains entrained to the LD cycle, irrespective of feeding schedule (Damiola et al., 2000; Hara et al., 2001; Stokkan et al, 2001). Similarly, animals housed in constant darkness (DD) that have their feeding restricted to the subjective day (inactive period)

show shifts in clock gene expression in the liver, but not the SCN (Damiola et al., 2000; Hara et al., 2001). Further, the SCN is not required for entrainment of the liver by feeding, as both sham and SCN lesioned animals showed equivalent shifts in liver clock gene expression in response to RF (Hara et al., 2001).

Interestingly, glucocorticoid signaling seems to be critical for modulating the speed of entrainment of the liver by RF (Le Minh et al., 2001). Adrenalectomized mice on RF demonstrated more rapid shifts in clock gene expression in the liver and kidney than intact animals, an effect mediated by glucocorticoid receptors (GR) as mutant mice lacking GR exclusively in the hepatocytes only demonstrated rapid phase shifting in the liver in response to RF, while other organs shifted more gradually. Again, the SCN was unaffected (Le Minh et al., 2001).

Of critical importance is the finding that RF also affects the brain, as suggested by an early experiment showing that RF caused a reversal of the day night rhythms of the neurotransmitters serotonin and norepinephrine in the hippocampus (Krieger, 1974). Similar to the finding from experiments looking at peripheral tissues, RF shifted the peak expression of *Per1* and *Per2* to daytime in the cortex and hippocampus of mice on a LD or DD cycle, again without having any effect on the SCN (Wakamatsu et al., 2001). This effect did not require an intact SCN, because it occurred equally in both sham and SCN-lesioned animals (Wakamatsu et al., 2001). Together these studies strongly suggest that the entraining effects of restricted feeding on the phase of clock gene expression in cells outside the master clock are not limited to the periphery, but also occur in the brain. In summary, circadian oscillator cells in the periphery and brain are able to respond to, and even anticipate, temporal changes in feeding. Entrainment in the peripheral tissues and

extra-SCN brain regions is necessary for rapid adjustment to temporal changes in feeding in order to maximize the efficient use of energy resources. In contrast, the SCN, which must regulate numerous circadian functions in accordance with the LD cycle, regardless of the timing of daily feeding, is unaffected by RF.

## What is the Function of Subordinate Brain Oscillators?

There is compelling evidence for the presence of circadian oscillators in the brain and periphery. Although the function of these oscillators has not been firmly established, they seem to be responsive to tissue specific signals. The retina and olfactory bulb, which are upstream of the SCN, are independent of the master clock and may play a role in its entrainment (Granados-Fuentes et al., 2004a, b; Goel, et al, 1998; Governale & Lee, 2001; Tosini & Menaker, 1996).

The liver and other peripheral tissues, on the other hand, are downstream of the SCN. Therefore, circadian rhythms of clock gene expression in peripheral tissues would be synchronized by the master clock when the LD cycle and other zeitgebers like feeding, are in phase, but could become uncoupled from the master clock when light and feeding were in opposition (Damiola et al., 2000; Hara et al., 2001; Stokkan et al, 2001). This type of coupled multiple oscillator system would be very flexible, allowing individual tissues to adjust as necessary to the changing environment, but remain co-coordinated over longer time scales; days, weeks and even years.

The presence of circadian oscillators in the brain in addition to the master clock (Abe et al., 2002) suggests a similar system of multiple coupled oscillators is needed to integrate information from multiple brain regions in order to fine-tune the circadian

control of physiology and behavior. This would include the integration not only of sensory information, metabolic state, and social cues, but also the internal emotional state. Evidence that this occurs is most apparent when the process of integration has broken-down. Some of the defining symptoms of seasonal affective disorder, depression, and bipolar disorder are circadian rhythms disturbances (Bunney & Bunney, 2000; American Psychiatric Association, 2000). Primary among these are sleep disturbances, but they also include increased nocturnal body temperature, daily mood swings, and disturbed melatonin and cortisol rhythms. In particular, an advanced phase of nocturnal cortisol level relative to time of sleep onset has been noted (Bunney & Bunney, 2000; Zerssen et al., 1985).

Treatments designed to improve the synchronization of circadian controlled behaviors and physiological functions have proven to be effective in the treatment of depression. For example, morning bright light therapy is an effective treatment for seasonal affective disorder, with patients often showing full recovery from all symptoms after several weeks of treatment (Bunney & Bunney, 2000; Terman et al., 2001). Although it is not as effective in the treatment of major depression, light therapy has also been shown to improve mood, attention, concentration, sleep, and cortisol and melatonin rhythms (Yamada et al., 1995). Phase advancing bedtime to reestablish synchronization between the already advanced cortisol and temperature rhythms and the sleep-wake cycle, either alone or in combination with one night of total sleep deprivation, has also proven effective in depressed patients who were resistant to pharmacotherapy (Berger et al., 1997; Riemann et al., 2002). Further, there is some evidence that antidepressant medications can alter clock gene expression in the hippocampus and striatim (Uz et al.,

2005). These results provide evidence for the interrelationship of emotional state and circadian rhythms.

Amir and Stewart (1998, 1999) demonstrated that emotional and motivational stimuli have an important influence on the circadian system. When an animal is maintained in constant darkness, exposure to a light pulse during the critical periods at the beginning and end of the subjective night can cause a phase shift in the free-running activity rhythm. Exposure to a light in a context where rats had previously received foot-shock attenuated the light-induced phase shift in free-running activity rhythms compared to animals that were exposed to the light in that context, but never received foot-shock (Amir & Stewart, 1998). In addition, they found a significant attenuation of the expression of the immediate early gene FOS in the SCN, a molecular correlate of clock resetting. They also demonstrated that light specifically paired with foot-shock caused attenuation of FOS in the SCN, but non-contingent presentation of light and shock did not (Amir & Stewart, 1999). These studies show that the emotional state of the animal is able to affect both circadian controlled running behavior and clock resetting. The association of the light with the foot-shock must be integrated with the signal coming to the master clock from the eye via the RHT. This could potentially occur in the limbic forebrain.

## The Present Thesis

The presence of circadian clock gene expression in the brain and most tissues of the periphery suggests that circadian control of physiology and behavior occurs at a local level in addition to more global control by the master clock. There are clock cells in the

eye and olfactory bulb that may modulate incoming sensory information to influence entrainment of the master clock, whereas the synchronization of clock cells in the liver to feeding time may allow for maximal metabolic efficiency. Following this line of reasoning, circadian oscillators in the brain may respond to region specific inputs allowing for a greater level of flexibility in the processing and integration of information resulting in ecologically appropriate behaviors. However, very little is actually known about extra-SCN brain oscillators. Therefore, the main goal of this thesis was to study circadian oscillators in the limbic forebrain to determine how they are regulated and what function they might serve in the integration of behavioral, physiological, and emotional states with the circadian system. We used immunocytochemistry for the clock gene protein product PER2 as a marker for clock gene oscillators in the limbic forebrain with the goal of determining if rhythmic expression of PER2 was present in the limbic forebrain, and if so, describing the patterns of PER2 expression, the relationship between the expression of PER2 in the limbic forebrain and SCN master clock, and determining what function putative circadian oscillators might serve in the limbic forebrain.

**The limbic forebrain.**

The limbic forebrain is made up of a number of anatomically distinct regions that together are responsible for the integration of sensory information, emotion, and memory with physiological and behavioral outputs and thus are critical for the survival of an animal. Among these, the BNST is a heterogeneous collection of nuclei (Dong et al., 2001a, b; Dong & Swanson 2003, 2004) collectively involved in fear learning, learned despair, sexual and maternal behavior, drug relapse, sympathetic processes, and regulation of the hypothalamic pituitary adrenocortical (HPA) axis (Ciccocioppo et al.,

2003; Claro et al., 1995; Davis, 1998; Erb et al., 2001a; Komisaruk et al., 2000; Lee & Davis, 1997; Leri et al., 2002; Ramos & DeBold, 2000; Schulz & Canbeyli, 2000; Walker et al., 2003; Zhu et al., 2001).

An interesting finding that suggests an important relationship between the SCN and BNST is that peak neural activity in these two regions is synchronized. As described earlier, most of the brain shows a circadian rhythm of electrical activity (Inouye & Kawamura, 1979), and clock gene expression (Abe et al., 2002). For example, the septum, nucleus accumbens, caudate putamen, and medial preoptic region, among others, demonstrate increased neuronal activity that is in phase with locomotor activity, peaking during the night or subjective night. In contrast, the SCN and BNST are 180° out of phase with locomotor activity and all other brain regions studied, with peak neuronal activity during the day (Yamazaki et al., 1998).

Of particular interest is the identification of the BNST in processes related to energy balance. Carr and colleagues (Carr et al., 1998, 1999) found that the expression of the immediate early gene protein FOS was generally higher in most brain areas in food-restricted rats than in *ad libitum* (AL) fed animals. The exception was the lateral dorsal division of the BNST, also known as the oval nucleus (BNST-OV), which showed higher FOS expression in AL fed animals, particularly after injections of naltrexone. Carr and colleagues suggested that the BNST-OV might be responsible for the compensatory ingestion that follows a period of fasting (Carr et al., 1998, 1999).

The amygdala is also an area of interest. Like the BNST, the amygdala is a heterogeneous collection of nuclei (Swanson, 2003) responsible for emotion, memory, sexual and social behavior, feeding, drug addiction and relapse, and modulation of

neuroendocrine functions (Alheid et al., 1995; Bennett et al., 2002; Cardinal et al., 2002; Conrad et al., 2004; Petrovich & Gallagher, 2003; Roozendaal & McGaugh, 1997; See et al., 2003; Shelley & Meisel, 2005; Stark, 2005). In terms of emotion, the amygdala is particularly important for fear and anxiety (Aggleton, 1992, 2000). Klüver-Bucy syndrome, first described in monkeys, was characterized by a loss of aggression, fearlessness, and visual agnosia (Klüver & Bucy, 1937, 1939), caused by bilateral temporal lobectomy. These symptoms were found to be due to lesion of the amygdala, rather than the overlying cortical tissue. Specifically, lesions of the central nucleus of the amygdala (CEA) cause rhesus monkeys to lose the fear of snakes and humans, and interferes with both the learning and expression of anxiety-like behaviors in the rat (Davis, 1992; Kalin et al., 2004; Kopchia et al., 1992; Paré et al., 2004).

Interestingly, the CEA and the BNST-OV are highly interconnected and morphologically and neurochemically similar (Day et al., 1999; Dong et al., 2001a, b; Ju & Swanson, 1989; Ju et al., 1989; Swanson, 2003). As a result of the interrelationship between the CEA and BNST-OV, the term 'central extended amygdala' has been adopted by some anatomists (Alheid, 2003; Alheid et al., 1995). The CEA and BNST are also functionally related. For example, the CEA is activated by stimuli that have been explicitly paired with an aversive stimulus, and therefore is involved in fear, while the BNST responds to less explicit fearful cues or environments presented for a longer duration, more akin to anxiety (Davis, 1998; Lee & Davis, 1997; Shulkin et al., 1998; Walker et al., 2003). Both areas are also involved in drug addiction and relapse (Day et al., 2001; Erb et al., 2001a, b; Leri et al., 2002) and salt appetite (Johnson et al., 1999; Zardetto-Smith et al., 1994).

In contrast, the CEA and basolateral nucleus of the amygdala (BLA) are anatomically and functionally distinct (Swanson, 2003). Except for the lack of a laminar structure, the BLA is more similar to the neighboring cortex than to the CEA (Alheid et al., 1995; Swanson, 2003). It does not project strongly to the BNST (Dong et al., 2001a), but instead projects mainly to the CEA and other amygdaloid nuclei, the cortex, and hippocampus, via the entorhinal cortex (Petrovich et al., 2001; Pikkarainen et al., 1999; Pitkanen et al., 1995; Savander et al., 1995). The BLA and CEA also show dissociated but complimentary functions. For example, Corbit and Balleine (2005) demonstrated that lesions of the BLA affected a rat's ability to respond to a change in the value of a reward, but general motivation was left intact, whereas the opposite pattern was observed after CEA lesions, suggesting that the BLA is responsible for association between conditioned stimuli and the value of unconditioned stimuli, and the CEA is responsible for the effects of conditioned motivation and arousal on behavior (Cardinal et al., 2002; Everitt et al., 2003). The BLA is important for the integration of hormonal influences on memory (Conrad et al., 2004; Bhatnagar et al., 2004; McGaugh et al., 2000; Roozendaal & McGaugh, 1997; Roozendaal et al., 2003, 2004) and direct stimulation of the BLA modulates synaptic plasticity in the hippocampus (Nakao et al., 2004).

Like the BNST, the BLA also seems to play a role in feeding behavior, particularly in making meaningful associations between feeding and food related cues (Petrovich & Gallagher, 2003). An example of this is conditioned taste aversion (Nachman & Ashe, 1974; Rolls & Rolls, 1973), where a novel taste is made aversive by pairing it with chemically induced nausea. Although the specific region of the amygdala responsible for conditioned taste aversion is a matter of debate (Lamprecht & Dudai,

2000), some studies have shown that lesions of the BLA eliminate conditioned taste aversion (Rolls & Rolls, 1973). Finally, the importance of the BLA for associative learning also seems to play a role in relapse to taking drugs of abuse (See et al., 2003).

Together with the BNST, BLA, and CEA, the hippocampus is likely to be critical for the integration of emotional and motivational state with circadian controlled behavior. The hippocampus sends extensive, topographically organized projections to most of the amygdala (Petrovich et al., 2001). As mentioned earlier, Wakamatsu and colleagues (2001) demonstrated that the hippocampus is sensitive to circadian entrainment by scheduled feeding. Although the function of feeding sensitive, rhythmic clock cells in the hippocampus is unknown, it may be related to the critical role of the hippocampus in learning and memory (Lynch, 2004; Shors, 2004). Synaptic plasticity in the form of long-term potentiation and neurogenesis in the dentate gyrus (DG) of the hippocampus are thought to be processes underlying learning and memory (Altman, 1963; Bliss & Lomo, 1973; Gould et al., 1999; Lynch, 2004). The hippocampus also seems to play a role in the modulation of emotional state, as reduction in hippocampal volume has been correlated with depression (Campbell & MacQueen, 2004; Sheline et al., 1996, 2003) and therapeutic effects of antidepressant therapy may be the result of hippocampal neurogenesis (Malberg & Duman, 2003; Malberg et al., 2000; Santarelli et al., 2003).

The hippocampus is also critical for the modulation of the HPA axis and is vulnerable to the damaging effects of chronically elevated glucocorticoid levels (Jacobson & Sapolsky, 1991; McEwen, 2001). The BNST acts as a relay between the hippocampus and amygdala and the hypothalamus, thus mediating hippocampal activation of the HPA axis (Foray & Gysling, 2004; Zhu et al., 2001). Interestingly,

stress activated glucocorticoids are thought to impair spatial memory via a BLA-dependant mechanism (Bhatnagar et al, 2004; Roozendaal & McGaugh, 1997; Roozendaal et al., 2003, 2004), suggesting an important functional relationship between the BLA and DG. Together the hippocampus, BNST and amygdala are responsible for the integration of memory and emotional state in the control of the physiology and behavior of the animal. Therefore, the presence of circadian oscillators in these critical regions could provide the means for learning, memory and emotional state to affect circadian rhythms downstream of the master clock.

**Experiments.**

The synchronization between the neural activity in the BNST and SCN (Yamazaki et al., 1998), suggested the possibility of synchronized oscillations of clock cells in these two regions. This was supported by the discovery in our laboratory of rhythmic expression of PER2 in the BNST-OV of the rat and mouse that was synchronized and in phase with that of the SCN. Chapter 1 describes the relationship between the BNST and SCN by examining the rhythm of the clock gene protein PER2 in both regions under different lighting conditions: LD, DD, LL, and in blind animals. This relationship was further characterized by examining the effect of SCN lesions, advance and delay of the LD cycle, and removal of adrenal hormones. The next step, described in Chapter 2, was to determine whether the synchrony in the rhythm of PER2 expression between the SCN and BNST-OV could be uncoupled using a schedule of restricted feeding. This was carried out under a 12 hour LD schedule, and under LL, a condition known to cause arrhythmicity of PER2 expression in the SCN (Beaulé et al., 2003a).

The daily rhythmic expression of PER2 was also examined in the amygdala and hippocampus, and described in Chapter 3. It was discovered that the CEA showed rhythmic PER2 expression that was in phase with that of the BNST-OV, but that the BLA and DG of the hippocampus showed rhythmic PER2 expression that was diametrically opposed to that of the CEA and BNST-OV. The influence of the master clock on the rhythmic oscillations of PER2 expression in the amygdala and hippocampus was characterized using targeted lesions of the SCN, as was the influence of adrenal hormones.

Finally, in Chapter 4, the relative contribution of the rewarding vs. the metabolic aspects of scheduled feeding to rhythmic PER2 expression in the limbic forebrain oscillators were examined. Animals were given either AL access to food or placed on a restricted feeding schedule under both LD and LL conditions, and the resulting effects on both behavior and PER2 expression in the SCN, BNST-OV, CEA, BLA, and DG were observed. In an attempt to discriminate the relative contributions of rewarding and metabolic effects of restricted feeding, animals were also given scheduled access to the rewarding liquids sucrose and saccharine, or saline after acute or chronic sodium depletion. Results indicate that the combination of reward and metabolic challenge that occur with restricted feeding, but neither one alone, have a profound effect on PER2 expression in the limbic forebrain.

## SECTION 1: RHYTHMIC EXPRESSION OF PER2 IN THE BNST-OV AND SCN

In Chapter 1, the circadian rhythm of expression of the clock gene protein PER2 in the BNST-OV and SCN was examined under LD, DD, and LL conditions, and in blind animals. In addition, the effects of SCN lesions, advance and delay of the LD cycle, and removal of the adrenal glands on PER2 expression in the BNST-OV were studied. In Chapter 2, the phase relationship of the rhythm of PER2 expression between the SCN and BNST-OV was examined in animals given AL access to food was compared to that of animals whose access to food was restricted to three hours during the daytime under a 12 hour LD schedule and under LL.

# CHAPTER 1

## 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

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**Abstract**

Circadian rhythms in mammals are regulated not only globally by the master clock in the suprachiasmatic nucleus (SCN), but also locally by widely distributed populations of clock cells in the brain and periphery that control tissue-specific rhythmic outputs. Here we show that the oval nucleus of the bed nucleus of the stria terminalis (BNST-OV) exhibits a robust circadian rhythm in expression of the Period2 (PER2) clock protein. PER2 expression is rhythmic in the BNST-OV in rats housed under a light/dark cycle or in constant darkness, in blind rats, and in mice, and is in perfect synchrony with the PER2 rhythm of the SCN. Constant light or bilateral SCN lesions abolish the rhythm of PER2 in the BNST-OV. Large abrupt shifts in the light schedule transiently uncouple the BNST-OV rhythm from that of the SCN. Re-entrainment of the PER2 rhythm is faster in the SCN than in the BNST-OV and it is faster after a delay than an advance shift. Bilateral adrenalectomy blunts the PER2 rhythm in the BNST-OV. Thus, the BNST-OV contains circadian clock cells that normally oscillate in synchrony with the SCN, but these cells appear to require both input from the SCN and circulating glucocorticoids to maintain their circadian oscillation. Taken together with what is known about the functional organization of the connections of the BNST-OV with systems of the brain involved in stress and motivational processes, these findings place BNST-OV oscillators in a position to influence specific physiological and behavioral rhythms downstream from the SCN clock.

## Introduction

The suprachiasmatic nucleus (SCN) of the hypothalamus is the primary circadian clock regulating daily rhythms in behavior and physiology in mammals (Klein et al., 1991). The cells of the SCN exhibit intrinsic circadian oscillations in gene expression and in electrical and metabolic activity (Inouye & Kawamura, 1979; Schwartz et al., 1980; Gillette & Reppert, 1987; Welsh et al., 1995; Reppert, 2000; Shearman et al., 2000; Beaulé et al., 2001; Reppert & Weaver, 2001), destruction of the SCN abolishes all circadian rhythms, and, most importantly, transplants of fetal SCN tissue, but not tissue from other brain regions can restore circadian behavioral rhythms in SCN-ablated animals (Ralph et al., 1990; Aguilar-Roblero et al., 1992; LeSauter et al., 1996; Li & Satinoff, 1998; Sollars & Pickard, 1998). How the oscillations of the SCN are translated into behavioral and physiological rhythms is not well understood. A leading hypothesis is that the gating of circadian information into specific rhythmic outputs is mediated by tissue-specific subordinate oscillators that are entrained by the SCN clock (Yamazaki et al., 2000; Buijs & Kalsbeek, 2001; Balsalobre, 2002; Reppert &Weaver, 2002; Schibler & Sassone-Corsi, 2002; Hastings et al., 2003).

The bed nucleus of the stria terminalis (BNST) is a complex and neurochemically heterogeneous basal forebrain structure that modulates a wide range of physiological and motivational processes. These include, but are not limited to, neuroendocrine, autonomic and behavioral responses to different types of stress and to drugs of abuse, ingestive behaviors, and reproductive and maternal behaviors (Fink & Smith, 1980; Casada & Dafny, 1991; Loewy, 1991; Gray, 1993; Davis et al., 1997; Van de Kar & Blair, 1999; Stefanova & Ovtscharoff, 2000; Erb et al., 2001a; Nijsen et al., 2001; Walker et al., 2001;

Figueiredo et al., 2003; Walker et al., 2003). Notably, many of the processes influenced by the BNST are under circadian control. Furthermore, there is anatomical and electrophysiological evidence that the BNST is linked to the SCN and that these structures exhibit synchronous rhythms in neural activity (Watts et al., 1987; Yamazaki et al., 1998; Leak & Moore, 2001). Such evidence, taken together with what is known about the functional organization of the connections of the BNST with systems of the brain involved in stress and motivational processes, places the BNST in a position to influence specific physiological and behavioral rhythms. To begin to explore this possibility, we asked first whether the BNST contains circadian clock cells that are controlled by the SCN. To this end, we studied the expression of the Period2 protein (PER2) in the BNST in rats. The rhythmic expression of PER2 is considered to be a defining feature of the molecular oscillators generating circadian rhythms in mammalian cells and analyses of the expression of *Per2* mRNA and PER2 have been used to identify clock cells in both neural and non-neural tissues (Oishi et al., 1998; Zheng et al., 1999; Field et al., 2000; Nuesslein-Hildesheim et al., 2000; Bittman et al., 2003; Shieh, 2003).

## 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. Adult male Wistar rats (275-300 g), bilaterally adrenalectomized Wistar rats, and adult male CF-1 mice (25-30 g) were purchased from Charles River (St-Constant, Québec). All animals had *ad libitum* access to food and water. Adrenalectomized rats were given

saline solution (0.9% NaCl) as a drinking fluid. Rats were housed individually in clear plastic cages equipped with running wheels. The cages were housed in ventilated, sound- and light-tight isolation chambers equipped with a computer-controlled lighting system (VitalView Mini Mitter Co. Inc., Sunriver, OR). Each running wheel was equipped with a magnetic microswitch connected to a computer. Wheel-running activity data were recorded with VitalView software (Mini Mitter Co.) and analyzed with Circadia Software. Mice were housed 3 per cage in isolation chambers.

**Surgery**

Rats were anesthetized with a ketamine (100mg/ml)/xylazine (20mg/ml) mixture given intraperitoneally (1.5ml/kg). Electrolytic lesions, aimed at the SCN (1.2 mm posterior to bregma; 1.9 mm lateral to the midline; 9.4 mm below the surface of the skull, at a 10° angle) were made by passing 2 mA current for 15 seconds through stainless steel electrodes (0.28mm in diameter), insulated except for the tip, using a Grass lesion maker (D.C. LM5A; Grass Instruments, West Warwick, RI). Neonatal enucleation was performed on 2-3 d-old male Wistar rat pups, under isoflurane anaesthesia, by severing the optic nerve, muscle, and other connective tissues and removing the eyes.

