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Contribution of serotonin 2C receptors to the regulation of circadian rhythm entrainment

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Contribution of serotonin 2C receptors to the regulation of circadian rhythm entrainment

by

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Abstract

Many aspects of physiology and behavior are driven by circadian rhythms that oscillate with a period of roughly twenty-four hours. These circadian rhythms are evolutionarily adapted to entrain to light and to food availability. Light-entrainable circadian rhythms are driven by a master pacemaker that resides in the suprachiasmatic nucleus (SCN), while food-entrainable circadian rhythms are driven by a separate food-entrainable oscillator (FEO), the neural basis of which remains unclear. Serotonin systems have been implicated in the regulation of circadian rhythms. However, the challenge in elucidating the mechanisms by which serotonin regulates circadian rhythms lay in the fact that serotonin exerts its effects via fourteen different serotonin receptors. This thesis investigates the role of serotonin$_{2C}$ receptors (5-HT2CRs) in the regulation of both light- and food-entrainable circadian rhythms, using a combination of genetics, behavioral assessment, and anatomical analysis. We present data that indicates an important contribution of 5-HT2CRs to the facilitation of light entrainable circadian rhythms, and to the gating of food-entrainable circadian rhythms.

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INTRODUCTION

One can imagine that in complex organisms, multiple biological processes are occurring at any given time. The temporal coordination of these processes to relevant environmental conditions is necessary for homeostasis and survival in the environment. Accordingly, circadian rhythms are used to coordinate physiological processes in almost all organisms, ranging from single-cell bacterial species to insects, birds, mammals, and humans (Bell-Pedersen et al., 2005). Circadian rhythms determine the temporal structure of almost all biological processes ranging from sleep/wake cycles, locomotor activity, feeding behavior, body temperature, brain activity, and glucocorticoid release, to molecular events such as cell division (Szabo et al., 1978; Czeisler and Klerman, 1999). In multicellular organisms, circadian rhythms can be demonstrated in peripheral tissues and cultured cells (Plautz et al., 1997; Balsalobre et al., 1998; Whitmore et al., 2000). Circadian rhythms are commonly assessed by analysis of overt rhythms of locomotor activity in model organisms such as fruit flies, mice, rats, hamsters, and humans.

The universality of circadian rhythms across diverse phylogenic branches reveals the central contribution of these rhythms to the maintenance of life on Earth. The evolutionary advantage of intact circadian rhythms has been demonstrated in several studies. For instance, Yan Ouyang and colleagues demonstrated that synchrony between endogenous rhythms and environmental cycles predicted reproductive fitness in competing cyanobacteria strains (Ouyang et al., 1998). In addition, *drosophila* strains with genetic ablations in the molecular drivers of circadian rhythms exhibited reduced reproductive fitness (Beaver et al., 2002). Moreover, ground squirrels with targeted brain lesions that specifically abolished circadian rhythmicity were significantly more likely to

be killed by predation in naturalistic settings (DeCoursey et al., 1997). The importance of circadian rhythms can also be highlighted by the adverse impact of circadian rhythm dysfunction on health. There is evidence that circadian rhythm dysfunction increases risk for cancer: as extended night-shift work has been associated with increased risk for breast cancer, and mice with genetic mutations in circadian clock genes are prone to tumor development and early death (Sephtona and Spiegel, 2003).

Circadian rhythms share a number of universal characteristics. By definition, circadian rhythms oscillate to a period of roughly twenty-four hours ("circadian" is translated as "about a day" in Latin). Under constant environmental conditions, circadian rhythms free-run with robust periods that deviate only slightly from twenty-four hours (Aschoff, 1960; Lowe et al., 1967), but also demonstrate entrainment to relevant environmental signals, particularly to light/dark cycles (Pittendrigh, 1993). In addition, circadian rhythms are robust to alterations by changes in the organism's natural environment. For instance, the invariance of circadian period in the face of large temperature shifts has been demonstrated in a number of species (Pittendrigh and Caldarola, 1973). The temperature independence and precision of free-running periods with only slight deviations from twenty-four hours were instrumental in proving that circadian rhythms of biological activity were driven by an internal clock and not by external variables.