**Tissue Preparation**

Rats were injected with an overdose of sodium pentobarbital (~100 mg/kg) and were perfused intracardially with 300 ml of cold saline (0.9% NaCl) followed by 300 ml of cold, 4% paraformaldehyde in a 0.1 M phosphate buffer, pH 7.3. Mice were perfused intracardially with 75 ml of cold saline followed by 75 ml of cold 4% paraformaldehyde. After perfusion, brains were postfixed in 4% paraformaldehyde and stored at 4° C

overnight. Serial coronal brain sections (50 μm) containing the SCN and BNST were collected from each animal using a vibratome.

**Immunocytochemistry**

Free floating sections were washed in cold 50 mM Tris buffered saline (TBS), pH 7.6, and incubated at room temperature for 30 min in a quenching solution made of TBS and 3% w/w $H_2O_2$. After the quenching phase, sections were rinsed in cold TBS and incubated for 1 hr at room temperature in a preblocking solution made of 0.3% Triton X-100 in TBS (Triton-TBS), 3% normal goat serum, and 5% milk buffer. After the preblocking phase, sections were transferred directly into an affinity-purified rabbit polyclonal antibody raised against PER2 (ADI, San Antonio, TX) diluted 1:1000 with a solution of Triton-TBS with 3% normal goat serum in milk buffer. Sections were incubated with the primary antibody for 48h at 4°C. After incubation in the primary antibody, sections were rinsed in cold TBS and incubated for 1 hr at 4 °C with a biotinylated anti-rabbit IgG made in goat (Vector Labs, Burlingame, CA), diluted 1:200 with Triton-TBS with 2% normal goat serum. After incubation with secondary antibody, sections were rinsed in cold TBS and incubated for 2 hr at 4°C with an avidin-biotin-peroxidase complex (Vectastain Elite ABC Kit; Vector Labs). After incubation with the ABC reagents, sections were rinsed with cold TBS, rinsed again with cold 50 mM Tris-HCl, pH 7.6, and again for 10 minutes with 0.05% 3,3'-diaminobenzidine (DAB) in 50 mM Tris-HCl. Sections were then incubated on an orbital shaker for 10 min in DAB/Tris-HCl with 0.01% $H_2O_2$ and 8% $NiCl_2$. After this final incubation, sections were rinsed in cold TBS, wet-mounted onto gel-coated slides, dehydrated through a series of alcohols, soaked in Citrisolv (Fisher Scientific, Houston, TX), and cover-slipped with

Permount (Fisher). Blocking experiments performed by adding the PER2 peptide (1mg/ml in PBS, pH7.4 with 0.02% sodium merthiolate, diluted 1:100) to the primary incubation solution prevented PER2 staining.

**Data Analysis**

Stained brain sections were examined under a light microscope and images were captured using a Sony XC-77 video camera, a Scion LG-3 frame grabber, and NIH Image (v1.63) software. Cells immunopositive for PER2 were counted manually using the captured images. For analysis, the mean number of PER2 immunoreactive cells per region was calculated for each animal from the counts of six unilateral images showing the highest number of labeled nuclei. Differences between groups were revealed with ANOVA. Significance threshold was set at 0.05 for all analyses.

## Results

**Synchronous rhythms in PER2 expression in the SCN and oval nucleus of the BNST.**

We first examined the expression of PER2 in the BNST and SCN of rats that were housed under a 12h light/dark (LD) cycle (300 lux at cage level) for at least 20 d and killed at one of eight equally spaced zeitgeber times (ZTs) (ZT0 denotes time of light on) over the day and night (n = 4 per time point). Analysis of PER2 expression in the BNST revealed dense nuclear staining within the oval nucleus (BNST-OV), an anatomically circumscribed and a neurochemically distinct structure located in the dorsal lateral region of the BNST (Dong et al., 2001b) (Figure 2). PER2 expression within the BNST-OV was strongly rhythmic, maximal at the beginning of the dark phase of the entraining

photocycle, at ZT12, and minimal at ZT0, the onset of the light phase (Figure 3a,b). In all other subregions of the BNST immunostaining for PER2 was exceedingly low at all times. The daily pattern of PER2 expression in the BNST-OV was identical to that seen in the SCN (Figure 3a,b), demonstrating that under entrained conditions the BNST-OV and SCN exhibit synchronous rhythms in expression of PER2.

**Synchronous rhythms in PER2 expression in the SCN and BNST-OV persist in constant darkness and in blind rats.**

The finding that the BNST-OV and SCN exhibit synchronous daily rhythms in expression of PER2 suggests strong coupling between the SCN clock and putative clock cells in the BNST-OV. Such coupling may be mediated intrinsically, independent of the light cycle, or it may be the result of a common effect of the light cycle on *Per2* gene expression in the two structures. This latter possibility is consistent with evidence that PER2 expression in the SCN is synchronized by the light cycle (Field et al., 2000), and by the finding that the BNST receives input from the eyes (Itaya et al., 1981; Cooper et al., 1994). To exclude the role of the photocycle, we assessed the expression of PER2 immunoreactivity in rats that were housed in constant darkness (DD) for 3 d and killed at one of eight equally spaced circadian times (CTs) [CT12 denotes the onset of daily activity (subjective night) under DD conditions] during the subjective day or night ($n = 4$ per time point). In addition, to examine the role of the eyes, themselves (Beaulé & Amir, 2003; Lee et al., 2003), we assessed the expression of PER2 in BNST-OV and SCN of adult blind rats that were enucleated during the neonatal period. PER2 expression in the BNST-OV of DD housed rats was strongly rhythmic, peaking at the beginning of the

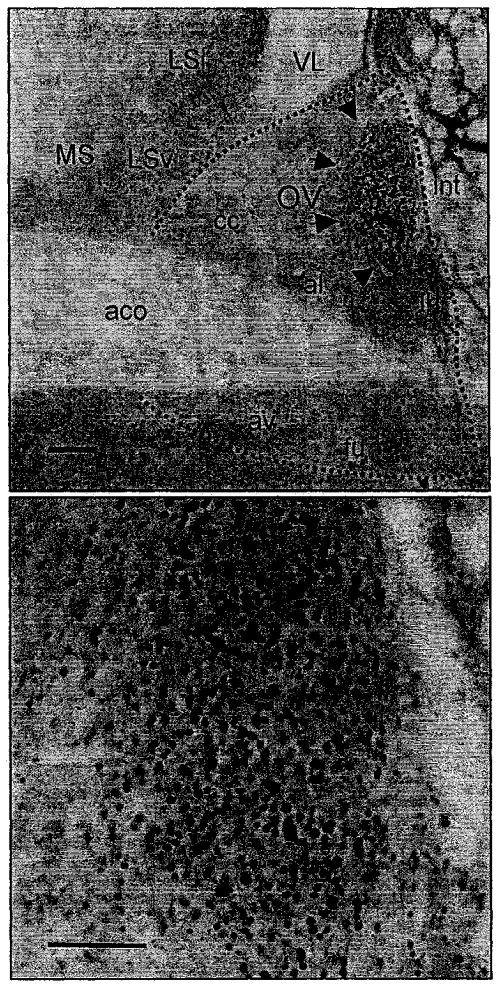


Figure 2. Expression of PER2 immunoreactivity in the BNST-OV. Top, Photomicrograph of a coronal brain section containing the BNST from a rat killed at ZT12. PER2 immunoreactivity is concentrated in the BNST-OV (marked by the black arrows). Expression in all other subdivisions of the BNST is exceedingly low. Scale bar, 200μm. Bottom, High magnification of BNST-OV showing PER2 immunostaining. Scale bar, 100μm. Abbreviations: aco, anterior commissure; av, anteroventral BNST; fu, fusiform nucleus of the BNST; al, anterolateral BNST; ju, juxtacapsular nucleus of the BNST; cc, anterolateral BNST central core; int, internal capsule; MS, medial septal nucleus; Lsv, ventral lateral septal nucleus; Lsi, intermediate part of the lateral septal nucleus; VL, lateral ventricle.

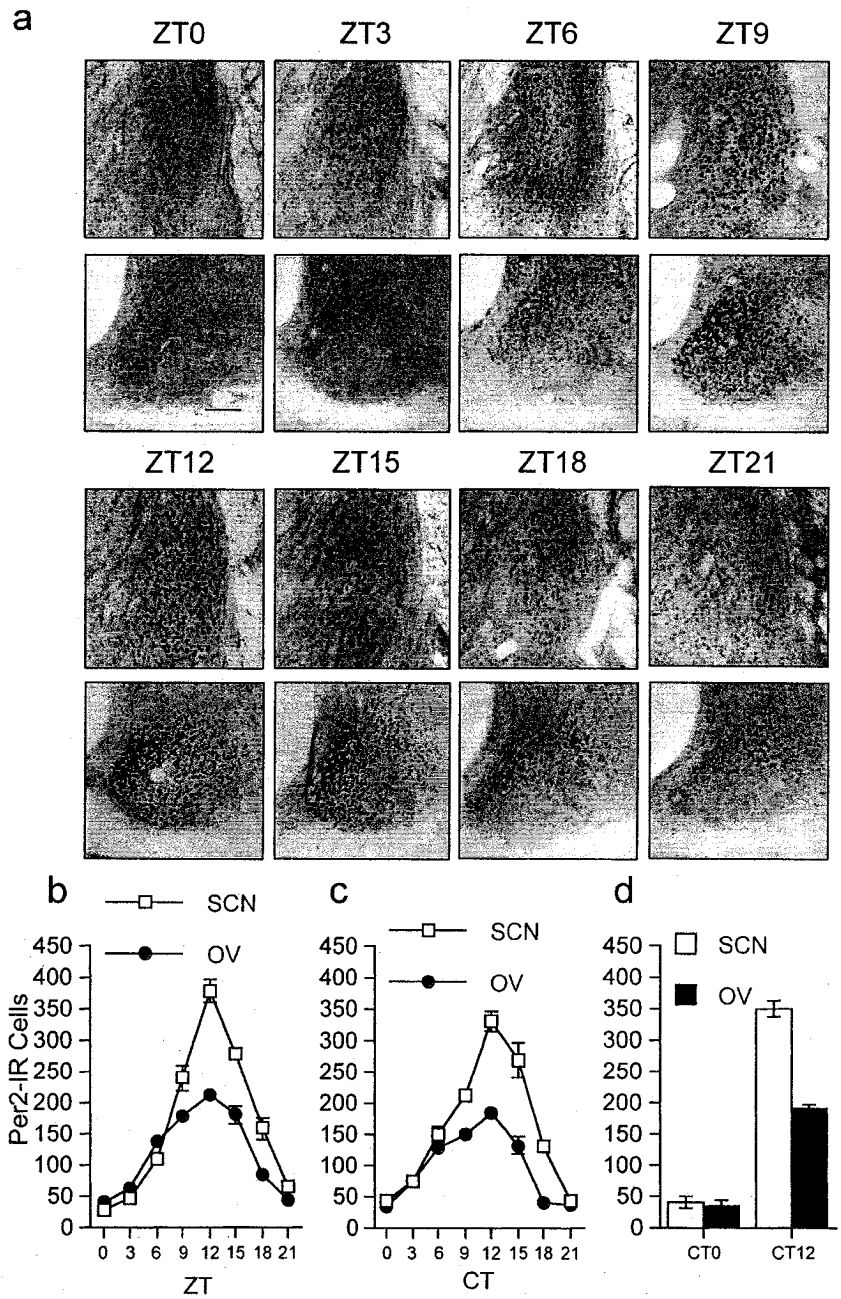


Figure 3. Synchronous rhythmic expression of PER2 in the BNST-OV and SCN. a, Photomicrographs showing examples of PER2 immunostaining in the BNST-OV and SCN from rats housed under a 12hr LD cycle and killed at different ZTs (ZT0, lights on; ZT12, lights off). Scale bar, 200μm. b, Mean ± SEM number of PER2-immunoreactive (Per2-IR Cells) nuclei in the SCN and BNST-OV of rats killed at different ZTs (n = 4 per time point). c, Mean ± SEM number of PER2 immunoreactive nuclei in the SCN and BNST-OV of rats that were housed in constant darkness and killed at different CTs (n = 4 per time point). d, Mean ± SEM number of PER2-immunoreactive nuclei in the SCN and BNST-OV of adult rats that were enucleated during the neonatal period and killed at CT0 or CT12 (n = 4 per time point).

subjective night, at CT12, and in complete synchrony with the rhythm of PER2 expression in the SCN (Figure 3c). Similarly, levels of PER2 expression in the SCN and BNST-OV of blind rats were high at CT12 and low at CT0 (Figure 3d), and similar in magnitude to those seen in sighted rats at the same circadian times (compare with Figure 3c). Thus, the BNST-OV and SCN exhibit synchronous rhythms in PER2 expression under conditions in the absence of photic time cues.

**Synchronous PER2 expression in the SCN and the BNST-OV of the mouse.**

To determine whether the rhythm in PER2 expression in the BNST-OV is species specific, we assessed the expression of PER2 in mice that were housed under a 12h LD cycle and killed at ZT0 or ZT12. Consistent with our finding in rats, in both the SCN and BNST-OV of light-entrained mice, the expression of PER2 was high at ZT12 and low at ZT0 (Figure 4), suggesting synchronous oscillations.

**PER2 oscillation in the BNST-OV depends on the functional integrity of the SCN.**

The subordinate nature of circadian clocks outside the SCN is evident from the finding that the oscillations in clock gene expression in most brain and peripheral tissues are not self-sustaining and dampen after a few cycle *in vitro* (Yamazaki et al., 2000; Abe et al., 2002; Balsalobre, 2002). Furthermore, it has been shown that SCN lesions that abolish circadian activity rhythms abolish circadian oscillations in *Per* gene expression in the brain and periphery in rats and in mice, *in vivo* (Oishi et al., 1998; Sakamoto et al., 1998; Wakamatsu et al., 2001). To study the role of the SCN in the control of PER2 oscillations in the BNST-OV, we assessed the expression of PER2 immunoreactivity in rats with bilateral electrolytic SCN lesions. To verify the effectiveness of the lesion, all the lesioned rats were housed in cages equipped with running wheels, under a 12h LD

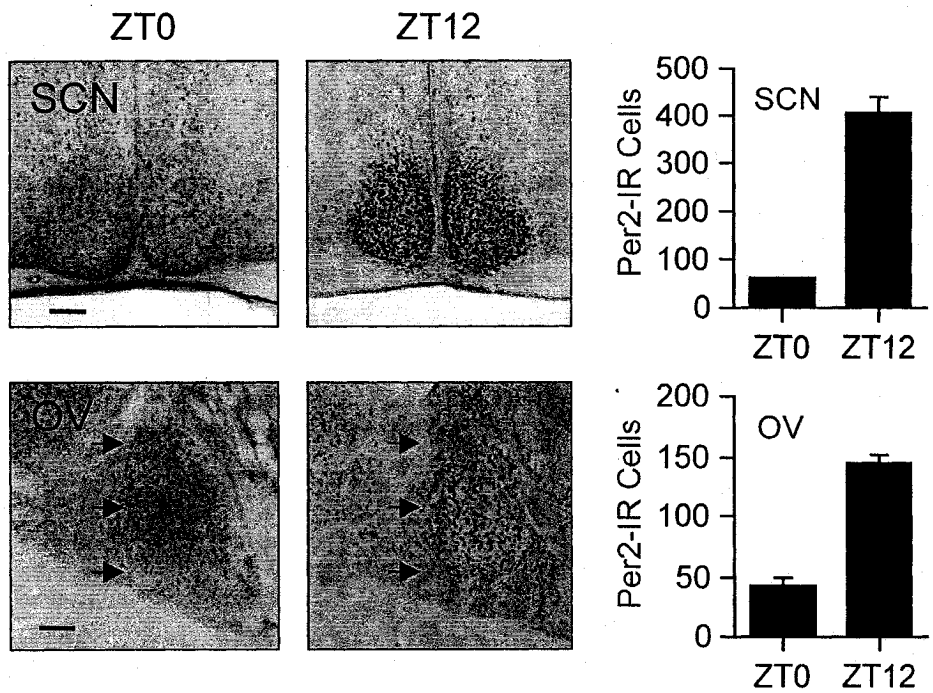


Figure 4. Synchronous expression of PER2 in the BNST-OV and SCN of the mouse. The photomicrographs show examples of PER2 immunostaining in the SCN and BNST-OV in mice housed under a 12hr LD cycle and killed at ZT0 or ZT12. Arrows point to the BNST-OV. Scale bars: SCN, 100μm; BNST-OV, 200μm. The bar graphs show mean ± SEM number of PER2 immunoreactive nuclei in the SCN and BNST-OV as a function of ZT (n = 3 per time point).

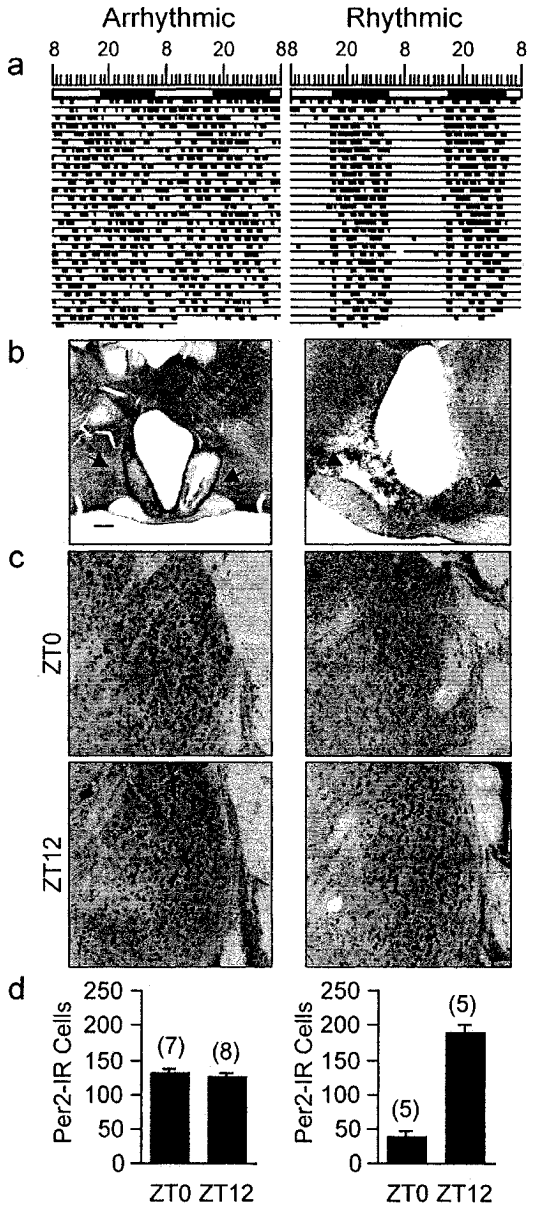


Figure 5. Bilateral SCN lesions that disrupt circadian activity rhythms abolish PER2 oscillations in the BNST-OV. a, Representative double-plotted actograms of wheel-running activity in an SCN-lesioned arrhythmic rat (left) and in a lesioned rat that remained rhythmic (right). The rats were housed under a 12hr LD cycle. The vertical marks indicate periods of activity of at least 10 wheel revolutions per 10 min. Successive days are plotted from top to bottom. b, Photomicrographs showing the largest extent of the SCN lesions of rats whose actograms are shown in a. Scale bar, 200μm. c, Photomicrographs showing examples of PER2 immunostaining in the BNST-OV from SCN-lesioned, arrhythmic rats (left) and from lesioned, rhythmic rats (right) as a function of ZT. Magnification, 20X. d, Mean ± SEM number of PER2-immunoreactive nuclei in the BNST-OV of SCN-lesioned arrhythmic rats (left) and of lesioned, rhythmic rats (right) as a function of ZT. The numbers in brackets indicate the number of rats per time point.

cycle, and their activity rhythms were monitored continuously for at least 60 d. Of the 25 lesioned rats, 15 became permanently behaviorally arrhythmic (Figure 5a). Histological inspection of brain sections from these arrhythmic rats revealed that all sustained considerable bilateral SCN damage. Of these, two rats had complete bilateral lesions that extended the entire length of the paired nuclei. All other arrhythmic rats sustained partial SCN damage that varied both in size and in location. In all arrhythmic rats the lesion extended from the SCN both laterally to the central part of the anterior hypothalamus, and dorsally, to the subparaventricular zone (Figure 5b). The remaining 10 lesioned rats did not become arrhythmic and were able to entrain to the LD cycle despite sustaining considerable damage in the SCN and surrounding tissues (Figure 5a,b). In these lesioned, but behaviorally rhythmic rats, PER2 expression in the BNST-OV was high at ZT12 and low at ZT0 (Figure 5c). This pattern of expression is identical to that seen in intact entrained rats at these times (Figure 3). In contrast, PER2 expression in the BNST-OV of the behaviorally arrhythmic lesioned rats did not vary as a function of time and was at an intermediate level. These results suggest that the rhythm in PER2 expression in the BNST-OV depends on the functional integrity of the SCN clock, as assessed by its ability to drive circadian locomotor activity rhythms. They also indicate that although the SCN clock appears to control the daily oscillation in PER2 expression in the BNST-OV, it is not required for local expression of the PER2 protein, per se.

**Constant-light-induced behavioral arrhythmicity blunts PER2 oscillations in SCN and BNST-OV.**

As is the case with bilateral SCN lesions, prolonged housing in constant light (LL) abolishes circadian activity and temperature rhythms in rats (Edelstein et al., 1995;

Edelstein & Amir, 1999). Furthermore, we have found recently that LL-induced behavioral arrhythmicity is associated with blunting of the PER2 rhythm in the SCN (Beaulé et al., 2003a; Sudo et al., 2003), a finding that is consistent with the idea that the behavioral effects of LL are mediated, at least in part, by a disruption of the molecular oscillation of the SCN clock. To study the importance of the SCN clock itself in the control of PER2 oscillations in the BNST-OV, we assessed the expression of PER2 immunoreactivity in the BNST-OV of rats that were housed in LL and displayed behavioral arrhythmicity for at least 40 days (Figure 6a). PER2 expression in both the SCN and BNST-OV of these arrhythmic rats was blunted and there were no significant variations in expression levels between the four equally-spaced time points used for the analysis (Figure 6b,c) (ANOVA: SCN, $F[3, 10] = 0.76$, $p = 0.54$; BNST-OV, $F[3,10] = 0.379$, $p = 0.77$). The finding that prolonged LL housing abolishes PER2 rhythmicity both in the SCN and in the BNST-OV underscores the importance of the SCN clock in the control of the rhythm of PER2 expression in the BNST-OV.

**PER2 oscillation in the left and right BNST-OV following unilateral SCN lesion.**

Several different mechanisms could mediate the effect of the SCN on PER2 oscillations in the BNST-OV. The rhythm in PER2 could be regulated by signals communicated from the SCN via neural projections (Watts et al., 1987). Alternatively, the rhythm may be regulated by a diffusible factor released from the SCN or by oscillating endocrine signals, such as CORT (Silver et al., 1996; Oishi et al., 1998; Balsalobre et al., 2000a). Finally, it may be under the control of one or more behavioral states that, themselves, are under circadian control, such as locomotor activity or feeding (Mrosovsky, 1996; Wakamatsu et al., 2001). To begin to explore the role of neural

connections, as well as to exclude the role of activity, we assessed the expression of PER2 immunoreactivity separately in the left and right BNST-OV in behaviorally rhythmic rats bearing a unilateral SCN lesion. Neural connections between the SCN and some of its targets are lateralized (de la Iglesia et al., 2003). Accordingly, we hypothesized that if the rhythm in expression of PER2 in the BNST-OV depends on neural connections from the SCN, then the rhythm in expression of PER2 in the BNST-OV ipsilateral to the lesion would be attenuated, and different from that in the contralateral side. Alternatively, if the rhythm of activity is the critical entraining agent, then PER2 expression in the ipsilateral and contralateral BNSTOV should not differ.