SCN-CONTROLLED CIRCADIAN RHYTHMS

In the 1970s, the SCN was proposed as the master pacemaker that entrains circadian rhythms to light/dark cycles and coordinates the circadian rhythms of the rest of the body. The SCN exhibits several characteristics indicating that this region could act as

the master pacemaker. The SCN receives direct and indirect retinal innervation (Moga and Moore, 1997; Abrahamson and Moore, 2001), and is thus well-positioned to receive entraining light/dark information. In addition, the SCN was shown to display strong circadian rhythms of glucose utilization *in vivo* (Schwartz and Gainer, 1977), and SCN tissue explants were found to exhibit sustained circadian rhythms *in vitro* . SCN lesions were shown to abolish overt circadian rhythms (Stephan and Zucker, 1972), which could be restored by grafting SCN-derived fetal tissue (Lehman et al., 1987). In addition, it was found that in hamsters made arrhythmic by targeted lesions of the SCN, transplantation of donor SCN restored circadian rhythms to the same period as the donor's former overt rhythm (Ralph et al., 1990). These and other studies helped establish the hypothesis that the SCN acts as the master circadian pacemaker.

Molecular basis of the circadian oscillator

Circadian oscillators across all species share similar molecular features, with positive feedback loops interacting with negative feedback loops to create self-sustaining circadian oscillations of transcriptional activity (King et al., 1997). For the purpose of this thesis, we will focus on the molecular basis of the mammalian circadian clock.

The CLOCK protein is a transcription factor in the bHLH-PAS family (Antoch et al., 1997; King et al., 1997; Gekakis et al., 1998), and forms heterodimers with a second bHLH-PAS protein named BMAL (Gekakis et al., 1998). CLOCK/BMAL heterodimers bind to E-box enhancers with the nucleotide sequence CACGTG to activate mRNA transcription of a number of circadian output genes (Gekakis et al., 1998; Jin et al., 1999). A forward mutagenesis screen in mice uncovered a loss-of-function mutation in the *clock* gene which abolished persistence of circadian rhythmicity in constant darkness (Vitaterna

et al., 1994). Later studies confirmed that loss of BMAL also abolished circadian rhythms (Bunger et al., 2000). These studies helped establish the role of CLOCK/BMAL heterodimers as the positive arms of the circadian transcriptional feedback loop.

But what of the negative feedback components of the mammalian circadian clock? In *drosophila,* the negative feedback component of the circadian oscillator is comprised of *Per* and *Tim* genes (Zeng et al., 1996). This mechanism was discovered to be similar to the mammalian circadian system. Three mammalian homologues of the *drosophila Per* gene (*mPer1, mPer2,* and *mPer3*) were discovered in mice (Shearman et al., 1997; Tei et al., 1997; Zylka et al., 1998; van der Horst et al., 1999; Zheng et al., 1999). In mammals, CLOCK induces transcription of *Per* genes; PER enters the nucleus to repress CLOCK-mediated transcriptional activity (Gekakis et al., 1998). David Weaver's group discovered that mice lacking functional *mPer1* and *mPer2* genes were unable to sustain circadian rhythms of locomotor activity in constant darkness, and that *mPer1/mPer2* double mutants were immediately arrhythmic in constant darkness (Bae et al., 2001). These studies support the notion that *Per* genes comprise key components of the circadian clock. Mice lacking functional *mPer2* displayed a robust attenuation of the rhythmic expression of *mPer1*, indicating that *mPer2* acts upstream of *mPer1* to positively regulate its expression (Zheng et al., 1999). In 1998, Joseph Takahashi's group discovered the mouse homolog of the drosophila *Tim* gene. Furthermore, it was found that, similarly to *drosophila*, mPER1 forms heterodimers with mTIM; these heterodimers translocate into the nucleus to inhibit CLOCK/BMAL-induced transactivation of *mPer1* gene expression (Sangoram et al., 1998). However, the magnitude of the inhibition was only ~50%, indicating that other mechanism contribute

substantially to the inhibition of CLOCK-mediated transcriptional activity (Sangoram et al., 1998).

Homologues of the *drosophila Cry* gene, which were implicated in the regulation of light entrainment, were also discovered in mice (van der Horst et al., 1999). The mouse homologue of the *drosophilia Cry* gene, *mCry1*, was discovered to be expressed in a circadian fashion in the SCN of wild-type but not *clock* mutant mice (Kume et al., 1999). A second homologue, *mCry2*, does not oscillate in a circadian fashion in the SCN; however, expression is reduced in *clock* mutant mice. These studies indicate that expression of both *Cry* homologues are regulated by CLOCK. mCRY1 and mCRY2 caused a nearly complete loss of CLOCK/BMAL induced transcription in cultured cells, and mutant mice lacking *mCry1* and *mCry2* are completely arrhythmic (Kume et al., 1999). Furthermore, mCRY proteins interact with mPER proteins to induce translocation in the nucleus (Kume et al., 1999). These data support the hypothesis that *Cry* and *Per* genes act as central components of the negative feedback loop of the circadian oscillator. Taken together, the studies described above helped establish the current model of the molecular circadian clock; in which CLOCK/BMAL, as the positive elements of the molecular clock, activate transcription of *Per* and *Cry* genes. PER and CRY accumulate in the cytoplasm and form heterodimers. These heterodimers translocate into the nucleus to inhibit CLOCK/BMAL-induced transactivation, shutting off the expression of *Per* and *Cry* genes as well as other circadian output genes, thus resetting the cycle (Figure 1).

SCN organization

The SCN is subdivided into two distinct regions: 1) a ventral “core” region that receives light information and is acutely sensitive to entraining light stimuli, and 2) a

dorsal “shell” region that does not receive directly light input and is not acutely sensitive to entraining light stimuli. (Leak et al., 1999; Abrahamson and Moore, 2001; Antle and Silver, 2005). The SCN core and shell are distinguished by neuropeptide expression markers. The SCN core expresses both gastrin-releasing peptide (GRP) and vasoactive intestinal peptide (VIP), while the SCN shell is characterized by lack of the above peptides and expression of arginine-vasopressin (AVP) (Abrahamson and Moore, 2001; Moore et al., 2002). Anterograde and retrograde tracer experiments demonstrate that the ventral SCN receives projections from three areas that receive light input; the retina, intergeniculate leaflet (IGL), and pretectal area (Moga and Moore, 1997). The ventral core is also innervated by serotonergic raphe nuclei (Moga and Moore, 1997). By contrast, the dorsal SCN receives sparse inputs from the raphe or visual areas, but receives projections from the limbic forebrain, cortex, and other hypothalamic regions (Moga and Moore, 1997). The SCN core and shell also have distinct but overlapping patterns of efferent projections, as determined by anterograde and retrograde tracer studies. The SCN core region sends dense projections mainly to the SCN shell and to peri-SCN regions such as the lateral SPVZ and VMH, and sparse projections to the lateral septum (LS) (Leak and Moore, 2001). The SCN shell region projects to the dorsomedial hypothalamic nucleus (DMH), preoptic area (POA), medial subparaventricular zone (mSPVZ), paraventricular thalamic nucleus (PVT), paraventricular hypothalamic nucleus (PVN), bed nucleus of stria terminalis (BST), and zona incerta (ZI), but does not project back to the SCN core (Leak and Moore, 2001). Overall, the SCN shell sends outward projections to a greater number of brain regions than the core, which indicates greater direct influence of shell oscillators to the

coordination of overt behavioral rhythms compared to the core oscillators. However, the dense projections from the core to the shell indicate that shell oscillators may be influenced by core oscillators. Furthermore, studies have shown that while the SCN shell exhibits strong endogenous rhythms of *Per* gene expression and weak induction by light stimuli, the ventral core demonstrates weak endogenous rhythms and strong induction by light (Yan et al., 1999; Karatsoreos et al., 2004). These studies helped establish the two-component model of circadian rhythm entrainment: in which ventral core oscillators are acutely responsive to light and serotonergic input, and mediate the communication of light and serotonin-related information to the dorsal shell, causing an eventual shift of the shell oscillators (Figure 2).

Characteristics and molecular correlates of circadian rhythm light entrainment

The entrainment of circadian rhythms to light may be studied by constructing a phase response curve of circadian phase shifts to light pulses applied at different times of day. Mice and rats exhibit a typical phase response curve to light stimuli, such that they display robust phase delays to early evening light pulses, weak phase advances to early morning light pulses, and are insensitive to light during the daytime (Daan, 1977).