All unilateral SCN lesioned rats (n = 12) exhibited stable photic entrainment of wheel-running activity rhythms and temporally appropriate levels of PER2 expression in the intact SCN (Figure 7a). Furthermore, both the ipsilateral and contralateral BNST-OV showed a daily pattern of PER2 expression, with lower levels at ZT0 and higher levels at ZT12 (Figure 7b,c). The magnitude of the daily difference, however, was blunted on the lesioned side. At ZT0, PER2 expression in the BNST-OV ipsilateral to the lesioned SCN was significantly greater ($p < 0.001$) than that in the contralateral side, whereas at ZT12, expression in the ipsilateral BNST-OV was significantly lower than that in the contralateral BNST-OV ($p < 0.001$). These results suggest that normal oscillations in PER2 expression in the BNST-OV depend, at least in part, on ipsilateral neural input from the SCN. Furthermore, the finding that unilateral SCN lesions attenuated the rhythm in PER2 expression in the ipsilateral BNST-OV, but had no effect on activity rhythms, excludes the involvement of activity, per se, in the regulation of PER2 expression in the BNST-OV.

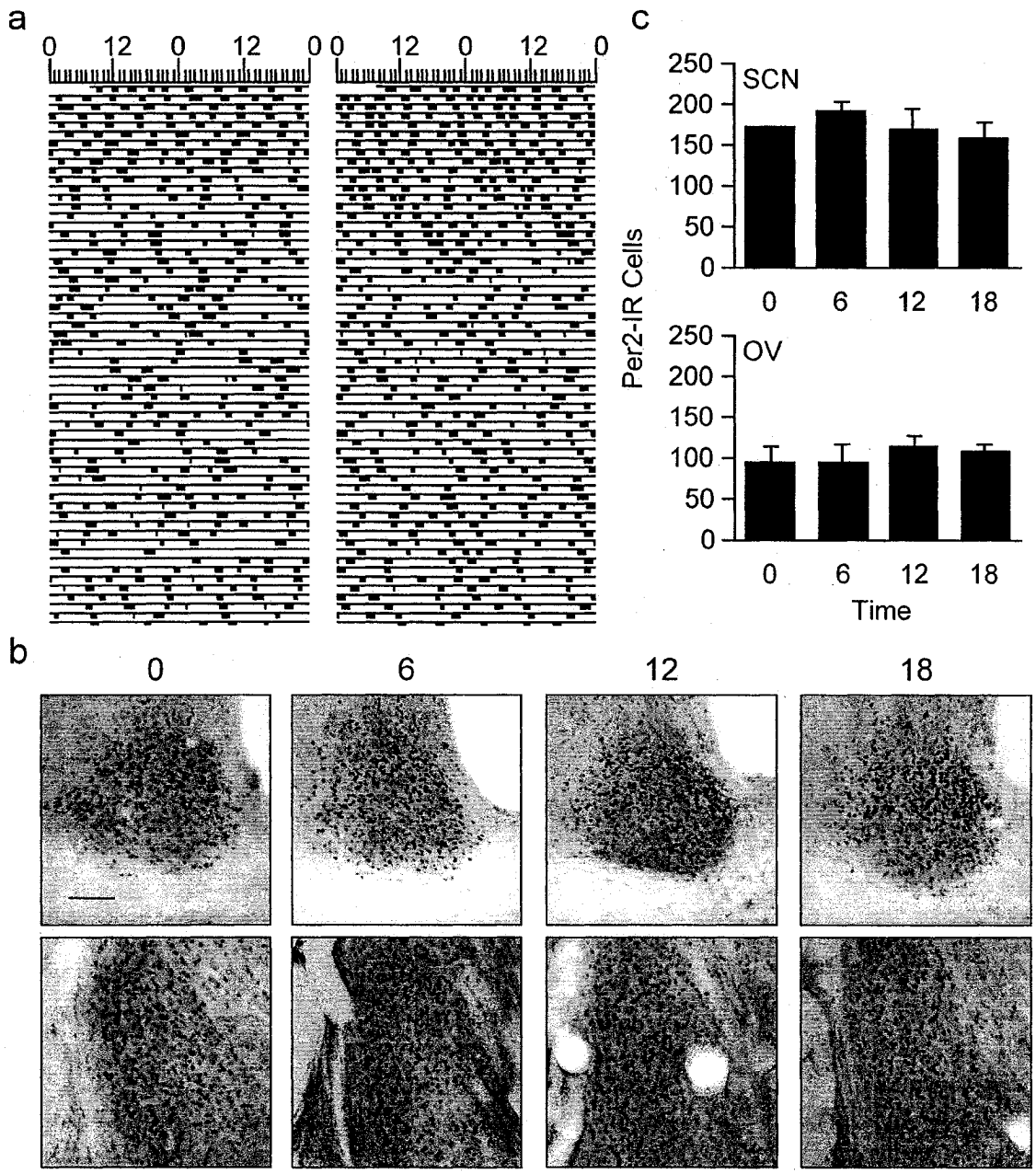


Figure 6. Constant-light housing disrupts circadian activity rhythms and abolishes PER2 rhythms in the SCN and BNST-OV. a, Representative double-plotted actograms showing wheel-running activity in two rats housed in LL. b, Photomicrographs showing examples of PER2 immunoreactivity in the SCN and BNST-OV of LL-housed rats killed at four equally spaced times around the clock. Scale bar, 200μm. c, Mean ± SEM number of PER2-immunoreactive nuclei in the SCN (top) and BNST-OV (bottom) of LL-housed rats (n = 3-4 per time point).

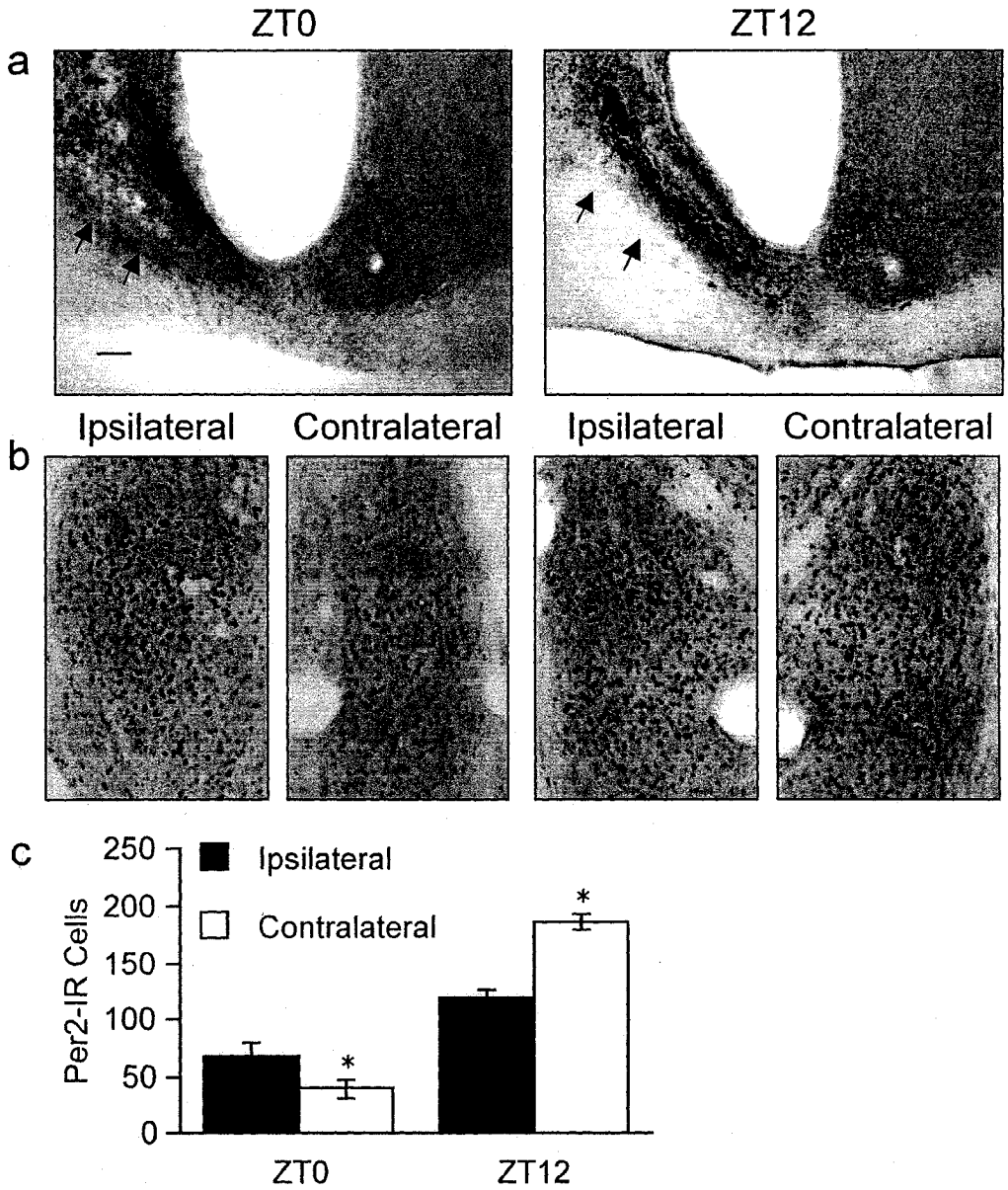


Figure 7. PER2 expression in the BNST-OV after unilateral SCN lesion. a, Photomicrographs showing examples of unilateral SCN lesions (arrows) in rats killed at ZT0 or ZT12. PER2 expression in the intact SCN is low at ZT0 and high at ZT12. Scale bar, 100μm. b, Photomicrographs showing examples of PER2 immunoreactivity in the BNST-OV ipsilateral and contralateral to the lesioned SCN. Magnification, 20X. c, Mean ± SEM number of PER2-immunoreactive nuclei in the ipsilateral and contralateral BNST-OV as a function of ZT. Asterisks indicate significant differences from the ipsilateral side ($p < 0.05$; $n = 5$-7 per time point).

**Uncoupling of PER2 expression between the SCN and BNST-OV after acute shifts in the entraining light cycle.**

Abrupt changes in the entraining light schedule alter the phase relationship between the SCN clock and subordinate clocks in the rest of the brain and periphery (Yamazaki et al., 2000; Abe et al., 2002). To further study the nature of the coupling between the SCN and BNST-OV, we assessed the expression of PER2 in LD housed rats that were subjected to an abrupt 8 hr advance or 8 hr delay in the onset of the entraining light cycle (Figure 8a). PER2 was assessed in groups of rats (n = 4 per group) that were killed 2, 4, 6, or 8 days after the imposed shift, at the new ZT12. The levels of PER2 in the SCN and BNST-OV in the shifted rats at the new ZT12 were compared to levels at ZT12 in entrained control rats. In rats subjected to an 8 hr advance of light onset, PER2 expression in the SCN returned to the high levels expected at ZT12 after 6 days, whereas in the BNST-OV, PER2 expression returned to the expected high levels after 8 d (Figure 8b). After an 8 hr delay in the time of light onset, PER2 expression in the SCN was at the expected high levels on day 2 and levels in the BNST-OV on day 6 (Figure 8c). These results demonstrate that large shifts in the entraining light cycle are attended by sequential changes in the phase of expression of PER2 in the SCN and BNST-OV, consistent with the hypothesis that the SCN synchronizes the BNST-OV rhythm. They also show that such shifts in the light cycle disrupt the synchrony in PER2 expression between the SCN and BNST-OV. The duration of this disruption depends on the direction of the shift in the light cycle, with overall longer desynchrony seen after an advance than a delay shift.

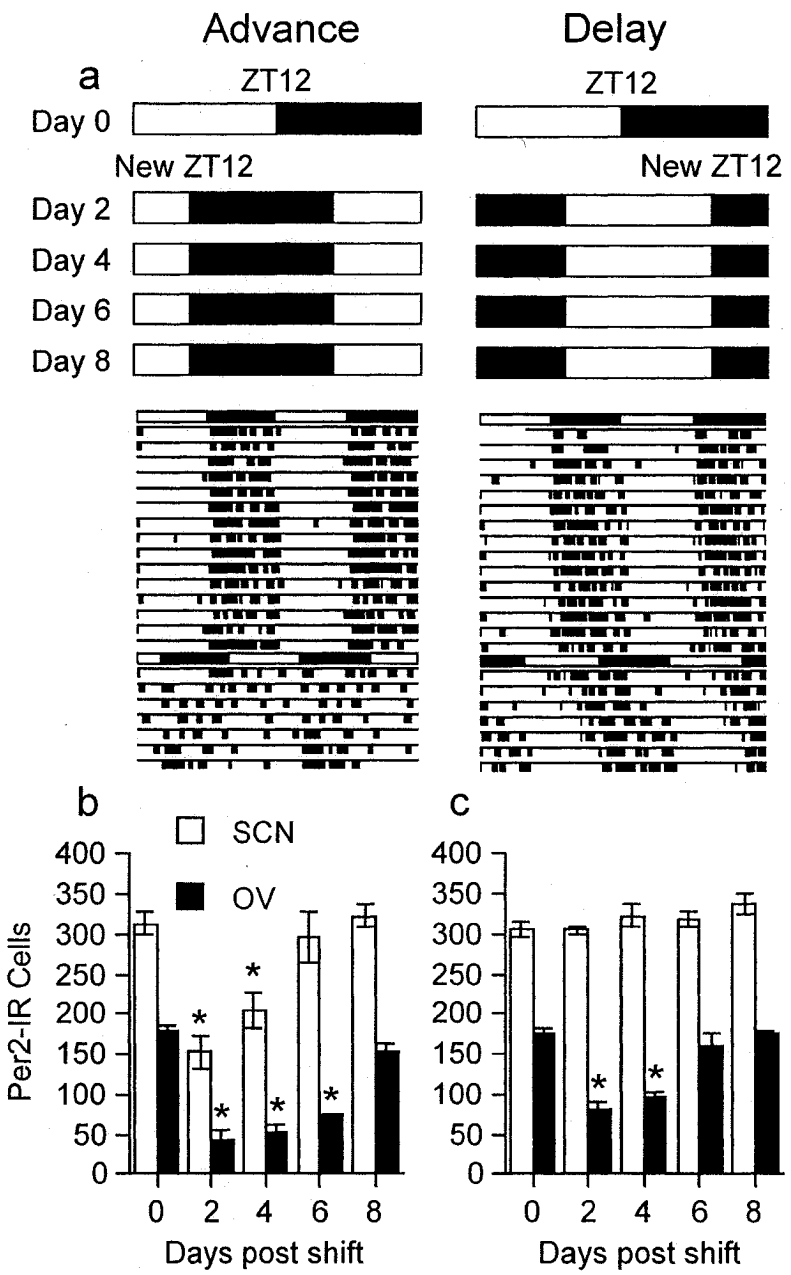


Figure 8. Uncoupling between PER2 expression in the SCN and BNST-OV after a shift in the light cycle. a, Schematic representation of the experimental design. The rats were entrained to a 12hr LD cycle. The onset of light was then advanced or delayed by 8 hr, and rats were killed 2, 4, 6, or 8 d later at the new ZT12. Representative actograms of rats from the advance and delay groups killed 8 d after the shift are shown. b, Mean ± SEM number of PER2-immunoreactive nuclei in the SCN and BNST-OV of rats killed at ZT12 without a shift (day 0) or 2, 4, 6, or 8 d after an advance shift at the new ZT12. c, Mean ± SEM number of PER2-immunoreactive nuclei in the SCN and BNST-OV of rats killed at ZT12 without a shift (day 0) or 2, 4, 6, or 8 d after a delay shift at the new ZT12. Asterisks indicate significant difference from Day 0 for each brain area.

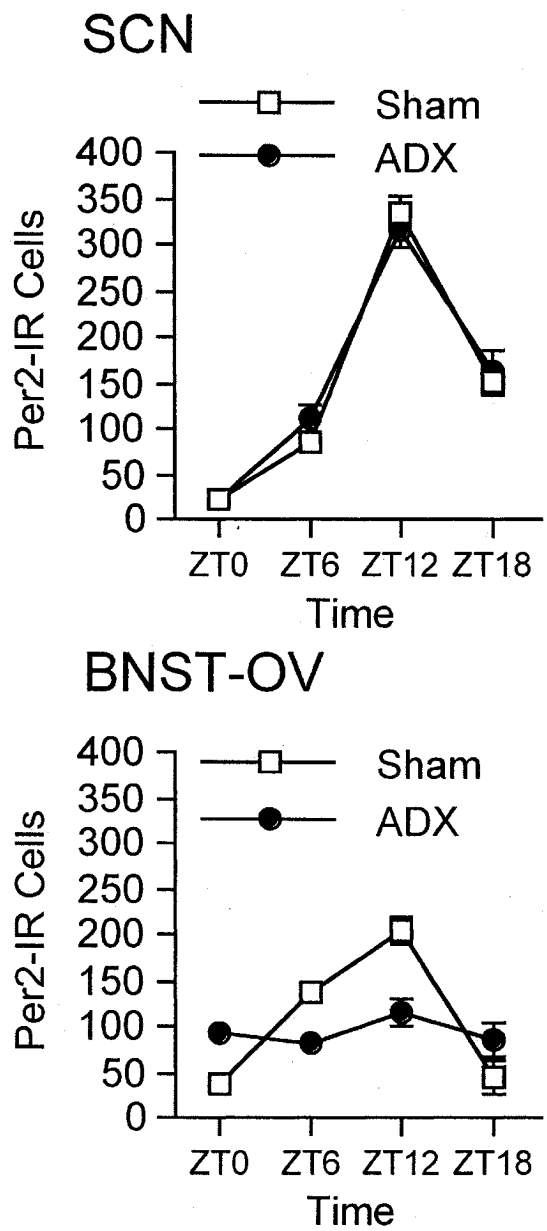


Figure 9. Effect of adrenalectomy on PER2 expression in the SCN and BNST-OV. Mean ± SEM number of PER2 immunoreactive nuclei in the SCN (top) and BNST-OV (bottom) of adrenalectomized and sham operated rats as a function of ZT (n = 5-7 per group per time point). Adrenalectomy had no effect on the rhythm of PER2 expression in the SCN, whereas it completely blunted the rhythm in the BNST-OV.

**PER2 oscillation in the SCN and BNST-OV after bilateral adrenalectomy.**

Basal secretion of glucocorticoid hormones from the adrenal glands follows a circadian rhythm that is under the control of the SCN clock (Szafarczyk et al., 1983). The adult SCN does not contain glucocorticoid receptors, and it is therefore unlikely that glucocorticoid hormones are involved in the regulation of clock gene expression in the SCN (Rosenfeld et al., 1988, 1993). In contrast, most peripheral tissues express glucocorticoid receptors, and administration of a synthetic glucocorticoid, dexamethasone, has been shown to induce *Per* gene expression in mouse liver, *in vivo*, and in cultured rat-1 fibroblasts (Balsalobre, 2002; Balsalobre et al., 2000a, b), suggesting that circulating glucocorticoids regulate *Per* gene expression in some peripheral tissues. Glucocorticoid receptors are expressed in the BNST (Lechner & Valentino, 1999), and CORT has been shown to modulate neuropeptide gene expression in the BNST (Makino et al., 1994b; Pompei et al., 1995; Watts & Sanchez-Watts, 1995). To begin to study the possibility that circulating glucocorticoids are involved in the control of PER2 in the BNST-OV, we assessed the expression of PER2 in the SCN and BNST-OV of bilaterally adrenalectomized (ADX) and sham-operated rats. All of the operated rats were housed in isolation boxes and maintained under a 12hr LD cycle for at least 3 weeks before use. As expected, bilateral adrenalectomy had no effect on PER2 expression in the SCN (Figure 9). In contrast, the rhythm in PER2 in the BNST-OV was blunted in the ADX rats ($F[3,19] = 1.24$, $p = 0.32$). PER2 expression in the BNST-OV in these rats remained at an intermediate level, suggesting that removal of the adrenals had a selective effect on the rhythm of PER2, but did not affect basal expression of PER2.

## Discussion

The finding that many brain and peripheral tissues exhibit circadian oscillations in clock gene expression has led to the hypothesis that circadian rhythms in mammals are regulated not only globally by the master SCN clock, but also locally by widely distributed populations of subordinate clock cells that control tissue-specific rhythmic outputs (Schibler & Sassone-Corsi, 2002; Hastings et al., 2003). Contrary to the SCN clock, oscillators outside the SCN are not self-sustaining and lose their rhythmicity after a few days when studied in isolated tissue *in vitro* and after SCN lesions *in vivo*. It has been proposed that both neural and endocrine pathways under the control of the SCN drive the rhythm and set the phase of these peripheral oscillators (Sakamoto et al., 1998; Yamazaki et al., 2000; Abe et al., 2002; Balsalobre, 2002; Kalsbeek & Buijs, 2002; Schibler & Sassone-Corsi, 2002; Bittman et al., 2003; Hastings et al., 2003; Kriegsfeld et al., 2003).

In the present study we found that neurons in the BNST-OV, a distinct subregion of the BNST, exhibit a robust daily rhythm in expression of PER2, consistent with the hypothesis that the BNST contains circadian clock cells that might play a role in local control of circadian rhythms. The expression of PER2 was rhythmic in the BNST-OV in rats housed under a LD cycle or in constant darkness, in blind rats, and in mice, emphasizing the ubiquitous nature of this region-specific molecular oscillation. Most importantly, in both rats and mice, the rhythm in PER2 expression in the BNST-OV was in synchrony with the PER2 rhythm in the SCN. Interestingly, studies in hamsters (Yamazaki et al., 1998) and in rats (Abe et al., 2002) have shown that the phase of the oscillations in neural activity and clock gene expression in most other brain regions is

distinct from the phase in the SCN. The finding that the BNST-OV and SCN exhibit synchronous rhythms in PER2 expression underscores the unique nature of the coupling between clock cells in the SCN and BNST and is consistent with the proposed role of the BNST in the gating of circadian information.

The rhythm in PER2 in the BNST-OV was eliminated by lesions of the SCN that abolished circadian activity rhythms and by constant-light housing that disrupts circadian activity rhythms and the oscillation in PER2 expression in the cells of the SCN without affecting the anatomical integrity of the SCN (Beaulé et al., 2003a). Thus, the oscillations in PER2 in the BNST-OV depend on the functional integrity of the SCN clock, consistent with other findings on the effect of SCN lesions on rhythms of *Per* genes in the brain and periphery in rats and mice (Sakamoto et al., 1998; Wakamatsu et al., 2001). Interestingly, unilateral lesions of the SCN that were without an effect on circadian activity rhythms also influenced the PER2 rhythm in the BNST-OV; however, only the rhythm ipsilateral to the lesioned side was attenuated. This finding argues against the importance of activity rhythms, per se, in the control of PER2 oscillation in the BNST-OV and demonstrates that transmission of information from the SCN to the BNST-OV is dependent at least in part on ipsilateral neural connections.

It has been shown previously that abrupt changes in the entraining light schedule disrupt the normal phase relationship between the SCN and circadian oscillators in the brain and periphery (Yamazaki et al., 2000; Abe et al., 2002). In our experiments we found that entrainment of the PER2 rhythm to a shifted light cycle was faster in the SCN than in the BNST-OV, and that in both structures it was faster after a delay than after an advance shift in the light cycle. These findings demonstrate that shifts in the light

schedule transiently disrupt the synchrony between the PER2 rhythm in the SCN and that seen in the BNST- OV. Such disruptions in the relationship between the phase of the SCN clock and subordinate clocks could lead to internal desynchronization among specific behavioral and physiological rhythms and could explain the disruptive physiological and behavioral symptoms associated with travel across time zones and shift work (Abe et al., 2002; Reddy et al., 2002). Interestingly, restricted feeding schedules, which entrain circadian activity rhythms via SCN-independent clocks (Stephan et al., 1979a), have also been shown to disrupt coupling between the SCN and other brain and peripheral oscillators (Damiola et al., 2000; Stokkan et al., 2001). Neurons in the BNST-OV are sensitive to food restriction (Carr et al., 1998; Carr & Kutchukhidze, 2000) and are activated by treatment with anorectic agents (Bonaz et al., 1993; Li & Rowland, 1995; Day et al., 1999), suggesting the intriguing possibility that oscillations of cells of the BNST-OV might be regulated directly by food-related stimuli.