Entraining light pulses rapidly induce characteristic profiles of gene expression in the SCN. c-*fos* is an immediate-early gene whose expression is used as a marker for neuronal stimulation (Dragunow and Faull, 1989). Light pulses that are known to induce phase shifts cause the acute induction of c-*fos* mRNA and protein in the retinorecipient ventral SCN region (Rusak et al., 1990). In addition, the magnitude of behavioral phase shifts in mice correlate with the magnitude of light-induced c-*fos* expression (Amy et al., 2000). Gene expression of both *mPer1* and *mPer2* are also acutely induced by night-time

light pulses (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997) . A detailed study of *mPer1* induction by light demonstrated that phase delaying light pulses promote the rapid induction of *mPer1* (Shigeyoshi et al., 1997). In addition, it was found that magnitude of light-induced *mPer1* expression was strongly predictive of behavioral phase shifts to the light stimulus (Shigeyoshi et al., 1997). Pretreatment with *mPer1* antisense oligonucleotides one hour prior to light exposure blocked expression of light-induced behavioral phase shifts, indicating that acute *mPer1* induction by light indeed contributes to the phase-shifting effects of light (Akiyama et al., 1999). A subsequent study demonstrated that early evening light pulses acutely induced both *mPer1* and *mPer2* gene expression but that early morning light pulses induced only *mPer1* expression (Yan and Silver, 2002, 2004). Also, mice lacking functional *mPer2* exhibited normal light-induced phase advances but severely attenuated phase delays; by contrast, mice lacking functional *mPer1* displayed normal light-induced phase delays but attenuated phase advances (Albrecht et al., 2001). These data support the hypothesis that light-induced phase delays and phase advances are regulated by distinct molecular components, with *Per2* and perhaps *Per1* contributing to light-induced phase delays, and *Per1* contributing to light-induced phase advances. It should be noted that while the acute responses of the SCN to phase-shifting light stimuli are well-characterized, the mechanisms by which these acute SCN responses are translated into long-term circadian phase shifts remain unclear.

FOOD ENTRAINMENT OF CIRCADIAN RHYTHMS

Behavioral and physiological characteristics of food-entrainable circadian rhythms

In times of limited food resources, the ability to adapt behavioral and physiological rhythms to times of maximal food availability is critical for survival. This adaptation to restricted food availability is studied most commonly by placing nocturnal rats and mice on a daytime restricted feeding schedule. This manipulation was found to induce anticipatory rhythms of locomotor activity (Richter, 1922; Edmonds, 1977), adrenal function, and body temperature (Krieger et al., 1977), and to shift sleep patterns (Mouret and Bobillier, 1971). Anticipatory rhythms entrain only to feeding schedules of roughly twenty-four hours, but not to 19 or 29-hour feeding schedules (Bolles and De Lorge, 1962; Bolles and Stokes, 1965). Furthermore, these anticipatory rhythms manifest whether the scheduled feeding occurs under standard light/dark cycles or under constant lighting conditions (Edmonds, 1977). Once established, these anticipatory rhythms persist under total food deprivation conditions (Mistlberger, 1994). This persistence of anticipatory rhythms in the absence of external cues (light or food), and their dependence on circadian feeding schedules, established the hypothesis that food-anticipatory rhythms represent the behavioral and physiological output of a food-entrainable circadian oscillator (FEO). The FEO was found to be independent of the SCN circadian oscillator. SCN lesions did not impair the ability of rats or mice to develop anticipatory rhythms of reinforced food-lever pressing (Boulos et al., 1980), corticosteroid secretion or body temperature (Krieger et al., 1977), or locomotor activity (Stephan and Becker, 1989; Marchant and Mistlberger, 1997).