The BNST-OV is highly responsive to CORT (Makino et al., 1994b; Pompei et al., 1995; Watts & Sanchez-Watts, 1995), and it has been shown that glucocorticoids can induce clock gene expression in peripheral tissues (Balsalobre et al., 2000b). The results of our last experiment showing that removal of the adrenal glands blunts the rhythm in PER2 expression in the BNST-OV, although not in the SCN, strongly suggest a role for adrenal glucocorticoid hormones in the control of PER2 oscillations in the BNST-OV. Moreover, and consistent with other evidence (Rosenfeld et al., 1993; Balsalobre et al., 2000b), they exclude a role for circulating corticosteroids in the regulation of clock gene expression in the SCN. Finally, and most significantly, the finding that removal of the adrenals blunts the rhythm in PER2 in the BNST-OV suggests that although neural

pathways from the SCN are necessary for circadian regulation of PER2 expression in this region, other factors, in this case adrenal hormones, which normally oscillate in a circadian fashion, play an equally important role.

Most cells of the BNST-OV express the inhibitory neurotransmitter, GABA (Sun & Cassell, 1993), suggesting that the rhythm in PER2 expression seen in the BNST-OV may be associated with oscillations in local GABAergic cell firing. Interestingly, GABA is the primary transmitter in the SCN (Moore & Speh, 1993; Moore et al., 2002), where it has been implicated both in the synchronization among individual clock cells as well in the transmission of circadian information from the SCN (Moore & Speh, 1993; Liu & Reppert, 2000; Buijs & Kalsbeek, 2001; Kalsbeek & Buijs, 2002; Moore et al., 2002). Furthermore, the BNST-OV contains several neuropeptides, including corticotropin-releasing hormone (CRH), enkephalin, neurotensin, somatostatin and dynorphin (Dong et al., 2001b). In preliminary studies we failed to observe any significant staining for PER2 in CRH immunoreactive cells in the BNST-OV, whereas cells that expressed enkephalin immunoreactivity also contained PER2. The enkephalin cells of the BNST-OV have been shown to be activated by certain types of stress stimuli, including immune and osmotic stimuli, and by amphetamine injections (Watts & Sanchez-Watts, 1995; Day et al., 1999; Day et al., 2001; Kozicz, 2002; Engstrom et al., 2003). It will be of interest, therefore, to determine whether such stimuli are capable of altering PER2 expression in the BNST-OV and thereby have an influence on specific, locally controlled circadian rhythms.

The BNST is involved in the modulation of physiological and behavioral processes that are known to be under circadian control, and it is linked, both anatomically

and functionally with the SCN (Watts et al., 1987; Yamazaki et al., 1998). The BNST-OV projects to virtually all other subregions of the BNST as well as to several brain regions outside the BNST (Dong et al., 2001b). The latter include the central nucleus of the amygdala (CEA), the tuberal region of the lateral hypothalamus, the parabrachial nucleus (PB) and the nucleus of the solitary tract (NTS), all of which have been shown to play important roles in controlling autonomic responses to homeostatic challenge and fear stimuli (Dong et al., 2001b). In addition, the BNST-OV has been shown to innervate the substantia innominata and nucleus accumbens both of which play key roles in the control of motivated behaviors driven by drugs and other incentive stimuli, as well as in the control of behaviors driven by homeostatic needs, such as food and water intake. Furthermore, the BNST-OV has been shown to receive afferents from many other subregions of the BNST and from the CEA, NTS, PB, locus coeruleus, dorsal raphe nucleus, ventral tegmental area, postpiriform transition cortical area, and insular cortex (Dong et al., 2001b). These neural connections, taken together with the evidence of glucocorticoid sensitivity, place the BNST-OV in a unique position to integrate stress and homeostatic and incentive stimuli, as well as to coordinate physiological responses and behaviors that are engaged by these stimuli. The present finding that the BNST-OV contains subordinate circadian clock cells provides a mechanism whereby the SCN could impose local circadian control over gene expression and neural activity and thereby affect information processing within the BNST as well as influence activity in neural circuits downstream from the BNST. Finally, the possibility that the putative clock cells of the BNST-OV might be sensitive to stress-, drug-, and food-related stimuli provides a

mechanism whereby such stimuli can affect circadian rhythms downstream from the SCN clock.

## Acknowledgements

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# CHAPTER 2

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

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**Abstract**

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

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

physiological arrhythmicity (Beaulé et al., 2003a; Sudo et al., 2003). We assessed locomotor activity and the expression of the clock protein Period2 (PER2) in the SCN and in one extra SCN structure, the oval nucleus of the bed nucleus of the stria terminalis (BNST-OV), where we have found that the rhythm of PER2 expression is normally synchronized with that in the SCN and, like the SCN, is abolished in LL (Amir et al., 2004).

## Experimental Procedures

### Animals and Housing

The experimental procedures followed the internationally recognized guidelines for the ethical use of laboratory animals of the Canadian Council on Animal Care and were approved by the Animal Care Committee, Concordia University. Care was taken to use the smallest number of animals possible and to minimize any suffering. Adult male Wistar rats (275-300 g) were housed individually in clear plastic cages equipped with running wheels. Each cage was place in a ventilated, sound and light tight isolation chamber equipped with a computer-controlled lighting system (VitalView Mini Mitter Co. Inc., Sunriver, OR). The rats were maintained either on a 12-h light/dark cycle ((LD; light: 300 lux at cage level) or LL. Wheel-running activity data were recorded with VitalView software (Mini Mitter Co.) and analyzed with Circadia software (Behavioral Cybernetics, St. Louis, MO, USA), as previously described (Amir et al., 2004). After at least 4 weeks of LD or LL housing with free access to food and water, half of the rats from each lighting condition were placed on a restricted feeding schedule for 10 days. Rats from the LD condition were allowed to eat for 3 h/day, during the daytime, from zeitgeber time (ZT) 4 to ZT7 (ZT0 denotes the onset of the light phase). Rats housed in

LL were allowed to eat for 3 h/day at the same time. Control rats from each lighting condition continued to have free access to food. All rats were perfused 11 days after the start of the restricted feeding or control schedules. Rats from the LD groups were perfused at ZT0, 6, 12, or 18. Rats from the LL groups were perfused 0, 6, 12, or 18 h after food presentation.

**Tissue Preparation**

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

**Immunocytochemistry**

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

TBS and incubated for 1 h at 4 °C with a biotinylated anti-rabbit IgG made in goat (Vector Labs, Burlingame, CA, USA), diluted 1:200 with Triton-TBS with 2% normal goat serum. Following incubation with secondary antibody, sections were rinsed in cold TBS and incubated for 2 h at 4 °C with an avidin-biotin-peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories). Following incubation with the ABC reagents, sections were rinsed with cold TBS, rinsed again with cold 50 mM Tris-HCl (pH 7.6), and again for 10 min with 0.05% 3,3'-diaminobenzidine (DAB) in 50 mM Tris-HCl. Sections were then incubated on an orbital shaker for 10 minutes in DAB/Tris-HCl with 0.01% $H_2O_2$ and 8% $NiCl_2$. After this final incubation, sections were rinsed in cold TBS, wet-mounted onto gel-coated slides, dehydrated through a series of alcohols, soaked in Citrisolv (Fisher Scientific, Ottawa, ON, Canada), and cover-slipped with Permount (Fisher).

**Data Analysis**

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

## Results and Discussion

In freely fed control rats housed under a 12-h LD cycle, running wheel activity was confined to the dark phase (Figure 10a, top), and, as previously described (Amir et al., 2004), PER2 expression in the SCN and BNST-OV was rhythmic and synchronous, peaking in the beginning of the dark phase, at ZT12 (Figure 10a, bottom). When rats housed in LD were placed on a restricted feeding schedule in which food was given for 3 hours each day, from ZT4 to ZT7, the normal pattern of nighttime activity was altered, and a second, distinct bout of activity of 2- to 3-h emerged in all rats before and during daytime food presentation (Figure 10b, top). Furthermore, this restricted feeding schedule uncoupled the rhythm of PER2 expression in the BNST-OV from that in the SCN. The peak of PER2 expression in the BNST-OV was delayed by 6 h relative to that in the SCN and peaked 12 h after food access (Figure 10b, bottom). The PER2 rhythm of the SCN was not affected by the RF schedule in these rats and expression remained entrained to the light cycle, peaking at ZT12 (Figure 10b, bottom).

In rats housed in prolonged LL with free access to food, activity rhythms and rhythms of PER2 expression in SCN and BNST-OV were abolished (SCN: $F[3,22] = 1.68$, $p = 0.19$; BNST-OV: $F[3,22] = 1.76$, $p = 0.18$) (Figure 11a). In another group of LL-housed rats, the schedule of restricted feeding led to the emergence of a distinct daily pattern of locomotor activity synchronized to the time of daily food presentation (Figure 11b). Importantly, in these rats we found a significant circadian rhythm of PER2 expression both in the SCN ($F[3,20]=14.33$, $p<.0001$) and in the BNST-OV ($F[3,20]=14.09$,$p<.0001$). Expression of PER2 in these regions was synchronous peaking 12 h after food access (Figure 11b).

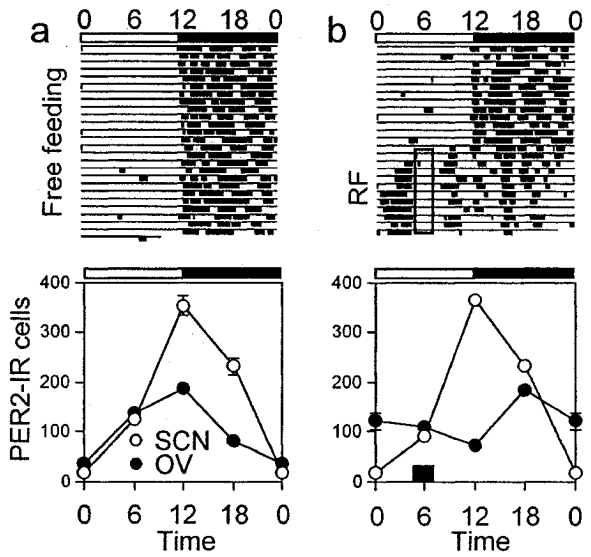


Figure 10. Representative actograms of locomotor activity, and mean (± SEM) number of PER2 immunoreactive nuclei measured in the SCN and BNST-OV as a function of time in (a) freely fed rats housed in LD (n = 5-6 per group), (b) restricted-feeding (RF) rats housed in LD (n = 7-10 per group). In each actogram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

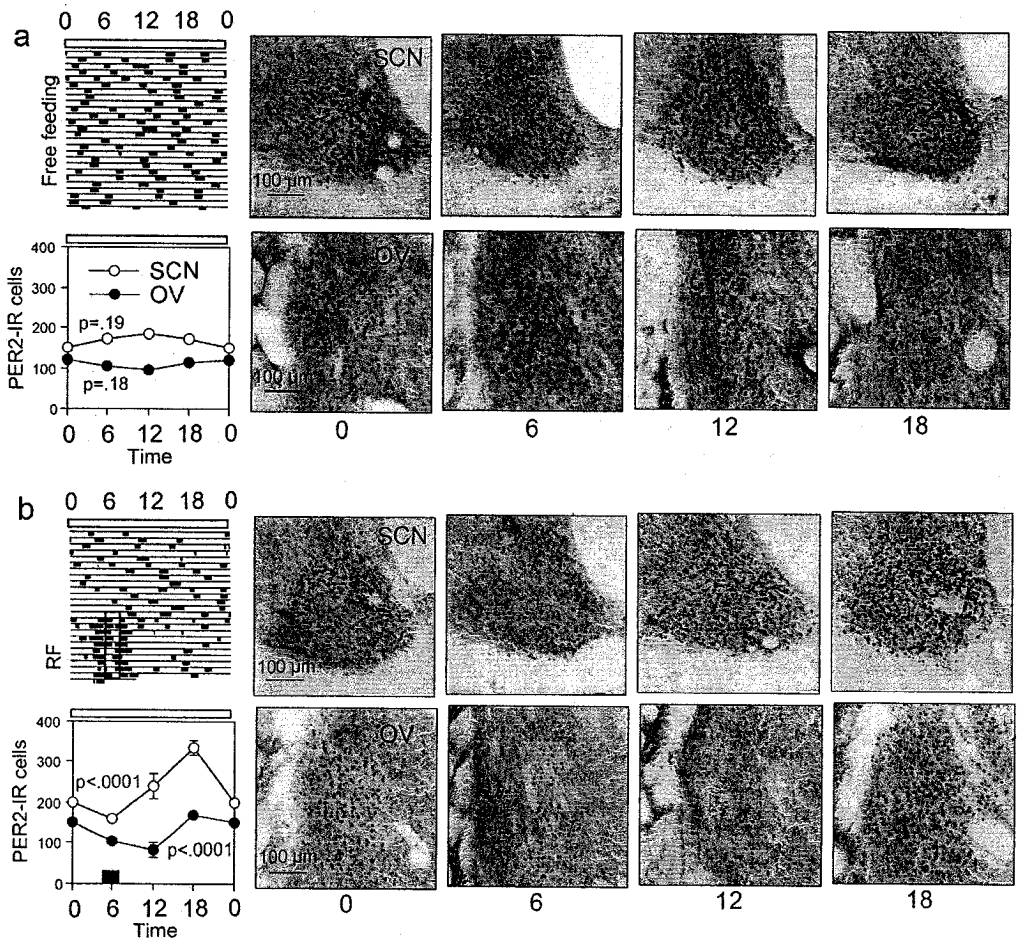


Figure 11. Representative actograms of locomotor activity and images showing PER2 expression in the SCN and BNST-OV and mean (± SEM) number of PER2 immunoreactive nuclei measured in the SCN and BNST-OV as a function of time in (a) freely fed rats housed in LL (n = 6–7 per group) and (b) restricted-feeding (RF) rats housed in LL (n = 6–7 per group). The vertical rectangle and black box in (b) mark the time of food presentation during the RF schedule. P-values indicate the significance of differences between time points (ANOVA).

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

In summary, we find that in arrhythmic rats a daily restricted feeding schedule not only restores and entrains an activity rhythm but also induces synchronized PER2 rhythms in the SCN and BNST-OV that peak 12 h after daily feeding. Thus scheduled meals can play an important function in entrainment of the SCN clock in situations where its oscillators become uncoupled leading to disruption of physiological and behavioral rhythms.

## Acknowledgements

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## SECTION 2: RHYTHMIC EXPRESSION OF PER2 IN THE CEA, BLA, and DG: SYNCHRONIZATION OF THE PHASE OF PER2 EXPRESSION IN THE LIMBIC FOREBRAIN BY RESTRICTED FEEDING

In Chapter 3, the pattern of expression of PER2 was examined in the CEA, BLA, and DG. It was shown that rhythmic PER2 expression in the CEA is in phase with that of the BNST-OV, but that the BLA and DG showed rhythmic PER2 expression that was 180° out of phase with that of CEA and BNST-OV. The effect of lesions of the SCN and adrenalectomy on PER2 expression in the CEA, BLA, and DG were also examined.
In Chapter 4 the phase of PER2 expression in the CEA, BLA, and DG were examined in animals given either AL or restricted access to food under both LD and LL conditions. In an attempt to discriminate the relative contributions of rewarding and metabolic effects of restricted feeding, animals were also given scheduled access to the rewarding liquids sucrose and saccharine, or saline after acute or chronic sodium depletion.

# CHAPTER 3

## The Central and Basolateral Nuclei of the Amygdala Exhibit Opposite Diurnal Rhythms of Expression of the Clock Protein Period2

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## Abstract

There is considerable evidence that circadian rhythms in mammals can be modulated by emotional state, but how emotional state modulates specific circadian outputs is poorly understood. We analyzed the expression of the circadian clock protein, Period2 (PER2) in three regions of the limbic forebrain known to play key roles in emotional regulation, the central nucleus of the amygdala (CEA), the basolateral amygdala (BLA), and the dentate gyrus (DG). We report here that cells in all three regions exhibit daily rhythms in expression of PER2 that are under the control of the master clock, the suprachiasmatic nucleus (SCN). The rhythm in the CEA and the rhythms in BLA and DG are diametrically opposite in phase and differentially affected by adrenalectomy. Adrenalectomy completely abolished the PER2 rhythm in CEA but had no effect on those in BLA and DG. We previously reported a rhythm in PER2 expression in the oval nucleus of the bed nucleus of the stria terminalis (BNST-OV) that is identical in phase and sensitivity to adrenalectomy to that found in the CEA. Together, these findings show that key structures of the limbic forebrain exhibit daily oscillations in clock gene expression that are controlled not only by input from the SCN but, importantly, by hormonal and neurochemical changes that normally accompany motivational and emotional states. Thus, cells within these areas are strategically positioned to integrate the inputs from the SCN and emotional states to modulate circadian rhythms downstream from the SCN clock.

In mammals, the suprachiasmatic nucleus (SCN) of the hypothalamus is recognized as a master clock responsible for the regulation of all behavioral and physiological circadian rhythms (Rusak & Zucker, 1979). The rhythm of the SCN, although sensitive to the daily cycle of light, is relatively invulnerable to environmental challenges and changes in physiology (Challet et al., 2003; Hara et al., 2001). Yet it is recognized that stressors and changes in emotional states can have disruptive effects on behavioral and physiological rhythms (Bunney & Bunney, 2000; Gorka et al., 1996; Jones, 2001; Meerlo et al., 1997; von Zerssen et al., 1985). Furthermore, disruptions of circadian rhythms have negative effects on mood and well being, as observed during jet lag or shift work (Cardinali, 2000; Cho, 2001).

Although the SCN is the only clock structure required for the generation of circadian rhythmicity, recent studies show that circadian rhythms in expression of clock genes, such as *Per1* and *Per2*, can be found in other brain regions that participate in the control of specific behavioral and physiological outputs. These include, but are not limited to, the striatum, paraventricular hypothalamic nucleus, arcuate nucleus, preoptic area, and olfactory bulbs (Abe et al., 2002; Granados-Fuentes et al., 2004a, b; Kriegsfeld et al., 2003; Masubuchi et al., 2000; Sakamoto et al., 1998; Shieh, 2003; Yamamoto et al., 2001). The study of clock genes in these and other functionally defined brain regions may help determine how the SCN clock controls specific circadian rhythms and, importantly, may contribute to an understanding of how diverse experiences and pathological conditions can affect circadian rhythms downstream from the SCN clock.

We found recently that the clock protein Period2 (PER2) is expressed rhythmically in the oval nucleus of the bed nucleus of the stria terminalis (BNST-OV)

(Amir et al., 2004), a region of the limbic forebrain known to be involved in the regulation of behavioral, autonomic and endocrine responses to motivationally and emotionally significant stimuli (Dong et al., 2001b; Johnson et al., 1999). This rhythm was synchronous with the PER2 rhythm in the SCN and was maintained in constant dark. Furthermore, both lesions of the SCN that abolished circadian locomotor activity rhythms and adrenalectomy completely dampened the PER2 rhythm in the BNST-OV. Based on these findings, we concluded that the BNST-OV contains circadian oscillators that could serve as an interface between the output of the SCN clock and emotional and motivational states (Amir et al., 2004).

The amygdala is another region of the limbic forebrain importantly involved in the control of motivational and emotional states (Cardinal et al., 2002; Everitt et al., 2003; See et al., 2003). Anatomically, the amygdala is subdivided into a number of distinct areas, of which two are particularly important in the regulation of motivational and emotional processes: the basolateral amygdala (BLA) and the central nucleus of the amygdala (CEA) (Cardinal et al., 2002; Everitt et al., 2003; Petrovich, Risold, & Swanson, 1996; Petrovich et al., 2000; Sah et al., 2003; Swanson, 2000). In view of our findings in the BNST-OV, it was of interest, therefore, to determine whether these regions of the amygdala might also exhibit daily rhythms of expression of PER2. Furthermore, we assess the expression of PER2 in the dentate gyrus (DG), a region of the hippocampus that is heavily influenced by the BLA (Abe, 2001; Akirav & Richter-Levin, 2002) and that has been implicated in emotional memory.

## 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 of Concordia University. Adult male Wistar rats (275-300 g) and bilaterally adrenalectomized Wistar rats were purchased from Charles River Breeding Laboratories. All animals had ad libitum access to food and water. Adrenalectomized rats were given saline solution (0.9% NaCl) as a drinking fluid. Rats were housed individually in clear plastic cages equipped with running wheels. The cages were housed in ventilated, sound- and light-tight isolation chambers equipped with a computer-controlled lighting system (VitalView, Mini Mitter, Sunriver, OR). Wheel-running activity data were recorded with VitalView software (Mini Mitter) and analyzed with Circadia, as previously described (Amir et al., 2004).

### Surgery

Rats were anesthetized with a ketamine (100mg/ml)/xylazine (20mg/ml) mixture given i.p. (1.5ml/kg). Electrolytic lesions, aimed at the SCN (1.2 mm posterior to the bregma, 1.9 mm lateral to the midline, and 9.4 mm below the surface of the skull, at a 10° angle) were made by passing 2 mA of current for 15 seconds through stainless steel electrodes (0.28 mm in diameter), insulated except for the tip, using a Grass lesion maker (DC LM5A, Grass Instruments, Quincy, MA). Loss of circadian locomotor activity rhythms in lesioned rats was determined from the wheel-running records obtained over 60 days using periodgram analysis (Circadia).

**Tissue Preparation**

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

**Immunocytochemistry**

Free-floating sections were washed in cold 50 mM Tris-buffered saline (TBS) (pH 7.6) and incubated at room temperature for 30 min in a quenching solution made of TBS and 3% w/w hydrogen peroxide ($H_2O_2$). After the quenching phase, sections were rinsed in cold TBS and incubated for 1 h at room temperature in a preblocking solution made of 0.3% Triton X-100 in TBS (Triton-TBS), 3% normal goat serum, and 5% milk buffer. After the preblocking phase, sections were transferred directly into an affinity-purified rabbit polyclonal antibody raised against PER2 (Alpha Diagnostic International, San Antonio, TX) diluted 1:1,000 with a solution of Triton-TBS with 3% normal goat serum in milk buffer. Sections were incubated with the primary antibody for 48 h at 4 °C. After incubation in the primary antibody, sections were rinsed in cold TBS and incubated for 1 h at 4 °C with a biotinylated anti-rabbit IgG made in goat (Vector Laboratories), diluted 1:200 with Triton-TBS with 2% normal goat serum. After incubation with secondary antibody, sections were rinsed in cold TBS and incubated for 2 h at 4 °C with an avidin-biotin-peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories). After incubation with the ABC reagents, sections were rinsed with cold TBS, then rinsed with cold 50 mM Tris-HCl (pH 7.6), and finally rinsed for 10 min with

0.05% 3,3'-diaminobenzidine (DAB) in 50 mM Tris-HCl. Sections were then incubated on an orbital shaker for 10 min in DAB/Tris-HCl with 0.01% $H_2O_2$ and 8% $NiCl_2$. After this final incubation, sections were rinsed in cold TBS, wet-mounted onto gel-coated slides, dehydrated through a series of alcohols, soaked in Citrisolv (Fisher), and coverslipped with Permount (Fisher).

**Data Analysis**

Stained brain sections (14-20 sections per area for each rat) were examined under a light microscope, and images were captured under a X20 magnification using a XC-77 video camera (Sony, Tokyo), a LG-3 frame grabber (Scion, Frederick, MD), and NIH Image (v1.63) software. Cells immunopositive for PER2 were counted manually on the captured images using a 400 X 400-μm template for the CEA and BLA and a 200 X 400-μm template for the DG. Within the DG, the template was placed horizontally over the CA3 region. The estimate of the number of PER2 immunoreactive cells per region was calculated from the counts of the 10 images showing the highest number of labeled nuclei unilaterally. Differences between groups were revealed with ANOVA. The significance threshold was set at 0.05 for all analyses.