Molecular basis of the food-entrainable oscillator

The role of various clock genes in the regulation of food-entrainable circadian rhythms has been a subject of intense interest. Although the SCN pacemaker has been

discounted as the site of the food-entrainable oscillator (FEO), it was still believed that the FEO would share the same molecular basis as the light-entrainable oscillator (LEO). Therefore, multiple studies have assessed the impact of clock gene mutations on food-anticipatory activity. Mice that lack functional *Per2* fail to exhibit FAA to a restricted feeding schedule, while mice that lack *Per1* retain normal FAA (Feillet et al., 2006b). In addition, mutant mice that lack functional *Cry* genes exhibit delays in the manifestation of FAA and FAA instability (Iijima et al., 2005). *Clock* mutant mice, which are completely arrhythmic in constant darkness, surprisingly exhibited enhanced FAA in both a standard light/dark cycle and constant darkness (Pitts et al., 2003). These results indicated the possibility that the FEO uses a separate bHLH-PAS transcription factor as the basis of its molecular clock, and that the FEO and LEO compete for control of behavior. A candidate bHLH-PAS factor is the transcription factor NPAS2, which shares similar structure to CLOCK, dimerizes with BMAL, binds to the same DNA E-box enhancers to promote transcription, and is similarly negatively regulated by CRY. However, NPAS2 is not found in the SCN, but is instead expressed widely throughout the forebrain, including sensory cortices, the basal ganglia, and limbic regions (Reick et al., 2001). NPAS2 mutant mice respond poorly to a daytime restricted feeding schedule, with impaired food intake and significantly greater body weight loss. In addition, these mice exhibit significant delays in the manifestation of FAA (Dudley et al., 2003). These findings suggest the possibility that under restricted feeding schedules, NPAS2 entrains an SCN-independent clock using sensory arousal as the principle Zeitgeber. Taken together, these studies have led investigators to propose that the FEO shares several molecular components in common with the LEO (such as *Per2* and *Cry*), but also

comprises distinct regulators (*npas2*). However, the role of any clock genes in the regulation of FAA is unclear, given recent studies that demonstrate normal FAA in mice with null mutations of *Bmal*, *Per1*, *Per2*, or *Per1+Per2* (PNAS, in press). Therefore, the molecular basis of food entrainment of circadian rhythms remains a mystery.

Neural substrates of the food-entrainable oscillator

The SCN-independence of food entrainment was an intriguing finding, and led researchers to search for the neural loci of the FEO using multiple approaches: 1) by assessing the impact of targeted lesions on food-anticipatory activity and 2) by visualizing the neural correlates of food-anticipatory activity using c-*fos* and circadian clock gene expression analyses. After three decades of such studies, there is no clear consensus about the neural substrates underlying food entrainment of circadian rhythms (Mistlberger, 1994; Stephan, 2002), although several brain regions have been implicated in the regulation of food entrainment.

Investigators have focused on hypothalamic regions known to be involved in the regulation of energy balance and locomotor activity. c-*fos* expression studies have demonstrated activation of several hypothalamic regions at time points correlating with FAA; which persists in food-deprivation conditions: the dorsomedial hypothalamic nucleus (DMH), lateral hypothalamic area (LHA), and perifornical region of the LHA, but not the ventromedial hypothalamus (VMH) or SCN (Angeles-Castellanos et al., 2004). The authors hypothesized that the DMH and LHA represent neural substrates of the FEO or the FEO's output effectors. Lesion studies have yielded inconsistent results, probably due to the nature of the lesions and/or assessment of food-anticipatory activity. For instance, Inouye found that electrolytic lesions of the VMH abolished food-

anticipatory activity to a two-week restricted feeding schedule (Inouye, 1982), however, Mistlberger's group found that similarly lesioned animals recovered their food-anticipatory activity 14-21 weeks after the surgical procedure, arguing against the hypothesis that the VMH acts as the sole FEO (Mistlberger and Rechtschaffen, 1984). In 2006, two reports implicated the DMH as one of the critical regions involved in food entrainment. Cliff Saper's group found that DMH lesions abolished food-anticipatory activity and that the magnitude of FAA correlated with the number of remaining DMH neurons (Gooley et al., 2006), while another group found that restricted feeding induced *de novo* expression of *Per2* specifically in the DMH, which persisted even after two days of food deprivation (Mieda et al., 2006). However, a second group found that DMH lesions did not abolish food-anticipatory activity in their hands (Landry et al., 2006). Two years later, it was found that BMAL knockout mice exhibited impaired FAA, and that viral-mediated BMAL rescue in the DMH but not SCN restored FAA (Fuller et al., 2008). However, those results have been thrown into question, due to a number of weaknesses in the evidence as presented [see Mistlberger et al, 2009 in press], and failure of other groups to confirm that *Bmal1* has any effect on FAA [Mistlberger et al, 2008; Storch and Weitz, 2009 in press; Pendergast et al, 2009, in press]. Consequently, although the DMH expresses circadian clock genes, and is clearly sensitive to feeding-related stimuli, its role in the regulation of FAA remains an open and hotly debated question. The LHA houses the orexin neuron system, which plays a critical role in the regulation of arousal and locomotor activity according to energy balance (Yamanaka et al., 2003). Genetic ablation of orexin neurons within the LHA were found to both abolish (Akiyama et al., 2004; Mieda et al., 2004) and have no effect (Mistlberger et al., 2003) on

food-anticipatory activity. The different results of these studies are most likely due to differences in the types of activity measured. However, orexin knockout mice exhibit reduced FAA (Kaur et al., 2008). These studies, while frustratingly inconsistent, support a general hypothesis that FAA is regulated at least in part by areas of the hypothalamus well known to participate in homeostatic regulation of feeding behavior and metabolism. Whether the LHA orexin neuron system or the DMH participate as components of the FEO or function in an FEO-efferent pathway remains to be clarified. A model of the neural circuitry underlying food entrainment, integrating both DMH and LHA contributions, is shown in Figure 3.

The contribution of extrahypothalamic regions to the regulation of food entrainment has not been well-characterized. For instance, while the LHA orexin neuron system is known to send widespread projections throughout the neuraxis to promote wakefulness and feeding (Peyron et al., 1998), the nature of the projections that mediate FAA expression are unclear. Restricted feeding paradigms that induce FAA have been shown to induce c-*fos* in a number of corticolimbic structures, such as the nucleus accumbens (NAc), amygdala, prefrontal cortex, paraventricular thalamic nucleus (PVT), and septal area, and to shift *Per1* rhythms in the accumbens. The NAc receives direct and indirect projections from LHA orexin neurons (Peyron et al., 1998; Fadel and Deutch, 2002; Vittoz et al., 2008). Furthermore, orexin administration enhances VTA-mediated dopamine neurotransmission in the NAc, and intra-NAc orexin A administration induces both feeding and locomotor activity (Thorpe and Kotz, 2005). These data suggest the possibility that the NAc acts downstream of the LHA to mediate expression of FAA. Lesion studies have yielded inconsistent results on the role of the

NAc in the regulation of FAA. Large lesions that encompass the entire nucleus have been reported to have no effect on FAA (Mistlberger and Mumby, 1992), while targeted lesions to the core and shell have revealed opposite roles of these subregions to the regulation of FAA (Mendoza et al., 2005).

SEROTONIN SYSTEMS AND CIRCADIAN RHYTHMS

A brief overview of serotonin system

Serotonin is a monoamine neurotransmitter that is synthesized in the raphe nuclei and released via widespread projections throughout the entire neuraxis. Brain serotonin systems have been implicated in the regulation of multiple aspects of behavior and affect, such as feeding behavior (Simansky, 1996), locomotor activity (Jacobs and Fornal, 1999), anxiety (Wu et al., 2008), and depression (Cryan and Mombereau, 2004). Serotonin exerts its effects via fourteen different serotonin receptors, each with distinct expression patterns, pharmacological profiles, and intracellular signaling mechanisms (Hoyer et al., 1994). Our lab is interested in investigating the neural mechanisms by which serotonin systems impact behavior, using genetic approaches to introduce perturbations of specific serotonin receptors.

One such receptor is the serotonin$_{2C}$ receptor (5-HT2CR). The 5-HT2CR is a 7-transmembrane, G_q-protein coupled receptor. Activation of 5-HT2CRs triggers a molecular cascade that results in release of intracellular calcium stores and protein kinase C activation, all of which promote neuronal activation (Hoyer et al., 1994). 5-HT2CRs, in addition to being responsive to endogenous serotonin, is unique among the serotonin receptors in that they may exhibit constitutive activity dependent on RNA editing (Herrick-Davis et al., 1999). Our lab has generated a mutant line of mice that lacks

functional 5-HT2CRs (Tecott et al., 1995), and work in our lab and others have demonstrated an important contribution of these receptors to the regulation of food intake, locomotor activity, dopamine neurotransmission, and seizure susceptibility (Tecott et al., 1995; Nonogaki et al., 1998; Rocha et al., 2002; Navailles et al., 2006; Navailles et al., 2008; Robinson et al., 2008).