## Results

**Two daily patterns of PER2 expression.**

The expression of PER2 in the CEA, BLA and DG was assessed in rats that were housed under a 12h:12h light-dark cycle (300 lux at cage level) for at least 20 days and killed at one of eight equally spaced zeitgeber times (ZTs) (ZT0 denotes time of light on) over the day and night (n = 4 per time point). A significant daily rhythm of nuclear staining for PER2 was seen in each of the three regions, but the time of peak expression

differed. In the CEA, PER2 was expressed primarily in the lateral subregion of the nucleus (Figure 12). Expression was maximal at the beginning of the dark phase of the entraining photocycle (at ZT13) and minimal at ZT1, the onset of the light phase (Figures 12 and 13). This daily pattern is identical to that seen previously in the BNST-OV and SCN of these same rats (Amir et al., 2004), demonstrating that the CEA, BNST-OV, and SCN exhibit synchronous rhythms in expression of PER2. In the BLA complex, the distribution of expression was fairly uniform, whereas in the DG, expression was greatest in pyramidal cell layer. In contrast to the CEA, PER2 expression in BLA and DG was maximal at the beginning of the light phase (at ZT1) and minimal at ZT13 (Figures 12 and 13). Thus, two synchronous but diametrically opposite rhythms in PER2 expression were revealed, one in the CEA and BNST-OV and the other in BLA and DG.

**The SCN controls PER2 oscillations in the CEA, BLA and DG.**

There is considerable evidence that the SCN plays a key role in the regulation of circadian rhythms in *Per* gene expression in the brain and periphery in rats and in mice (Abe et al., 2002; Oishi et al., 1998; Sakamoto et al., 1998). Furthermore, we reported previously that SCN lesions that abolished circadian locomotor activity rhythms blunted the rhythm in PER2 expression in the BNST-OV, whereas incomplete lesions that were without effect on circadian locomotor activity rhythms had no effect on PER2 expression in the BNST-OV (Amir et al., 2004). The results of the present analyses were similar; lesions that abolished circadian locomotor activity rhythms abolished PER2 rhythms in the CEA, BLA, and DG, whereas SCN lesions that had no effect on locomotor activity rhythms had no effect on PER2 rhythms in these regions (Figure 14). These results show

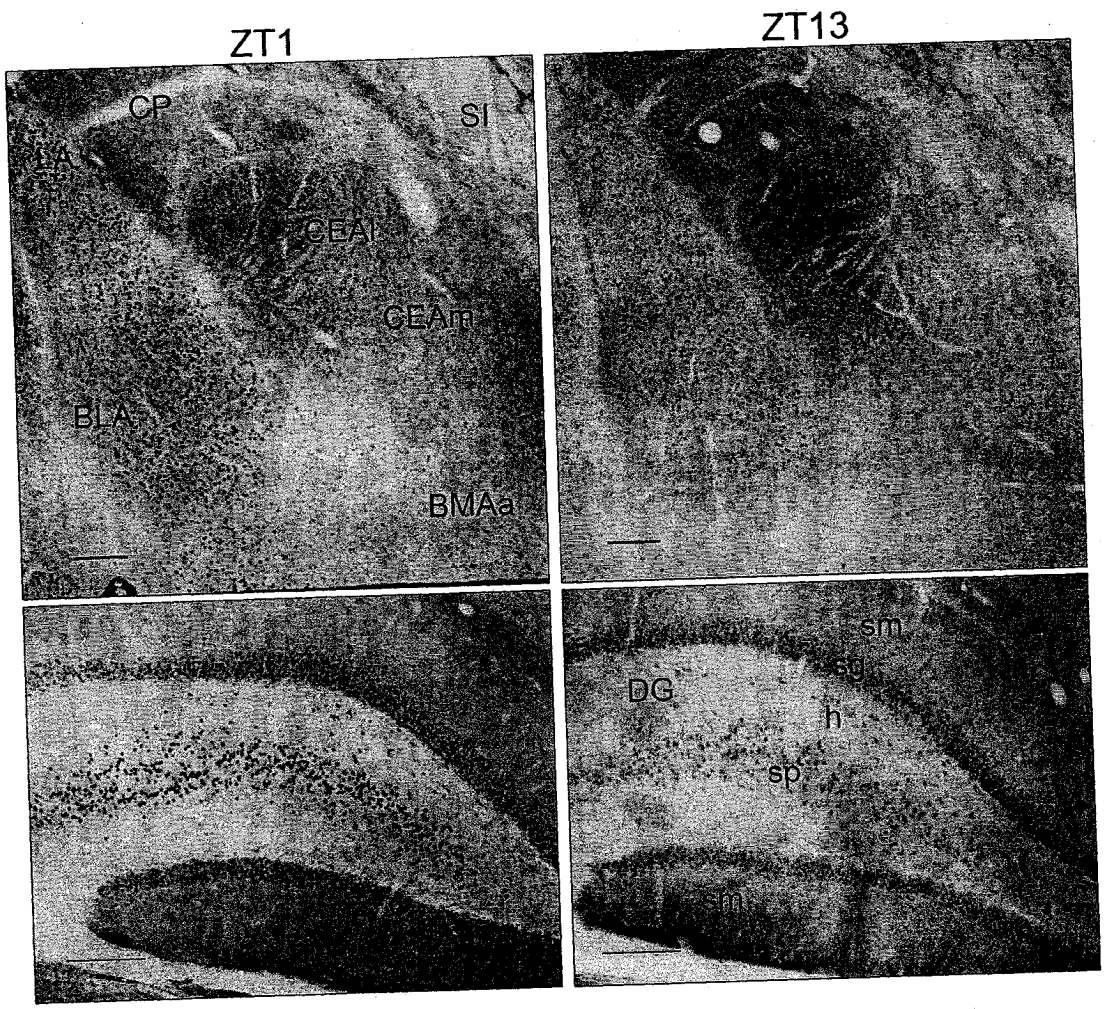


Figure 12. Expression profiles of PER2 immunoreactivity in the CEA, BLA and CA3 region of the DG of rats killed at ZT1 or ZT13. (Scale bars: 200μm). CP, caudoputamen; LA, lateral nucleus of the amygdala; SI, substantia innominata; CEAl, central nucleus of the amygdala, lateral part; CEAm, central nucleus of the amygdala, medial part; BMAa, basomedial nucleus of the amygdala, anterior part; sm, molecular cell layer; sg, granular cell layer; h, hilus; sp, pyramidal cell layer.

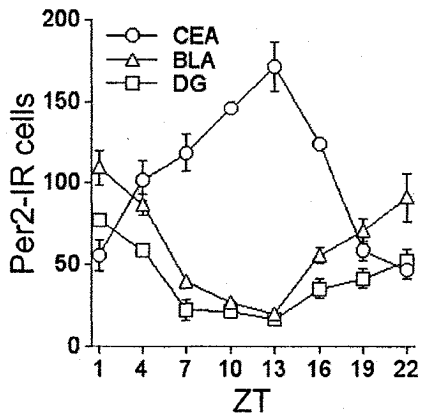


Figure 13. Mean (± SEM) number of PER2-immunoreactive nuclei in the CEA, BLA and CA3 region of the DG of rats killed at different ZTs (n=4 per time point). Cells immunopositive for PER2 were counted manually using a 400x400-μm template for the CEA and BLA and a 200X400-μm template for the DG. The number of PER2 immunoreactive cells per region was calculated for each animal from the counts of 10 images showing the highest number of labeled nuclei.

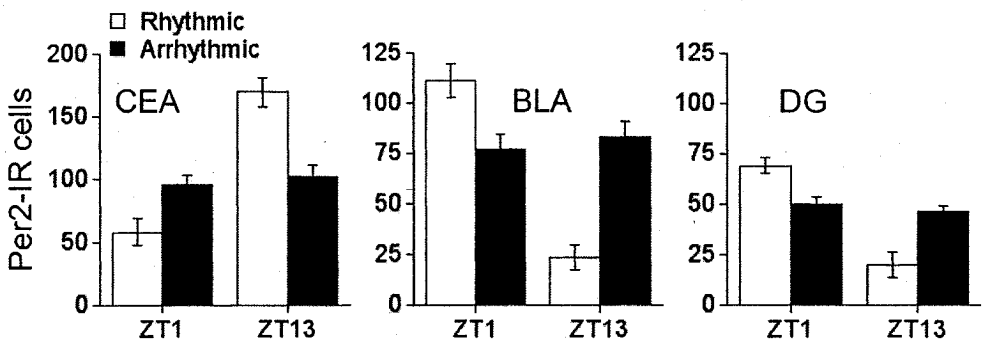


Figure 14. Mean (± SEM) number of PER2 immunoreactive nuclei in the CEA, BLA and CA3 region of the DG of SCN-lesioned rhythmic and SCN-lesioned arrhythmic rats as a function of ZT (n = 6-8 per group).

that, as is the case with the BNST-OV, the rhythms in PER2 expression in the CEA, BLA, and DG depend on the functional integrity of the SCN clock as determined by its ability to drive circadian locomotor activity rhythms.

**PER2 oscillation in the CEA, BLA, and DG after bilateral adrenalectomy**

Cells in the CEA, BLA, and DG express glucocorticoid receptors and exhibit a range of responses to endogenous and exogenous glucocorticoids (Conrad et al., 2004; Honkaniemi et al., 1992; Makino et al., 1994a; Morimoto et al., 1996; Roozendaal & McGaugh, 1997; Sousa & Almeida, 2002). Here, we found that in adrenalectomized rats, the rhythm in PER2 in the CEA was abolished ($F[3,18] = 0.91$, $p = 0.45$) (Figure 15). In contrast, adrenalectomy had no effect on PER2 expression in the BLA and DG. In BLA, PER2 expression was rhythmic in adrenalectomized rats ($F[3,18] = 7.34$, $p = 0.002$), and in the DG, the rhythm of expression approached significance ($F[3,18] = 2.83$, $p = 0.06$). These rhythms were indistinguishable from those in the BLA and DG of sham operated control rats (Figure 15). These finding suggest that PER2 expression in these regions is differentially sensitive to glucocorticoids. Our previous findings showing that removal of the adrenal glands blunts the rhythm in PER2 expression in the BNST-OV led us to suggest a role for adrenal glucocorticoid hormones in the control of PER2 oscillations in the BNST-OV (Amir et al., 2004). The present results show that the rhythm of expression of PER2 in the CEA is similarly controlled by the adrenal gland, whereas that in the BLA and DG are not influenced by adrenal hormones.

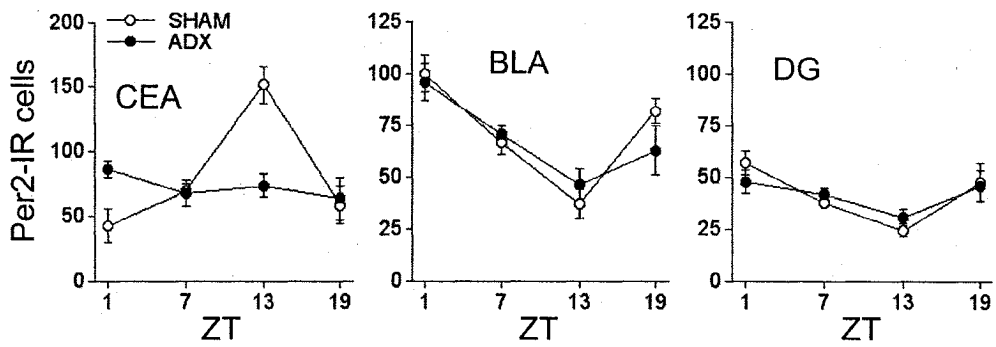


Figure 15. Mean (± SEM) number of PER2 immunoreactive nuclei in the CEA, BLA and CA3 region of the DG of adrenalectomized and sham operated rats as a function of ZT (n = 5-7 per group per time point).

## Discussion

The BLA and CEA are known to play distinct but integrative roles in the regulation of emotional state. BLA receives multimodal sensory input from the cortex and thalamus, and studies of phenomena as diverse as fear learning and drug reward suggest that the primary function of the BLA is to integrate information from conditioned and unconditioned stimuli and to communicate this information to areas of the brain involved in motivational and emotional responses (Cardinal et al., 2002; Everitt et al., 2003; Petrovich et al., 1996). A major projection of the BLA is to the CEA, whose primary function is to gate information from the BLA, and from a host of other cortical and subcortical areas, to the limbic-forebrain, hypothalamic, and brainstem regions controlling specific endocrine, autonomic, arousal, and behavioral responses (Petrovich & Swanson, 1997; Pitkanen et al., 1995; Savander et al., 1995; Veening et al., 1984). Furthermore, there is considerable evidence that information from the BLA is also transmitted to the hippocampus by means of its projections to the entorhinal cortex and can modulate neural plasticity in the DG (Nakao et al., 2004; Pikkarainen et al., 1999). The BLA modulates neural plasticity in the DG, and this effect has been proposed as a mechanism whereby emotional stimuli affect hippocampal memory processes (Abe, 2001; Akirav & Richter-Levin, 2002; Paré, 2003). Several transmitter systems and hormones and a large number of cellular and molecular mechanisms have been proposed to control and modulate neural transmission and plasticity within each of these regions (Pape & Stork, 2003). In the present study, we found that cells in the CEA, BLA, and DG exhibit daily rhythms in expression of the clock protein PER2, a key component of the molecular oscillator generating circadian rhythms in cells and tissues of mammals

(Bae et al., 2001; Zheng et al., 1999). Our finding of circadian oscillations of PER2 in the CEA, BLA, and DG adds a previously unrecognized level of complexity to the control and modulation of activity within these regions.

We identified two temporally distinct patterns of PER2 expression: one in the CEA, where expression peaks in the evening, and one in the BLA and DG, where expression peaks in the morning. Significantly, PER2 expression in the CEA is confined to the lateral part of the nucleus, which closely resembles the cytoarchitecture and chemoarchitecture of the BNST-OV (Cassell et al., 1986; Dong et al., 2001a, b; Ju & Swanson, 1989; Ju et al., 1989). Furthermore, the circadian pattern of PER2 expression in the CEA is identical to that which we found previously in the BNST-OV (Amir et al., 2004). In contrast, the patterns of expression in the BLA and DG resemble those that we have observed in other hippocampal subregions and in cortical areas such as cingulate, entorhinal, piriform, and insular cortices (data not shown).

The two diametrically opposite rhythms observed in the CEA and BNST-OV and in the BLA and DG were similarly controlled by the SCN clock, as shown by the finding that lesions of the SCN that abolished circadian locomotor activity rhythms also abolished the rhythms of PER2 expression. Indirect projections from the SCN to these regions by means of, for example, the paraventricular nucleus of the thalamus, have been described and could carry circadian information to these structures (Moga et al., 1995; Peng & Bentivoglio, 2004). Loss of PER2 rhythmicity in the CEA, BLA, and DG after functional disruption of the SCN clock suggests that rhythmic expression of PER2 in these structures is driven by the SCN. Alternatively, the SCN may function merely as a

synchronizer of rhythms generated in these regions by multiple self-sustaining cellular circadian oscillators (Yoo et al., 2004).

Another important finding, and one with particular significance to the regulation of circadian oscillations within these regions, is that the rhythms in PER2 expression were differentially sensitive to adrenalectomy. As previously reported for the BNST-OV (Amir et al., 2004), the rhythm in PER2 in the CEA depended on the presence of the adrenal glands, whereas the rhythms in the BLA and DG were unaffected by adrenalectomy. The finding that the PER2 expression in the CEA and BNST-OV is abolished by adrenalectomy suggests that the rhythm of PER2 is modulated by glucocorticoids, which is consistent with evidence that *Per* gene expression in peripheral tissues is sensitive to glucocorticoids (Balsalobre et al., 2000 a, b). Furthermore, these regions contain high densities of glucocorticoid receptors, and glucocorticoids are known to modulate cell morphology and the expression of peptides such as corticotropin-releasing factor (CRF) and enkephalin (ENK) in these regions (Ahima et al., 1992; Honkaniemi et al., 1992; Rosenfeld et al., 1993 Makino et al., 1994b; Mulders et al., 1997; Pompei et al., 1995; Shulkin et al., 1998; Watts & Sanchez-Watts, 1995). Preliminary evidence indicates that CORT added to the drinking water can restore PER2 rhythms in the CEA and BNST-OV of adrenalectomized, SCN-intact rats. It is important to note, however, that we have yet to determine whether the presence of CORT in itself is permissive or whether it is the rhythm of intake that brings about this effect. An additional observation that we made is that cells within the CEA and BNST-OV that express PER2 are immunoreactive to ENK but not to CRF. This observation is interesting in light of findings that ENK cells of the CEA and BNSTOV are particularly

sensitive to the effect of stressors and drugs as assessed by *c-fos* gene expression (Day et al., 1999, 2001; Kozicz, 2002).

Contrary to the CEA and BNST-OV, the rhythm of PER2 expression in the BLA and DG was insensitive to adrenalectomy. This finding is surprising, given the overwhelming evidence of the importance of these regions in the effects of glucocorticoids on physiology and behavior. For example, although the BLA has only a moderate level of glucocorticoid receptors, it has been shown that it is critical for the effect of CORT on emotional memory (Roozendaal et al., 2003). The hippocampus contains high levels of glucocorticoid receptors and is well known for its role in stress-induced CORT-mediated feedback regulation of the hypothalamic-pituitary-adrenal (HPA) axis (Jacobson & Sapolsky, 1991; Morimoto et al., 1996). Thus, in these regions, there appears to be a dissociation between the effect of glucocorticoids on memory processes and the regulation of the HPA axis and its effects on PER2 expression.

The current view of circadian organization in mammals is that rhythms are controlled by a hierarchical system of circadian oscillators in the brain and periphery that are subordinate to, or enslaved by, the master clock in the SCN (Schibler & Sassone-Corsi, 2002). Although the function of specific subordinate oscillators is yet to be determined, it is assumed that they serve to gate information from the master clock into tissue-specific rhythmic outputs. In accordance with these ideas, we propose that the cell groups that exhibit rhythms in PER2 expression that we have identified in the amygdala, BNST, and hippocampus are strategically situated to play a role in the circadian modulation of processes normally mediated by these regions. Furthermore, we propose that disturbances of the coupling between the master clock and these cell groups, such as

those we have observed after adrenalectomy and after shifts in the entraining light cycle, will affect emotional state and cognitive functioning. Finally, our findings may suggest a mechanism to explain how stressful experiences and changes in emotional state can act downstream from the SCN to affect specific behavioral and physiological circadian rhythms.

## Acknowledgements

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# CHAPTER 4

## Synchronization of Subordinate Circadian Oscillators in the Limbic Forebrain by Scheduled Feeding

Elaine Waddington Lamont, Valerie Harbour, Barry Robinson, Jane Barry-Shaw, Laura Renteria Diaz, Jane Stewart, & Shimon Amir

Unpublished Manuscript

## Abstract

Rhythmic expression of the clock gene protein Period2 (PER2) suggests the presence of subordinate circadian oscillators in the limbic forebrain, specifically in the oval nucleus of the bed nucleus of the stria terminalis (BNST-OV), the central (CEA) and basolateral (BLA) nuclei of the amygdala, and the dentate gyrus (DG) of the hippocampus. Previously, we demonstrated that scheduled restricted feeding (RF), limiting food access to the day, caused a shift in the phase of PER2 expression in the BNST-OV in rats on a 12 hour light dark (LD) cycle, and restored the rhythm of PER2 expression abolished by long-term exposure to constant light (LL). The phase of PER2 expression in the master clock, located in the suprachiasmatic nucleus (SCN) was unaffected by RF in LD, but became synchronized with the rhythm of PER2 expression in the BNST-OV after RF in LL. We now report that RF also causes a shift in the phase of PER2 expression in the CEA, BLA, and DG, such that all three areas become synchronized, peaking 12 hours after the time of food presentation. This effect was not due to the rewarding aspect of the feeding schedule, as daily access to sucrose or saccharine did not change the phase of PER2 expression. A restricted saline access schedule in rats in a state of salt depletion was also ineffective in altering the phase of PER2 expression. We conclude that RF in a negative energy balance state is required for synchronization of limbic forebrain oscillators.

## Introduction

Feeding that is restricted to the middle of the light phase of the cycle or of the subjective day leads to anticipatory behavioral activity that precedes the occurrence of daily food (see Stephan, 2002 for a review), an anticipatory peak in CORT (Honma et al.,1983; Krieger, 1974), and a number of physiological changes that normally occur in preparation for feeding (Díaz-Muñoz et al., 2000). These behavioral and physiological changes seen in rodents do not depend on the activity of the suprachiasmatic nucleus (SCN), the master circadian clock (Davidson & Stephan, 1999a; Krieger et al., 1977; Stephan et al., 1979a, b), and in fact can actually re-entrain the master clock when it is intact, but arrhythmic as a result of chronic exposure to constant light (Lamont et al., 2005b).

Recently, we demonstrated in the rat the existence of circadian oscillations in the expression of the clock gene protein Period2 (PER2) in parts of the limbic forebrain including the oval nucleus of the bed nucleus of the stria terminalis (BNST-OV; Amir et al., 2004; Lamont et al., 2005b), the central nucleus of the amygdala (CEA), the basolateral nucleus of the amygdala (BLA) and the dentate gyrus of the hippocampus (DG; Lamont et al., 2005a). Furthermore, we found that under some conditions these rhythms can be uncoupled from the rhythm of PER2 in the SCN. For example, we found in rats maintained on a restricted daily feeding schedule that the peak of the rhythm of expression of PER2 in the BNST-OV was shifted in time and uncoupled from that seen in the SCN (Lamont et al., 2005b). These findings have led us to explore further the effects of the restricted feeding schedule on the rhythms of PER2 expression in all of these brain regions.

One question that arises from our previous findings, and from those of others on the effects feeding schedules on clock gene expression in peripheral organs, is whether food deprivation is a necessary condition. Earlier work has suggested that sugar, rather than fat, is effective in inducing behavioral anticipation in food deprived animals (Pecoraro et al., 2002; Stephan & Davidson, 1998). Additionally, previous research shows that ingesting a large, palatable, nutritionally balanced meal during the daytime can cause food anticipatory activity even when the animals have *ad libitum* (AL) access to standard lab chow (Mistlberger & Rusak, 1987), and induce activation of the immediate early gene *c-Fos* in brain reward related brain areas (Mendoza et al., 2005) suggesting that reward may be as important as deprivation in the effect of feeding on the circadian system. In order to explore this issue in the context of our experiments on anticipatory activity and PER2 expression in the brain, we studied the effects of a similar schedule of availability of two highly palatable substances, either sucrose or saccharine, in non-deprived rats. In order to determine whether a deprivation state other than food deprivation would induce changes in the rhythms of PER2 expression, we compared the effects of a restricted feeding schedule to those of a restricted saline access schedule in rats in a state of salt depletion. Finally, we assessed the effects of a restricted feeding schedule in rats with abnormal feeding patterns and known metabolic abnormalities as a result of neonatal treatment with monosodium glutamate (MSG).

## Materials and Methods

### Animals and Housing

All procedures were done in accordance with the Canadian Council on Animal Care guidelines and approved by the Animal Care Committee of Concordia University. Adult male Wistar rats (300-400g; Charles River, St. Constant, QC, Canada) were individually housed in clear plastic cages in light and soundproof ventilated boxes. In cages equipped with running wheels, activity was transmitted via a magnetic micro switch to a computer and recorded using VitalView software (Mini Mitter Co. Inc., Sunriver, OR). All rats except for those housed in constant light (see below) were housed on a 12h/12h light dark schedule throughout the experiments. The lighting schedules were controlled by computer using the VitalView software, or by Noma timers in cages without running wheels. Rats received standard lab chow *ad libitum* (AL) throughout the experiment, except during scheduled restricted feeding (SRF), described below. Tap water was continuously available, with the exception of rats in the scheduled salt access experiment, which had AL access to low sodium food and distilled water. All animals were weighed regularly throughout the experiment either before or after experimental manipulations. Sucrose (32%), saccharine (0.2%), and saline (2%) solutions (Fisher Scientific, Ottawa, ON, Canada) were made with distilled water. Furosemide was diluted from a 50mg/ml (Lasix, CDMV, St. Hyacinthe, QC, Canada) stock solution to 10 mg/kg/ml using 0.9% saline.

### Postnatal Monosodium Glutamate Treatment

Pups were given a subcutaneous injection of 2mg/g monosodium glutamate (MSG, Sigma-Aldrich, Oakville, ON, Canada) in purified distilled water on day 1, 3, 5, 7, and 9 post-partum. Optic nerve atrophy was confirmed at the time of perfusion.

## Procedure

### Scheduled Feeding Experiments

On the day before an experiment began, food was removed from the overhead food hopper near the end of the light period for animals in the RF condition. Standard lab chow pellets were placed in the cage in a small container for 3 hours a day for 10 days at zeitgeber time (ZT) 4-7, where ZT0 is lights on and ZT12 is light off. For AL fed rats, the sound attenuating boxes were opened at times corresponding to presentation and removal of food. A separate group of rats was housed in constant light for 2 months prior to the beginning and throughout the experiment. During the experiment food was presented for 3 hours each day as described for the rats on the 12h/12h schedule. The rats used for these experiments were the same as those used in a previous report (Lamont et al., 2005b) In the case of the MSG-treated rats that become obese on ad lib feeding schedules, all were given restricted access to food to control large differences in weight between groups. Those on the schedule of restricted feeding during the day were treated as described above. For the others food was available for 3 hours during the night from ZT 16-19. It is known that scheduled feeding at night during the period when rats normally eat does not affect the relation between peripheral oscillators and the SCN master clock (Damiola et al., 2000).

**Scheduled Treat Experiments**

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

**Scheduled Salt-Access Experiments**

To induce acute daily salt appetite, rats were given a daily subcutaneous injection of 10mg/kg of furosemide, a diuretic, at ZT5, one hour prior to presentation of 2% saline solution. Animals in the control condition received an injection of physiological saline. Chronic salt appetite was induced by implanting rats with Alzet (Cupertino, CA) osmotic mini-pumps subcutaneously filled with either furosemide or physiological saline under isoflurane anesthesia. The dosage was adjusted to deliver approximately 10mg/kg of furosemide per day. All rats in these experiments were given access to a 2% saline solution for 1 hour daily at ZT6 for 10 days.

**Tissue Preparation**

Groups of rats were injected with an overdose of sodium pentobarbital (100 mg/kg) on Day 11 (scheduled feeding experiment and salt-access experiments) or Day 15 (scheduled treat) at times corresponding to the beginning (ZT1) or middle (ZT7) of the light phase, or the beginning (ZT13) or middle (ZT19) of the dark phase. Scheduled feeding rats killed at ZT1 had no food before perfusion, whereas those killed at ZT7 did. Rats were perfused intracardially with cold isotonic saline (0.9% sodium chloride at 4°C) followed by cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3). Brains were removed and postfixed overnight in 4% paraformaldehyde at 4°C. Serial coronal sections

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

**Immunocytochemistry**

Free floating sections were rinsed (3 X 10 minutes) in cold 50 mM Trisma buffered saline (TBS; pH 7.6) then incubated in a hydrogen peroxide (3% $H_2O_2$ in TBS) quenching solution for 30 minutes at room temperature (RT). Sections were again rinsed in TBS, preblocked in a solution of 0.3% Triton X 100 in TBS (Triton-TBS), 3% normal goat serum (NGS), and 5% milk buffer (MB), then transferred directly into a rabbit polyclonal PER2 antibody (ABI, San Antonio, TX) with 0.3% Triton-TBS, 3% NGS and 5% MB and incubated for approximately 48 hours at 4° C. Sections were then rinsed for 3 X 10 minutes in cold TBS and incubated for one hour (4° C) in a secondary antibody solution made from a biotinylated anti-rabbit IgG made in goat (Vector Labs, Burlington, ON, Canada), diluted to a concentration of 1:200 with 0.3% Triton-TBS and 3% NGS, rinsed in TBS, and transferred to a tertiary phase consisting of an avidin biotin peroxidase complex in TBS (Vectastain Elite ABC Kit, Vector Labs) for 2 hours at 4 °C. Finally, sections were rinsed with TBS, and then rinsed again with cold 50 mM Tris-HCl (pH 7.6) for 10 minutes. Sections were then incubated for 10 minutes with 0.05% DAB in 50 mM Tris-HCl, then a further 10 minutes in DAB/50mM Tris HCl with 0.01% H2O2 and 8% NiCl on an orbital shaker. After this final incubation, sections were rinsed in cold TBS, wet-mounted onto gel coated microscope slides, dehydrated in a series of alcohols, soaked in Citrisolve (Fisher) for 30 minutes, and coverslipped with Permount (Fisher).

**Immunocytochemical Data Analysis**

PER2 immunoreactivity was examined under a light microscope (Leitz Laborlux

S) and recorded for each animal. Brain sections through the BNST-OV, SCN, CEA, BLA and HIP were digitized using a Sony XC-77 Video Camera connected to a Scion LG-3 frame grabber using the NIH Image Software package (version 1.62). The number of PER2 positive cells was counted within a 400X400 μm square using Image SXM (version 1.73) and the average was calculated for the 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.

**Behavioral Data Analysis**

Activity data for individual animals were recorded continuously and displayed in 10-min bins using the Dataquest III software package (Mini Mitter, Sunriver, OR). Double plotted actograms were used to monitor running-wheel activity rhythms at each stage of the experiments.

## Results

**Restricting feeding to the middle of the day causes limbic forebrain oscillators to become uncoupled from the SCN, but synchronized with each other.**

As found previously in the BNST-OV (Lamont et al., 2005b), rats restricted to feeding in the middle of the day and housed on a 12h/12h light/dark cycle showed anticipatory activity for 1-3 hours before the presentation of food and had a rhythm of PER2 expression in the CEA, BLA and DG that peaked at ZT19. Figure 16 shows the PER2 rhythms in each of these regions for rats in the FR group compared to those for rats in the control group. It can be seen that the rhythm peaked at ZT19 (12 hours after the time of feeding) in all regions in FR group, whereas in the AL group it peaked at ZT13 in

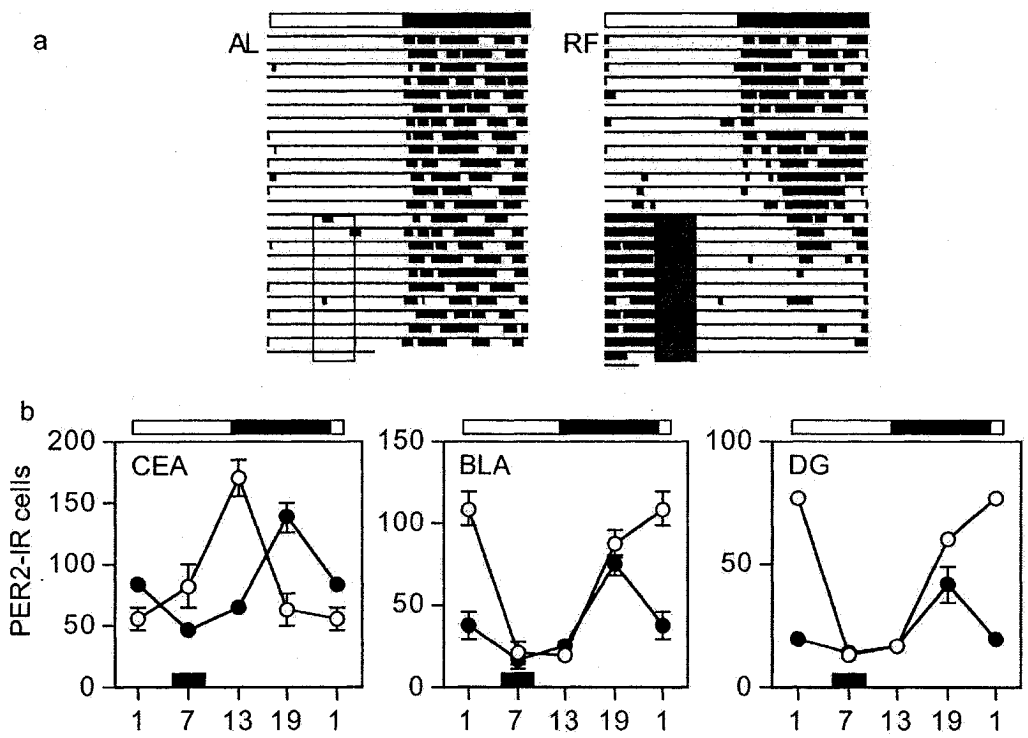


Figure 16. Representative single plotted actograms (a) and mean (± SEM) number of immunoreactive nuclei (b) found in the CEA, BLA, and DG as a function of time in freely fed (open circles) or restricted feeding (closed circles) rats housed in L/D (n = 5-6 per group). Vertical rectangles (a) and black boxes (b) indicate the time of food availability.

the CEA and ZT1 in the BLA and DG (CEA: main effect of time, $F[3,23] = 9.457$, $p < 0.0003$; time X treatment interaction, $F[3,23] = 26.064$, $p < 0.0001$; BLA: main effect of time, $F[3,23] = 39.296$, $p < 0.0001$; time X treatment interaction, $F[3,23] = 10.926$, $p < 0.0001$; DG: main effect of time, $F[3,23] = 42.516$, $p < 0.0001$; time X treatment interaction, $F[3,23] = 19.290$, $p < 0.0001$). Thus not only did the restricted feeding schedule lead to a shift in the time of peak of the rhythm of PER2 expression in all regions, but the rhythms in BLA and DG became synchronized to those in CEA and BNST-OV. This was a surprising finding in that in the AL group the CEA and DG rhythms were 180° out of phase with those in the CEA and BNST-OV.

This experiment supports and extends research demonstrating that when feeding is restricted to the day, there was a 12 hour shift in the peak of *Per1* and *Per2* mRNA expression in the cortex and hippocampus without affecting the SCN (Wakamatsu et al., 2001). Similar shifts in clock gene mRNA expression, up to 12 hours, have been found in the liver and other organs, whereas gene expression in the SCN remained entrained to the light-dark cycle (Damiola et al., 2000; Stokkan et al., 2001). Damiola et al. (2000) also found a similar result when animals were housed in constant dark (DD) and had their feeding restricted to the subjective day (inactive period).

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

Long-term exposure to constant light produces behavioral arrhythmicity and a loss of PER2 expression rhythm in the SCN (Beaulé et al., 2003a; Lamont et al., 2005b; Muñoz et al., 2005; Ohta et al., 2005; Sudo et al., 2003), and in the BNST-OV (Amir et al., 2004). We reported recently, that SRF restores both circadian running wheel activity

and PER2 rhythm in the BNST-OV and, surprisingly, the SCN, demonstrating that the master clock is sensitive to restricted feeding when it is not in a state of endogenous synchronicity (Lamont et al., 2005b). In this experiment, we sought to determine whether FR would induce similar changes in PER2 rhythms in all regions studied in rats housed in constant light.

As was seen previously in the SCN and BNST-OV in rats housed in constant light and fed ad lib (Lamont et al., 2005b), the rhythm of PER2 expression in the BLA, and DG (main effect of time: BLA: $F[3,8] = 1.915$, $p = 0.2057$; DG: $F[3,8] = 1.456$, $p = 0.2975$) was abolished. A rhythm remained in the CEA (CEA: $F[3,8] = 9.071$, $p < 0.0059$), but was greatly attenuated (Figure 17b). When food was restricted to 3 hours at the same time each day, the rhythm of PER2 expression peaked at ZT18 (12 hours after feeding) in the CEA and DG (CEA: $F[3,8] = 13.609$, $p < 0.0017$; DG: $F[3,8] = 6.715$, $p < 0.0141$). A two way ANOVA between the AL and RF groups revealed a significant effect of treatment ($F[3, 16] = 34.331$, $p < 0.0001$), and a treatment by time interaction in the CEA ($F[3, 16] = 5.977$, $p < 0.0062$), suggesting that RF did have a significant effect on the rhythm of PER2 expression in this area, even though the rhythms was not completely abolished by long term exposure to LL, as it was in the other areas studied (Lamont et al., 2005b). A rhythm of PER2 expression was not restored in the BLA (BLA: $F[3,8] = 1.888$, $p = 0.2101$). However, the two way ANOVA between the AL and RF groups did reveal a significant main effect of treatment ($F[3, 16] = 13.393$, $p < 0.0021$), suggesting that although the PER2 rhythm was not restored, RF did have a significant effect in the BLA (Figure 17b).

This synchronization of PER2 rhythmicity was accompanied by increased wheel

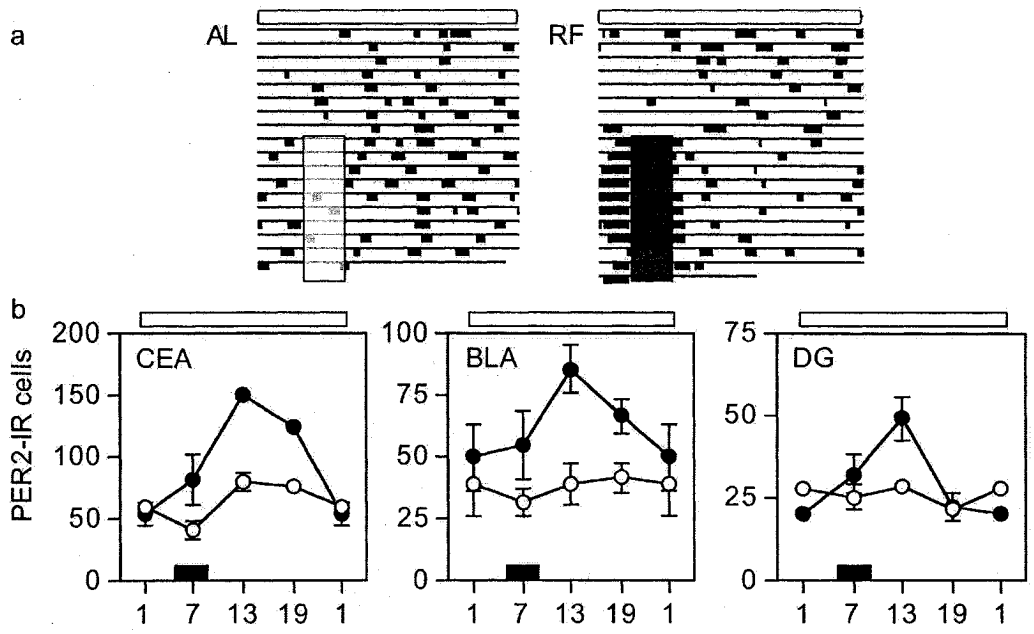


Figure 17. Representative single plotted actograms (a) and mean (± SEM) number of immunoreactive nuclei (b) found in the CEA, BLA, and DG as a function of time in freely fed (open circles) or restricted feeding (closed circles) rats housed in LL (n = 3 per group). Vertical rectangles (a) and black boxes (b) indicate the time of food availability.

running activity for 1 to 3 hours around the time of food availability (Figure 17a). Anticipatory activity tended to be less robust than that observed in animals on a normal light dark cycle and activity in general was quite low.

**Restricted access to a scheduled treat is not sufficient to change the rhythms of PER2 expression in subordinate oscillators.**

Feeding in a deprived state is rewarding for rats (Berridge, 1996). Sucrose and saccharine are also rewarding and non-deprived rats will consume them in approximately equal quantities (Ågmo & Marroquin, 1997; Messier & White, 1984), but only the sucrose, which has nutritive value, induces conditioned place preference (Ågmo & Marroquin, 1997; White & Carr, 1985). Therefore, the goal of this study was to evaluate the effect of nutritive and non-nutritive rewarding substances on the rhythms of PER2 expression in the limbic forebrain in the absence of deprivation. Animals that were given limited access to sucrose and saccharine readily consumed these liquids, suggesting that they did find them rewarding (sucrose M = 19.114 ml, SEM = 0.634, saccharine M = 9.172 ml, SEM = 0.326, water M =3.568, SEM = 0.168). However, neither sucrose nor saccharine animals showed the same behavioral anticipation seen in the restricted food access experiments (Figure 18a). Significant differences in PER2 expression were found in the BNST-OV (significant effect of treat group $F[2, 47] = 4.892$, $p < 0.012$; group by time interaction, $F[6, 47] = 3.912$, $p < 0.003$) and BLA (significant effect of group, $F[2, 46] = 3.269$, $p < 0.047$). There were no apparent phase shifts but only differences in absolute levels of PER2 expression, which was very different from the synchronization among all of the limbic forebrain regions seen with scheduled restricted feeding (Figure 18b).

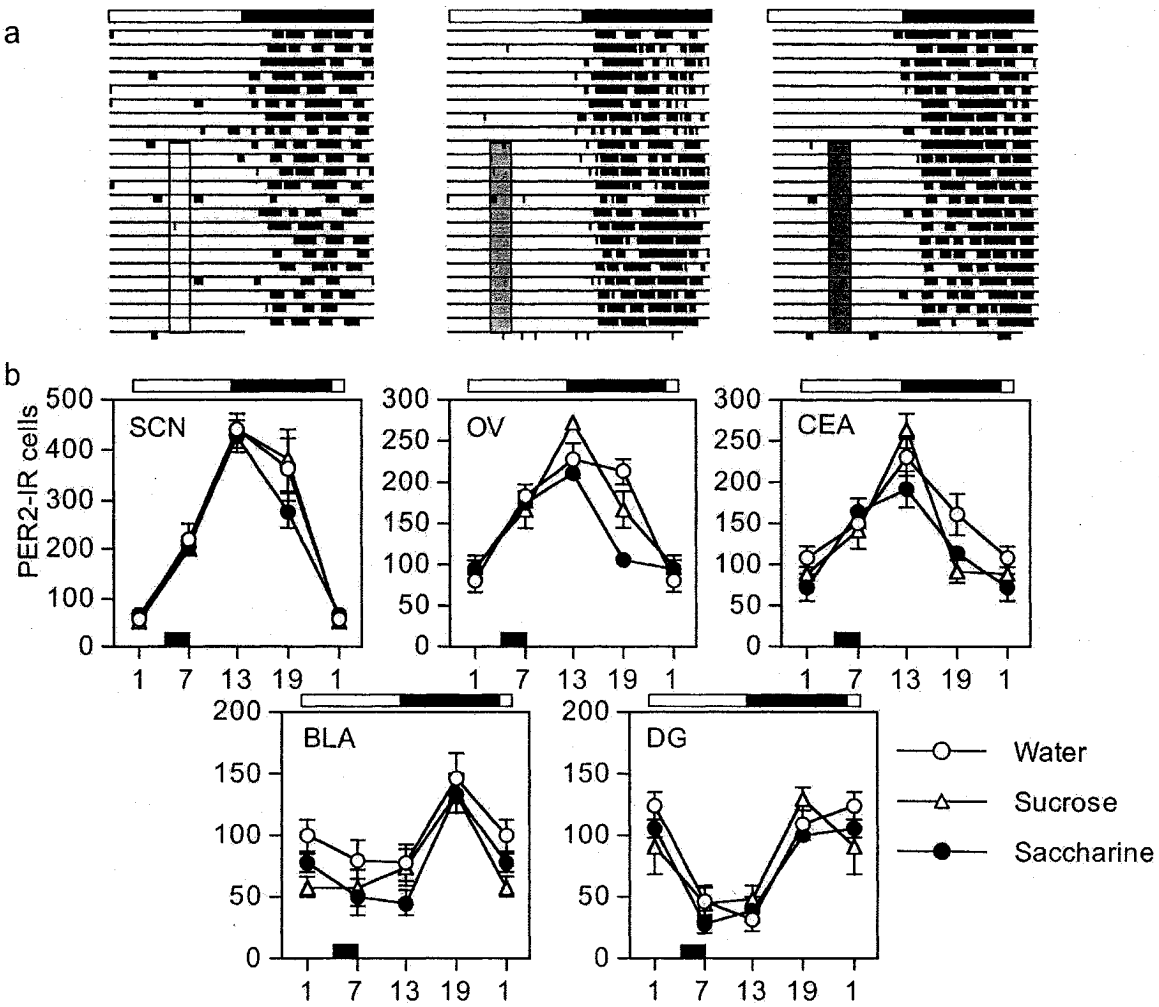


Figure 18. Representative single plotted actograms of locomotor activity (a) and mean (± SEM) number of immunoreactive nuclei (b) found in the CEA, BLA, and DG as a function of time in freely fed rats housed in L/D and given access to water (open circles), sucrose (open triangles), or saccharine (closed circles) for 2 hours each day for 11 days (n = 4-5 per group). Vertical white (water), light gray (sucrose), and dark gray (saccharine) rectangles (a) and black bars (b) indicate the time of treat availability.

**Induction of a negative metabolic state alone is not sufficient to produce synchronization of subordinate oscillators.**

Providing scheduled access to saline in a salt deprived anima produces a situation that more closely parallels scheduled feeding because both a reward stimulus and a state of deprivation are present (Berridge, 1996; Johnson & Thunhorst, 1997). In view of this, we hypothesized that scheduled access to saline could provide the necessary metabolic signals that would cause synchronization of these oscillators and uncouple them from the master clock. Daily injection of the diuretic furosemide produced a repeated acute salt appetite, causing animals to drink more of a 2% saline solution in a 1-hour period each day than saline injected controls (salt appetite group M = 6.96 ml, SEM = 0.621, control group M = 3.81 ml, SEM = 0.268). However this had no effect on PER2 expression in any of the brain regions examined (Figure 19).

Chronic salt appetite, induced by continuous administration of furosemide via osmotic mini-pumps, also produced salt appetite (salt appetite group M = 7.91 ml, SEM = 0.604, control group M = 4.61 ml, SEM = 0.358). Unlike scheduled feeding, there was little evidence of behavioral anticipation prior to salt access (Rosenwasser, et al., 1985, 1988) (Figure 20a). There was a significant reduction in PER2 expression in the BLA (main effect of treatment group $F[1, 28] = 5.637$, $p < 0.025$) around the time of salt access (Figure 20b, 21). However, contrary to our prediction, there was no effect on the BNST-OV, CEA, or DG oscillators.

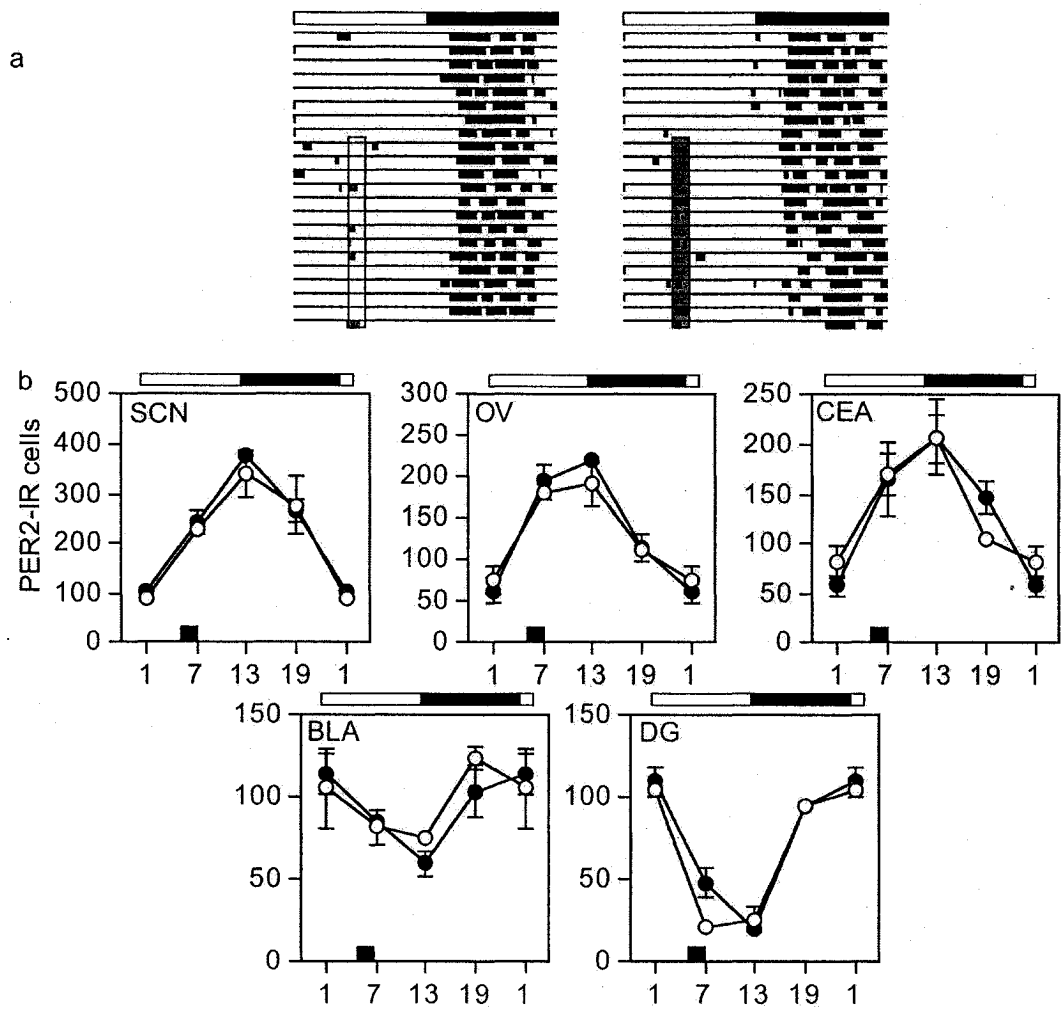


Figure 19. Representative single plotted actograms of locomotor activity (a) and mean (± SEM) number of immunoreactive nuclei (b) found in the CEA, BLA, and DG as a function of time in freely fed rats housed in L/D, given daily injections of saline (open circles), or 10 mg/kg furosemide (closed circles), then allowed access to 2% saline for 1 hour each day for 11 days (n = 6 per group). Vertical rectangles (a) and black bars (b) indicate the time of saline availability.

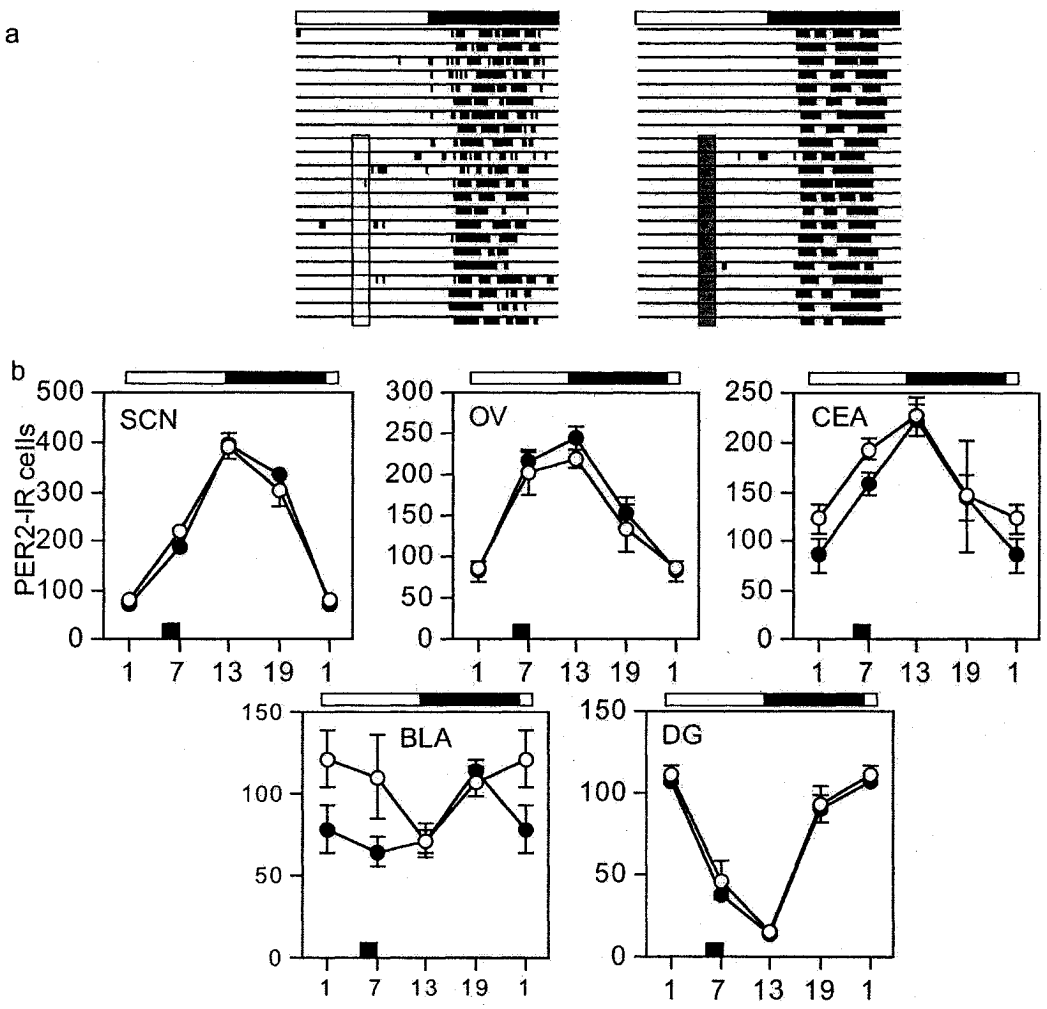


Figure 20. Representative single plotted actograms of locomotor activity (a) and mean (± SEM) number of immunoreactive nuclei found in the CEA, BLA, and DG as a function of time in freely fed rats housed in L/D, implanted with osmotic minipumps delivering saline (open circles), or approximately 10 mg/kg/day of furosemide (closed circles), then allowed access to 2% saline for 1 hour each day for at least 11 days (n = 6-8 per group). Vertical rectangles (a) and black boxes (b) indicate the time of saline availability.

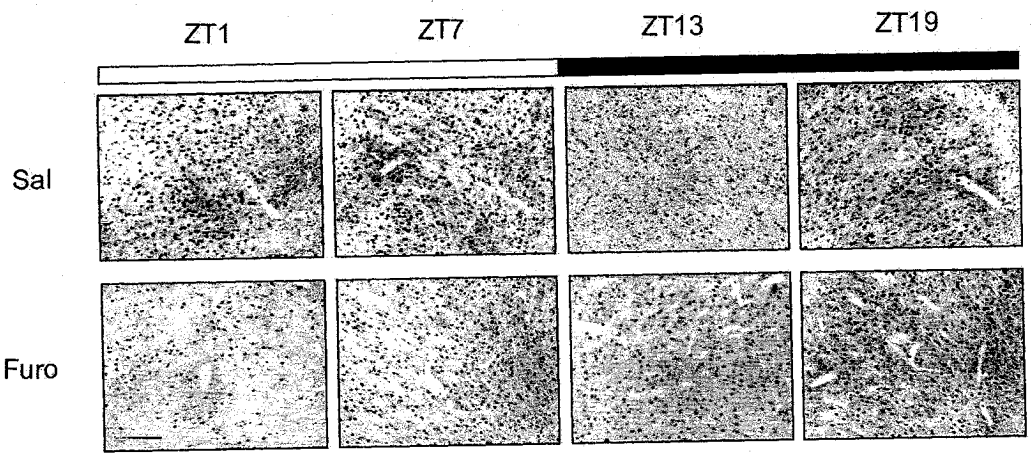


Figure 21. Representative photomicrographs showing PER2 expression in the BLA as a function of time in rats housed in L/D, implanted with osmotic minipumps delivering saline (Sal), or approximately 10 mg/kg/day of furosemide (Furo), then allowed access to 2% saline for 1 hour each day for at least 11 days (n = 6-8 per group). Scale bar, 100 μm.

**Synchronization of subordinate oscillators by food is not impaired by MSG induced lesions of the arcuate nucleus.**

In the previous experiments we found virtually no effects on PER2 rhythms in rats given access to food treats in the middle of the day without food restriction. Similarly, there was only a minimal effect on these rhythms in rats given access to a palatable salt solution in salt deprived rats, suggesting that alterations in energy metabolism induced by food restriction were critical to the effect. We asked therefore whether the effects of a restricted feeding schedule would be similar in rats with known abnormal feeding patterns and metabolic abnormalities as a result of neonatal treatment with monosodium glutamate (MSG). Neonatal treatment with MSG causes severe damage to the orexigenic population, co-expressing neuropeptide Y (NPY) and agouti-related gene product (AgRP; Broberger et al., 1998; Cowley et al., 1999; Meister et al., 1989; Zigman & Elmquist, 2003) leading to hypophagia, but also to obesity due to loss of negative feedback regulation of body weight (Dawson et al., 1997). MSG treated rats show normal and even accentuated food anticipatory activity, but somewhat abnormal feeding patterns under ad lib conditions (Mistlberger & Antle, 1999; Stricker-Krongrad et al., 1998).

Regardless of their behavioral and physiological abnormalities, we found that MSG treated animals on the RF schedule showed food anticipatory activity (Figure 22a) and changes in the rhythms of PER2 expression in the BNST-OV, BLA, and DG, and a trend in the CEA. The rhythms were synchronized and peaked at ZT19, about 12 hours after food presentation (OV: main effect of time, $F[3,38] = 3.952$, $p < 0.0151$; time X treatment interaction, $F[3,38] = 5.471$, $p < 0.0032$; CEA: main effect of time, $F[3,37] =$

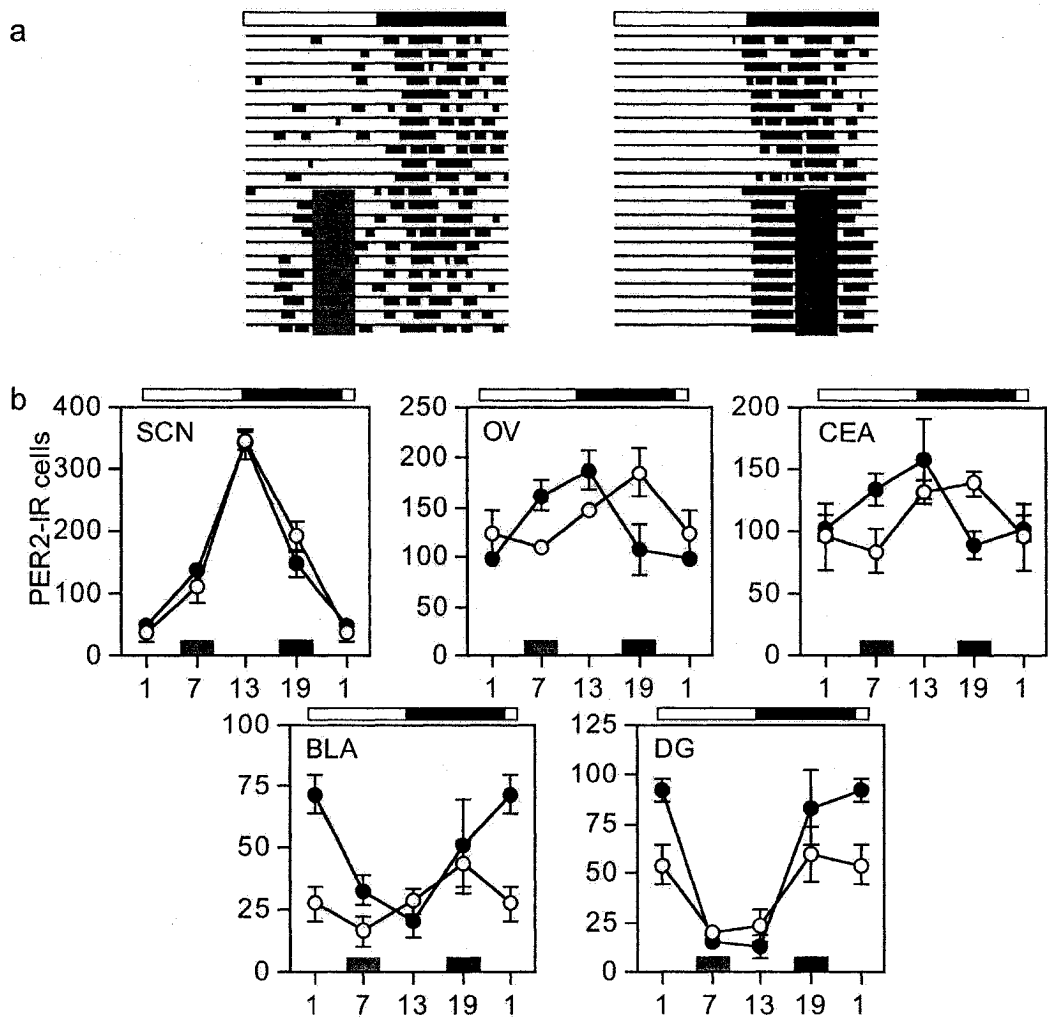


Figure 22. Representative single plotted actograms of locomotor activity (a) and mean (± SEM) number of immunoreactive nuclei found in the SCN, BNST-OV, CEA, BLA, and DG as a function of time in MSG treated rats housed in LD (n = 4-7 per group), and given three hours of access to food during the day (open circles) or night (closed circles). Vertical rectangles (a) and boxes (b) indicate day (light gray) or night (dark gray) feeding times.

2.752, p = 0563; time X treatment interaction, F[3,37] = 2.444 p = 0.0793; BLA: main effect of time, F[3,37] = 5.124, p < 0.0046; time X treatment interaction, F[3,37] = 3.758, p < 0.0188; DG: main effect of time, F[3,37] = 21.329, p < 0.0001; time X treatment interaction, F[3,37] = 2.929, p < 0.0463). PER2 in the SCN was unaffected (main effect of time, F[3,38] = 103.239, p < 0.000; time X treatment interaction, NS) (Figure 22b). Furthermore, the findings from the MSG rats given food during the day, contrast sharply with those from the MSG rats given food access for three hours during the night. This later group showed high activity levels at night with an interruption during food presentation, but PER2 rhythms in all regions studied maintained their phase relationship with the SCN, and were indistinguishable from those of ad lib fed normal rats (compare Figure 22b with Figure 16b, and Chapter 2, Figure 10) (Amir et al., 2004; Lamont et al., 2005a,b).

## Discussion

The findings from these experiments show that a restricted feeding schedule that is sufficient to produce behavioral anticipation also can uncouple the phase of PER2 expression in the limbic forebrain from that of the SCN and produce synchronization of the phase of PER2 expression between brain regions that are normally opposite in phase. Furthermore, the synchronization of the phase of PER2 expression between the limbic forebrain oscillators must be the result of the combined effects of a rewarding stimulus with negative energy balance, as neither daily presentation of a nutritive (sucrose) or non-nutritive (saccharine) treat without food deprivation, nor chronic salt deprivation with daily access to saline were effective in producing this effect.

Given that the BNST-OV, amygdala, and hippocampus have various functions related to incentive salience, motivation, reward, memory, and energy balance, it is likely that the synchronization of PER2 expression in these distinct, but interconnected brain regions represents a coordinated response to the presentation of the most ecologically relevant stimulus, food, presented outside of the normal active period.

A question emerging from these experiments is what is the mechanism that causes synchronization of the phase of these putative circadian oscillators? One possibility is that limbic forebrain oscillators synchronize each other via direct anatomical projections. This seems possible within the central extended amygdala, due to the high level of interconnectivity between the BNST-OV and the CEA (Dong et al., 2001a, b), but is less likely with the other limbic forebrain regions. The BLA sends few if any projections to the BNST-OV, but instead projects to the CEA (Dong et al., 2001a, b; Savander et al., 1995). The hippocampus does project to most subregions of the of the amygdala (Petrovich et al., 2001). As well, there is evidence that synaptic plasticity in the DG can be modulated by stimulation of the BLA (Nakao et al., 2004), suggesting a functional relationship between the BLA and DG. However, there is little evidence of direct anatomical connections from the BLA to the DG except via the entorhinal cortex or parasubiculum (Nakao et al., 2004; Petrovich et al., 2001; Pikkarainen et al., 1999). Furthermore, if the phase of PER2 expression between limbic forebrain regions could be synchronized by direct anatomical interconnections, we might expect these brain regions to remain synchronized with each other after SCN lesion. Currently, there is no evidence that this can occur, as PER2 rhythms are abolished by SCN lesions that produce behavioral arrhythmicity (Amir et al., 2004; Lamont et al., 2005a). Therefore, the

maintenance of the phase relationship of PER2 expression in the limbic forebrain regions is not likely due to anatomical connections within the limbic forebrain.

Daily scheduled feeding in food restricted animals seems to be sufficient to induce uncoupling of peripheral oscillators (Damiola et al., 2000; Le Minh et al., 2001) and extra-SCN brain oscillators (Lamont et al., 2005a; Wakamatsu et al., 2001) from the SCN, and synchronize the phase of rhythmic PER2 expression in brain regions that are normally opposite in phase, whereas the scheduled presentation of rewarding stimuli or metabolic challenge alone affect the amount, but not phase, of PER2 expression. This suggests that some stimulus capable of altering the phase of clock gene expression in the periphery and brain, but not the SCN, occurs as a result of the change in meal timing from the night to day. There is evidence that adrenal hormones play a role in the effect of RF on clock gene expression in the brain and periphery, but not in the SCN.

Glucocorticoids are critical for resetting peripheral clocks in the liver, kidney, and heart, (Balsalobre et al., 2000a). The glucocorticoid hormone analogue dexamethasone can induce rhythmic expression of clock and clock controlled genes in rat-1 fibroblasts, and either advance or delay the phase of clock gene expression in the liver, kidney and heart *in vivo*, depending on the time of day it is administered. Furthermore, the phase resetting of clock gene expression in the liver by dexamethasone was found to be dependent on functional glucocorticoid receptors (GR's), as mutant mice with inactive GR's restricted to the liver hepatocytes ($GR^{alfp}$Cre mice) showed phase shifts and induction of Per1 expression in the heart and kidney, but not the liver, following dexamethasone injection (Balsalobre et al., 2000a). As expected, clock gene expression

in the SCN, which lacks GR's in adult animals (Rosenfeld et al., 1988; 1993), was unaffected by dexamethasone administration.

Under conditions of restricted feeding, glucocorticoids seem to be responsible for inhibiting, rather than enhancing food-induced phase shifts in peripheral oscillators. Normally the shift in clock gene expression in the peripheral tissues induced by day-time feeding takes several days, similar to light-induced phase shifts in the SCN. In adrenalectomized animals, feeding-induced phase shifts in peripheral organs occur almost immediately, suggesting that glucocorticoids slow down the entrainment of peripheral gene expression to a day-time feeding schedule that is in conflict with the normal nocturnal feeding pattern of the animal. Again this effect required functional GR's, as $GR^{alfp}Cre$ mice showed shifts in clock gene expression in the liver after only 2 days, while other tissues with functional GR's required a week to show the same shifts. Wild-type mice showed similar clock gene expression in all peripheral tissues at both time points (Damiola et al., 2000). Although these results seem somewhat contradictory, it is clear that glucocorticoids act as modulators of the phase of peripheral oscillations, and may also play a role in the phase relationship of PER2 expression in the limbic forebrain.

A further piece of evidence that glucocorticoids may be critical in the synchronization of the phase of PER2 expression in the limbic forebrain is the dramatic change in the CORT rhythms that occurs under scheduled feeding. Normally, nocturnal animals on an AL feeding schedule show a peak in CORT levels around the offset of light and onset of activity. Daytime fed animals show either a shift in the phase of CORT or a second peak in CORT levels just before feeding onset (Damiola et al., 2000; Davidson & Stephan, 1999b; Díaz-Muñoz et al., 2000; Honma et al., 1983; Krieger,

1974; Morimoto et al., 1977). This finding has been replicated numerous times and seems to be related both to the timing of the meal, and a state of negative energy balance. Pecoraro and colleagues (2002) demonstrated that a rise in CORT level occurs in anticipation of sucrose in food deprived rats, but not in AL fed animals (Pecoraro et al., 2002). Therefore, it may be a change in the timing of CORT secretion that is causing a synchronization of PER2 expression in the extra-SCN brain oscillators as well as the rapid uncoupling of the SCN from peripheral and extra-SCN oscillators. Consistent with this hypothesis is the critical role of glucocorticoid regulation in energy balance (Richard et al., 2000). In fact even relatively moderate alterations in the circadian timing of glucocorticoid concentrations can lead to significant fat storage (Dallman et al., 2000). Also consistent with this hypothesis is the finding of synchronization of brain oscillators in MSG treated rats. Although MSG animals are obese and have severe lesions of most of the neurons critical for energy balance in the arcuate nucleus of the hypothalamus (Broberger et al., 1998; Meister et al., 1989), evidence suggests that these animals have a somewhat blunted circadian rhythm of CORT (Magarinos et al., 1988) but show normal changes in corticotrophin releasing hormone (CRF; Kiss et al., 1999) and adrenocorticotropic hormone (ACTH; Larsen et al., 1994) in response to an acute stressor, normal dexamethasone suppression of CORT, and enhanced CORT secretion in response to stress (Larsen et al., 1994; Magarinos et al., 1988). It is likely, therefore that MSG rats would show the same prefeeding increase in CORT seen in normal animals on RF.

On the surface, the idea that CORT mediates the synchronization of peripheral and extra-SCN oscillators is appealing. There is certainly evidence for a blood borne

signal that is capable of synchronizing peripheral oscillators (Guo et al., 2005; Silver et al., 1996). We have also shown that adrenal hormones are critical for the rhythm of PER2 in both the CEA and BNST-OV (Amir et al., 2004, Lamont et al., 2005a). Intact adrenals also seem to be critical for the circadian rhythm of electrical activity in the hypothalamus (Terkel et al., 1974). There are, however, findings that do not support this hypothesis. Evidence from this laboratory shows that the oscillators of the BLA and DG are unaffected by adrenalectomy; they remain rhythmic and maintain their phase relationship with the SCN (Lamont et al., 2005a). In addition neither adrenalectomy (Stephan et al., 1979a) nor hypophysectomy (Davidson & Stephen, 1999b) prevents behavioral anticipation of scheduled feeding. These findings do not completely exclude the possibility that changes in glucocorticoid levels play a role in the synchronization of oscillators outside the SCN. It is possible that even if the BLA and DG do not require adrenal hormones for their rhythmicity, they still respond to changes in peak CORT levels. Rhythmic CORT seems to be necessary for rhythmic PER2 expression in the BNST-OV and CEA, but not BLA and DG (Segall et al., 2005, SFN Abstract). Similarly, a shift in the CORT rhythm may responsible for the synchronization of the phase of PER2 expression seen in the limbic forebrain after RF. If this is so, then preventing the shift in CORT rhythm by placing adrenalectomized animals on a restricted feeding schedule should result in a lack of PER2 rhythm in BNST-OV and CEA, while the BLA and DG would remain rhythmic but show no shift in the phase of PER2 expression. Experiments examining the effects of scheduled feeding in adrenalectomized animals would be required to test this question.

In the present experiment, changes were found in PER2 protein levels in the BLA in response to saline access in animals with chronic salt appetite, and in the BLA, CEA, and BNST-OV in ad lib fed animals given daily access to sucrose and saccharine. This suggests daily access to a treat and salt appetite can cause changes in PER2 expression of specific limbic forebrain regions, but are insufficient to produce shifts in the phase of PER2 expression. Perhaps these alterations in the level of PER2 expression in these regions are simply molecular correlates of neuronal activation. For instance, the BNST, CEA, and BLA are important for the control of sodium appetite and lesions of these areas specifically affect salt appetite in response to sodium depletion (Nachman & Ashe, 1974; Johnson et al., 1999; Zardetto-Smith et al., 1994). In addition, the BLA is critical for conditioned taste aversion (Nachman & Ashe, 1974; Rolls & Rolls, 1973), and may play a vital role in memory for the significance of food (Gallagher, 2000; Petrovich & Gallagher, 2003). Further research using experimental manipulations that allow for the independent manipulation of the BNST-OV, CEA, BLA, and DG may provide insight into the function of circadian oscillators in each of these regions.

The presence of rhythmic PER2 expression in the limbic forebrain suggest that these regions may act as subordinate circadian oscillators, and may be responsible for the modulation of circadian rhythms of emotional and motivational state, downstream of the SCN master clock. Under conditions where feeding, CORT rhythm, and activity occur together during the dark phase of the light dark-cycle, the SCN and limbic forebrain regions maintain their typical phase. However, when these conditions are violated, as in the case of scheduled restricted feeding, the phase relationship among these oscillators changes and even the master clock can become entrained to a non-photic cue (Castillo et

al., 2004; Lamont et al., 2005a). This has significant implications for meal timing as a means of improving adjustment to shift work and jet lag.

## Acknowledgements

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# GENERAL DISCUSSION

A number of significant findings emerged from the experiments presented in this thesis. The first is that there are two distinct and opposite patterns of PER2 expression in the limbic forebrain. One pattern occurs in the BNST-OV and CEA. In these two regions, PER2 expression peaks around the beginning of the night or subjective night, and is synchronized and in phase with the SCN. The other pattern, synchronized with the SCN but 180° out of phase, occurs in the BLA and DG, as well as in cortical areas (data not shown). The rhythm of expression of PER2 in all of these limbic forebrain regions was abolished by SCN lesions and LL, suggesting that it was dependent on an anatomically and functionally intact SCN. However, these regions were differentially sensitive to the effects of adrenalectomy, in that the BNST-OV and CEA required adrenal hormones for the maintenance of PER2 rhythmicity, whereas PER2 expression in the BLA and DG remained rhythmic and maintained its phase relationship to the SCN even in adrenalectomized animals.

There are at least two possible explanations of these effects. One is that the rhythm of PER2 expression is abolished because the molecular clock mechanism ceases to operate and the individual neurons become arrhythmic. The other possibility is that each neuron continues to rhythmically express PER2, but these neurons no longer act in concert with each other either within, or between brain regions, with the result that a coherent rhythm in PER2 expression is not apparent using immunocytochemistry, which provides only a "snap-shot" of the number of cells immunopostive for a protein at a specified point in time, but does not reveal changes within a cell over time. In addition, it is important to point out that pooling data across animals could "wash-out" individual

differences in PER2 levels over time. This is particularly true in behaviorally arrhythmic animals where animals are grouped according to clock time, without any reference to circadian time.

One way of addressing this problem is to observe the putative clock cells over time to determine whether individual cells and tissues are rhythmic. Studies using bioluminescence to monitor the dynamics of the molecular clock mechanisms in real time suggest that individual SCN cells continue to have circadian rhythms of gene expression, but become desynchronized from each other in animals that are behaviorally arrhythmic as a result of extended exposure to LL (Ohta et al., 2005; Yamaguchi et al., 2003). Examining the BNST-OV, CEA, BLA, and DG of *Per1::Luc* rats or PER2::LUC mice, both *in vitro* and *in vivo* would allow direct observation of these regions after SCN lesion, LL exposure, or adrenalectomy and reveal whether the cells in these regions have become arrhythmic or desynchronized.

The finding that the essential clock gene protein PER2 is rhythmically expressed in a circadian manner in these limbic forebrain regions; that this expression is synchronized with that of the established circadian master clock, the SCN; and that the SCN is required for sustained rhythmicity of PER2 in these regions, suggest that the BNST-OV, CEA, BLA, and DG are subordinate circadian oscillators. If the rhythmic expression of PER2 in the limbic forebrain indicates that these neurons have all of the molecular machinery necessary for a functional circadian oscillator, then presumably that would result in measurable changes in the outputs of those cells in terms of electrical activity and neurotransmitter secretion, like that observed in the SCN (Gillette & Reppert, 1987; Inouye & Kawamura, 1979; Yamazaki et al.,1998). *In vitro* experiments

measuring the electrophysiological activity and neurotransmitter secretion of individual neurons from each of these regions would be necessary to determine whether the circadian oscillations in PER2 expression observed in these limbic forebrain regions are actually correlated with the activity being generated at the level of the individual neurons. Some evidence suggests that this is the case for the BNST, which has a circadian rhythm of neural activity that is in phase with that of the SCN (Yamazaki et al., 1998). However, an approach that combined individual cell recordings with a marker for clock gene expression would provide a convincing demonstration that the changes in PER2 levels as a function of time observed in the experiments presented here are correlated with changes in the electrical activity and neurochemical output of neurons in the limbic forebrain.

The function of putative subordinate circadian oscillators in the limbic forebrain is unknown. However, an investigation of the neuronal architecture and connectivity of the PER2 immunopositive neurons in the BNST-OV, CEA, BLA, and DG could point toward future directions for experiments exploring this issue. We do not have data on the identity of neurons that express PER2 in the BLA or DG. In the BNST-OV and CEA, neurons are primarily GABA-ergic, and the majority of these co-express CRH or ENK, suggesting one or both of these two cell types could also express rhythmic PER2 (Day et al., 1999; Dong et al., 2001b; Cassell et al., 1986; Honkaniemi et al., 1992). Preliminary finding using immunocytochemistry to double label cells in the BNST-OV and CEA indicate that PER2 is expressed in cells positive for ENK, but show no evidence of PER2 co-expression with CRH.

Preproenkephalin mRNA is significantly reduced by adrenalectomy in the rat forebrain, including the CEA (Ahima et al., 1992). In addition, the majority of met-ENK-

ergic neurons in the CEA (Honkaniemi et al., 1992), and presumably also in the BNST-OV, demonstrate GR-like immunoreactivity, suggesting that glucocorticoids could act via GR receptors to modulate the rhythm of PER2 expression in the central extended amygdala. Experiments are currently underway to further explore the effects of adrenalectomy and CORT replacement on the rhythm of expression of PER2 in the BNST-OV and CEA (Segall et al., 2005).

ENK-ergic neurons in the BNST-OV and CEA have been shown to be highly activated by stressors including immune system and osmotic challenges, loud noise, restraint, or injections of amphetamine, whereas few CRH positive cells respond to the same stressors (Day et al., 1999, 2001, 2005; Engström et al., 2003; Kozicz, 2002). This suggests an important role for these neurons in the modulation of the stress response to a wide variety of stressors. Interestingly, ENK mRNA levels are also increased in CEA neurons of animals re-exposed to a context previously associated with footshock (ENK mRNA in the BNST-OV was not measured) (Petrovich et al., 2000), suggesting that the neurons expressing PER2 may be sensitive to both conditioned and unconditioned stress.

Of particular interest is the possibility that fear conditioning could be used to discriminate between the functions of the putative oscillators in the BNST-OV and CEA. These two regions share important similarities including strong anatomical connections (Dong et al., 2001b; Ju & Swanson, 1989; Ju et al., 1989), similar chemo- and cytoarchitecture (Day et al., 1999; Dong et al., 2001a, b; Swanson, 2003), similar responses to stress at the level of CRH-ergic (Makino et al., 1994a,b; Santibañez et al., 2005) and ENK-ergic neurons (Day et al., 1999, 2001, 2005; Engström et al., 2003; Kozicz, 2002), and identical changes to the pattern of rhythmic PER2 expression after

adrenalectomy. Preliminary results from our laboratory also show identical changes in the phase and level of PER2 expression in the BNST-OV and CEA in response to changes in gonadal steroid hormones over the esterous cycle and following gonadectomy in both male and female rats (Perrin et al., 2005). A procedure that would reveal differential effects on the BNST-OV and CEA would be useful in helping to understand the function of the rhythmic PER2 expression in these two related but distinct regions. Davis and colleagues (1997) have shown that the central nucleus of the amygdala is critically involved in the expression of conditioned fear, and also postulate a related but different role for the BNST. Specifically, they suggest that the CEA is essential for the behavioral effects (freezing, fear potentiated startle, etc.) observed after the presentation of a cue that has previously been paired with an aversive stimulus. In contrast, lesions of the BNST that include the oval nucleus have little effect on conditioned fear, but produce impairments in the enhancement of acoustic startle by light or intracerebroventricular administration of CRH (Davis, 1998; Davis et al., 1997; Treit et al., 1998; Walker et al., 2003). Stated simply, the CEA is involved in the expression of conditioned fear, whereas the BNST appears to be involved in nonspecific anxiety (Walker et al., 2003).

Although there is little research on circadian rhythms of the expression of fear and anxiety, there is evidence for a circadian rhythm of startle reactivity, with rats showing highest startle amplitudes during the subjective night (Frankland & Ralph, 1995). There is also an interesting experiment showing evidence for a "time-stamping" effect of aversive learning. In this case, hamsters showed the strongest avoidance of the aversive context 24 and 48 hours after training, but relatively little avoidance at 32 and 40 hours after training (Cain et al., 2004). These examples suggest that both unconditioned startle

and memory for a fearful context are modulated by circadian time. If this is the case, fear conditioning may also vary as a function of the time of day. Given the differential roles of the BNST-OV and CEA in non-specific anxiety and conditioned respectively, fear conditioning could be a way to study differential roles for the BNST-OV and CEA and perhaps help reveal the function of rhythmic PER2 expression in these regions.

The second significant finding reported here is that under both LD and LL conditions, rats on a restricted feeding schedule show food anticipatory activity and synchrony of the phase PER2 expression between the BNST-OV, CEA, BLA, and DG, such that all four regions show peak PER2 expression approximately 12 hours after the presentation of food. Because the SCN is relatively invulnerable to the effects of restricted feeding under a 12-hour LD schedule, there is an uncoupling of the limbic forebrain oscillators from the SCN leading to a shift in the phase of PER2 expression in the limbic forebrain, while the phase of PER2 in the SCN remains entrain to the LD cycle. In contrast, long term exposure to LL disrupts the synchrony among SCN clock cells (Ohta et al., 2005) rending them vulnerable to entrainment by restricted feeding and causing a restoration of the rhythm and synchronization of the phase of PER2 expression in the SCN and limbic forebrain regions, with a peak level occurring 12 hours after food presentation.

Although the experiments presented here did not reveal the mechanism by which RF synchronizes limbic forebrain oscillators, they did exclude some possibilities. Daily scheduled feeding has at least two important components. Food is a rewarding stimulus that animals will work to obtain, and while on this schedule, the animal is in a negative energy balance state. Both sucrose and saccharine, are rewarding (Ågmo & Marroquin,

1997; Messier & White, 1984), but failed to synchronize the phase of PER2 expression in the limbic forebrain when given to non deprived rats (i.e., not metabolically challenged). The presentation of a reward to rats in any negative metabolic state was also insufficient; daily access to a saline solution in rats with furosemide induced salt-appetite also failed to cause synchronization the phase of PER2 expression the limbic forebrain regions. This suggests that the synchronizing cue responsible for changes in rhythmic PER2 expression must be something that is altered by scheduled feeding, but not by reward or non-energy related metabolic challenge.

As discussed at the end of the previous chapter, a change in the levels of CORT is a possible synchronizing cue. The BNST-OV, CEA, BLA and DG all have modest to high levels of GR mRNA (Morimoto et al., 1996). Therefore, CORT could potentially access PER2 positive neurons in the limbic forebrain via glucocorticoid receptors, causing synchronization among the BNST-OV, CEA, BLA, and DG, similar to the resetting of the circadian phase of peripheral tissues seen in response to dexamethasone (Balsalobre et al., 2000a). Daytime feeding causes a shift in the timing of peak CORT levels from the beginning of the night to just before feeding onset (Damiola et al., 2000; Davidson & Stephan, 1999b; Díaz-Muñoz et al., 2000; Honma et al., 1983; Krieger, 1974; Morimoto et al., 1977). Furthermore, this shift in the peak of CORT is related to both to the timing of the meal, and a state of negative energy balance (Honma et al., 1983; Pecoraro et al., 2002). Therefore the change in the circadian timing of this hormonal signal may be sufficient to uncouple the peripheral and limbic forebrain oscillators from the SCN. An interesting finding that strengthens this hypothesis is that the clock gene *Per1* may be a direct target of glucocorticoid signaling (Balsalobre et al.,

2000a, b; Hida et al., 2000; Le Minh et al., 2001). In this case, CORT could potentially advance or delay the phase of the molecular clock mechanism by altering *Per1* transcription, and in turn altering the rhythm of PER2 expression.

In the adult brain, the SCN itself does not contain glucocorticoid receptors (Rosenfeld et al., 1988; 1993), and would be insensitive to alterations in the timing of CORT release. This fits well with the lack of effect of RF on the rhythm of clock gene expression in the SCN under a LD schedule observed here, and by others (Damiola et al., 2000; Wakamatsu et al., 2001). However, this does not explain the restoration and synchronization of phase of PER2 rhythmicity among the BNST-OV, CEA, BLA, DG, and SCN that occurs when the same restricted feeding schedule is given in LL. The absence of glucocorticoid receptors on SCN neurons would mean that CORT would not be sufficient to alter PER2 expression in the SCN, even under LL conditions. Perhaps the effect of restricted feeding on the SCN is secondary to the synchronization of the BNST-OV, CEA, BLA and DG with each other. Knowing the time course of the effect of restricted feeding on PER2 expression in the different brain regions of LL-housed rats would answer this question and could provide more insight into the mechanism responsible for both the behavioral and molecular aspects of scheduled feeding.

Among the experiments described here, scheduled restricted feeding was the only manipulation that produced robust behavioral anticipation and synchronized the phase of PER2 expression in the limbic forebrain. However, it is also interesting that restricted access to sucrose, saccharine, or saline did not change the phase of PER2 expression. The only dramatic change that resulted from these experiments was a reduction in the level of PER2 expression in the BLA of animals in the chronic salt deprivation group

around the time of saline access (ZT1 and ZT7, see Figures 19 and 20). A potential explanation for this finding comes from research suggesting a role for the BLA in the learned motivational properties of food and flavored liquids.

Conditioned taste aversion is thought to be dependant on the amygdalar complex, particularly the BLA (Lamprecht & Dudai, 2000). In addition, the BLA seems to be essential for conditioned potentiation of eating, a procedure in which hungry rats are presented with a conditioned stimulus (CS) paired with food delivery (CS+) and a second unpaired cue (CS-). During the test stage, sated rats will eat significant amounts of food when presented with the CS+, but relatively little with the CS- or no CS (Petrovich & Gallagher, 2003; Weingarten, 1983). CEA lesions have no effect on feeding during the CS, whereas lesions of the BLA selectively disrupt conditioned potentiation of feeding, but have no effect on unconditioned feeding. (Gallagher, 2000; Petrovich & Gallagher, 2003). This suggests the BLA is necessary for the learned motivational properties of feeding. If a learned association was made between the time of day of saline presentation and its significance for the satiation salt appetite, perhaps this would require the participation of the BLA and be sufficient to alter the expression of PER2 in this region, but not in the BNST-OV, CEA, or DG.

The experiments presented here suggest that a negative energy balance state is necessary for the changes in PER2 expression observed in the BNST-OV, CEA, BLA, and DG after RF. Findings by other labs also highlight the importance of feeding itself. A recent study by Kobayashi and colleagues demonstrated that while fasting does not affect the phase of rhythmic expression of *Per1* and *Per2* in the liver and heart, it does produce changes in the levels of the mRNA of these clock genes that are only restored by

feeding (Kobayashi et al., 2004). Furthermore, feeding, but not fasting, enhanced the speed of re-entrainment to a 12 hour shift in the light dark cycle (Kobayashi et al., 2004). This suggests that both metabolic state and feeding are important for the regulation of clock gene expression.

The DNA binding affinity of both CLOCK::BMAL1 and NPAS2::BMAL1 vary with the redox state of nicotinamide adenine dinucleotide co-factors (Rutter et al., 2001). Therefore, it has been suggested that cellular redox state might directly entrain the molecular clock (Rutter et al., 2001). In support of this assertion, NPAS2 deficient mice were less able to adapt to a daytime feeding schedule than their wild type littermates, and many became sick and died (Dudley et al., 2003). Given that NPAS2 was found in most of the limbic forebrain regions described in this thesis, the effect of cellular redox state of CLOCK or NPAS2 could account for the sensitivity of the rhythm of PER2 expression seen in the BNST-OV, CEA, BLA, and DG seen as a result of restricted feeding.

Several important questions remain regarding the mechanisms for the integration of circadian information from the SCN and limbic forebrain oscillators. Results from the lesion experiments presented here suggest that in the case of the BNST-OV, rhythmic PER2 expression depends on the integrity of the ipsilateral SCN, suggesting that there is an ipsilateral projection from the SCN to the BNST-OV. This is supported by other anatomical evidence (Leak & Moore, 2001; Watts & Swanson, 1987; Watts et al., 1987), but the direct projection from the SCN, primarily originating in the shell, to the BNST is relatively minor. Likewise, there is some evidence for a modest projection from the SCN to the medial amygdala (Watts et al., 1987), but little or no evidence for any direct projections to the other nuclei of the amygdala, the hippocampus or cortex. Therefore,

the integration of information from the SCN and limbic forebrain is not likely due to direct reciprocal connections between these regions.

In contrast, there is ample evidence that the limbic forebrain receives indirect input from the SCN via the major SCN output regions, the paraventricular nucleus of the thalamus (PVT), the paraventricular (PVN), dorsomedial (DMH), and lateral hypothalamic (LH) nuclei, and the subparaventricular zone (SPVZ; Leak & Moore, 2001; Moga et al., 1995; Watts & Swanson, 1987; Watts et al., 1987). The PVT is a major relay for circadian information, receiving information not only from the SCN, but also from the SPVZ, intergeniculate leaflet and retina (Moga, et al., 1995; Moore et al., 2000; Watts et al., 1987). In turn, the PVT projects to the BNST, CEA and BLA, and hippocampus indirectly via the septum, as well as the SCN and SCN output areas, the SPVZ, DMH, and LH (Moga et al., 1995; Peng & Bentivoglio, 2004). In addition, all of these areas project back to the PVT (Chen & Su, 1990), suggesting interconnections between the SCN, thalamus, and limbic system, reminiscent of the thalamo-striato-cortical loops critical for higher-level integration of cortical processing (McFarland & Haber, 2002). Therefore, the PVT can be seen as an "entrainment pathway", taking relevant information from the SCN and relaying it to the rest of the brain (Moga et al., 1995; Peng & Bentivoglio, 2004).

The hypothalamus has been proposed as an integrator of circadian rhythms (Chou et al., 2003; Saper et al., 2005). As stated above, the DMH, SPVZ, and PVN, as well as the medial preoptic area (MPA) all receive major input from the SCN (Deurveilher & Semba, 2003, 2005; Leak & Moore, 2001; Moga et al., 1995; Watts & Swanson, 1987; Watts et al., 1987), and targeted lesions of these areas selectively disrupt circadian

rhythms of melatonin, sleep/arousal, and body temperature (Chou et al., 2003; Moore & Danchenko, 2002). The DMH, LH, and PVN, along with the arcuate nucleus, are part of the hypothalamic control center for the regulations of energy balance, feeding, and satiety (Jobst et al., 2004; Saper et al., 2002). The PVN also has autonomic and endocrine functions, for example the release of CRH, which causes release of CORT (Chou et al., 2003; Guillemin, 2005; Lowey, 1991; Saper et al., 2005; Thompson & Swanson, 2003). The hypocretin/orexin and melanin-concentrating hormone neurons of the LH, which are involved in both arousal and feeding, receive direct projections from the SCN, as well as indirect projections from the DMH, SPVZ, and MPA (Abrahamson et al., 2001; Chou et al., 2003; Deurveilher & Semba, 2003, 2005; Willie et al., 2001).

These SCN outputs to hypothalamic nuclei converge with projections from all parts of the brain including the CEA and BLA, which project to the LH; the BNST-OV, which projects directly to the PVN, and indirectly to the LH and DMH via the fusiform nucleus of the BNST; and the hippocampus (Jacobson & Sapolsky, 1991; Mulders et al., 1997; Petrovich et al., 2001, 2002). Integrated SCN and limbic forebrain inputs are then relayed to major arousal and sleep promoting regions (Abrahamson et al., 2001; Chou et al., 2003; Deurveilher & Semba, 2005); to areas involved in the control of feeding, metabolism, predation, reproductive, maternal, and defensive behaviors, to the PVT and thalamocortical systems, and to brainstem motor and autonomic control areas (Comoli, et al., 2005; Lowey, 1991; Saper et al., 2002; Thompson & Swanson, 2003; Walker et al., 2001). Even a very brief review of the anatomy and function of the hypothalamus reveals a complex system that integrates circadian, limbic, homeostatic, and autonomic inputs to control nearly every ecologically relevant physiological function and behavior (Petrovich

et al., 2001; Thompson & Swanson, 2003). Circadian modulation of behavioral state does not take place in any single, discrete location, but is accomplished by the interaction of multiple integrators and relays coordinating numerous and varied inputs from all over the brain and periphery. Although this is some distance from a single clock model, it clearly allows for much greater flexibility and more accurately reflects the complexity and bidirectionality of circadian controlled behavior and physiology.

The discovery of putative circadian oscillators in the limbic forebrain provide support for the hypothesis that the SCN is not so much a master clock, but master orchestrator, providing a synchronizing signal to subordinate oscillators (Davidson et al., 2003). Under normal circumstances, the difference between these two is theoretical, as the synchronization of neural, hormonal, emotional, motivational, and behavioral inputs are mutually supporting, each synchronized with each other and the LD cycle. Only when the LD cycle and other non-photic cues are not in phase would subordinate oscillators become uncoupled from the master orchestrator. Unfortunately, with trans-meridian flight, rotating shift work, light pollution, all night drive-throughs, heavy stress loads, and psychotropic drugs, these "unusual circumstances" have become typical. For this reason, an understanding of extra-SCN and peripheral subordinate oscillators is critical to a deeper understanding of circadian rhythms, and especially disorders of circadian rhythms.

The presence of putative circadian oscillators in the limbic forebrain invites speculation about the role of these areas in the symptoms associated with jet lag and maladaptation to shift work. The phase shift experiment described in Chapter 1 suggests that there is a delay between the re-entrainment of the SCN to the new light schedule and

the resynchronization of the BNST-OV with the SCN. Although not specifically tested here, the results with the other brain regions are likely similar; after a large shift in the light dark cycle, there is probably a period of several days during which the limbic forebrain oscillators are out of phase with the SCN and each other. It is possible that the desynchrony among the SCN, extra-SCN, and peripheral oscillators accounts for the general feeling of malaise, inability to concentrate, loss of appetite, and other symptoms associated with jet lag and rotating shift work.

This speculation also applies to circadian related symptoms associated with depression and mental illness. If one of the limbic forebrain oscillators became uncoupled from the SCN and other extra-SCN oscillators, this could produce some of the symptoms associated with depression, such as disregulation of the sleep-wake cycle, loss of appetite, or inability to feel pleasure. The differential response to adrenalectomy between the BNST-OV/CEA and the BLA/DG oscillators suggest that this type of uncoupling among the limbic forebrain oscillators is possible. This combined with the finding that PER2 expression seems to occur in ENK-ergic cells, which are sensitive to a wide range of stressors, suggests that the synchronized circadian oscillations in the central extended amygdala may be relevant to depressive symptoms. Examining the role of the BNST-OV and CEA in the circadian modulation of mood would likely be a fruitful line of investigation.

Although the work presented here provides some insight into the specific stimuli that the extra-SCN oscillators of the limbic forebrain are responsive to, more work will be necessary to understand the function of circadian oscillators in these regions. For example, while it is possible to speculate that a metabolic cue that is altered as a result of

scheduled restricted feeding is responsible for synchronizing the phase of PER2 expression in the limbic forebrain oscillators, it is difficult to determine the purpose of this synchronization, or the behavioral outcome if the phase synchronization of some or all of the limbic forebrain oscillators were prevented from occurring. Previous research has shown that large electrolytic or chemical lesions of the limbic forebrain regions have no appreciable effect on food related anticipatory activity (Mistlberger & Mumby, 1992). However, using small RNA interference to prevent the circadian oscillation of individual cells may reveal real deficits in behavioral and physiological adaptation to altered feeding schedules.

The discovery of two opposite but synchronized patterns of PER2 expression in the limbic forebrain suggest a level of complexity in the regulation of circadian rhythms that was previously unappreciated, but is not unexpected. Uncovering the function of these clock cells in reward and motivation, energy balance, sexual and maternal behavior, mood regulation, stress, and many unforeseen area will be a very interesting and rewarding pursuit.

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