Regulation of light-entrainable circadian rhythms by serotonin and 5-HT2CRs

Multiple lines of evidence support the hypothesis that serotonin systems play a major role in the resetting of circadian rhythms by light. Serotonin neurons from midline raphe nuclei provide both direct and indirect inputs to the SCN (Meyer-Bernstein and Morin, 1996). The median raphe send serotonergic fibers directly to the SCN, while the dorsal raphe sends indirect inputs to the SCN via the IGL (Meyer-Bernstein and Morin, 1996). Furthermore, anatomical studies indicate that serotonergic innervation of the SCN is concentrated in the retinorecipient ventral core region of SCN (Abrahamson and Moore, 2001).

Pharmacological and lesions studies indicate that serotonin systems contribute to the regulation of circadian rhythm entrainment to light. Lesions of SCN serotonergic fibers, through bilateral infusion of 5,7-dihydroxytryptamine into the SCN, enhanced light-induced phase delays in mice (Bradbury et al., 1997). In hamsters, serotonin depletion altered the phase response curve to light, such that the phase delay region of the curve was significantly larger in lesioned animals versus sham-lesioned controls (Morin and Blanchard, 1991). Furthermore, pretreatment with a non-specific serotonergic antagonist potentiated light-induced phase shifts in hamsters (Lall and Harrington, 2006).

Multiple serotonin receptors are expressed in the rodent SCN, including the 5-HT1A, 5-HT1B, 5-HT2A, and 5-HT2C receptor (Prosser et al., 1993; Roca et al., 1993; Moyer and Kennaway, 1999; Belenky and Pickard, 2001). 5-HT2C receptor expression has been reported to be expressed at much higher levels that 5-HT1B or 5-HT2A receptors (Roca et al., 1993; Moyer and Kennaway, 1999), indicating that 5-HT2C receptors may play an important role in regulating circadian rhythms. Indeed, non-selective 5-HT2C receptor agonists have been shown to mimic the phase-delaying effects of an early evening light pulse on circadian rhythms of body temperature and melatonin (Kennaway and Moyer, 1998), as well as on c-*fos* and *Per* gene expression in the SCN (Varcoe et al., 2003; Varcoe and Kennaway, 2008). In chapter 2, we assess the contribution of 5-HT2C receptors to the regulation of SCN-controlled circadian rhythms, using behavioral analysis of 5-HT2C receptor knockout mice, analysis of 5-HT2C receptor gene expression within SCN subdivisions, and analysis of SCN gene expression in response to phase-shifting light stimuli.

Dearth of evidence for the regulation of food-entrainable circadian rhythms by serotonin

There are few studies that assess the contribution of serotonin systems to the regulation of FAA. In one study, fluvoxamine (a selective serotonin reuptake inhibitor) administration did not impact FAA (Yokoyama et al., 2007). In another study, old rats exhibited impairments in developing FAA to restricted feeding schedules; however, treatment with non-selective 5-HT2 receptor antagonists restored FAA in old rats to levels comparable to young animals (Shibata et al., 1995). We had found that 5-HT2C receptors are expressed in several brain regions implicated in the regulation of FAA, such

as the DMH, LHA, and NAc (Tecott lab, unpublished observations) and are thus well-positioned to influence FAA. In chapter 3, we assess the contribution of 5-HT2C receptors to the regulation of food-anticipatory activity, using a combination of behavioral and physiological analyses and c-*fos* expression studies.

Figure Legends:

Figure 1: Model of Mammalian Clock Feedback Loop in the SCN incorporating the proposed functions of the mCRY, mPER, and mTIM Proteins. (Kume et al., 1999)

Figure 2: Model of SCN organization. The SCN core receives dense inputs from the visual system and projects to the shell SCN to phase shift the shell's rhythms. (Yan et al., 1999)

Figure 3: Model of food-entrainable neural circuit. The DMH receives circadian-related information from indirect SCN projections and energy balance-related inputs from the VMH and arcuate nucleus. The DMH sends outward projections to the LHA to influence wakefulness, locomotor arousal, and feeding. Figure from (Saper et al., 2005b)

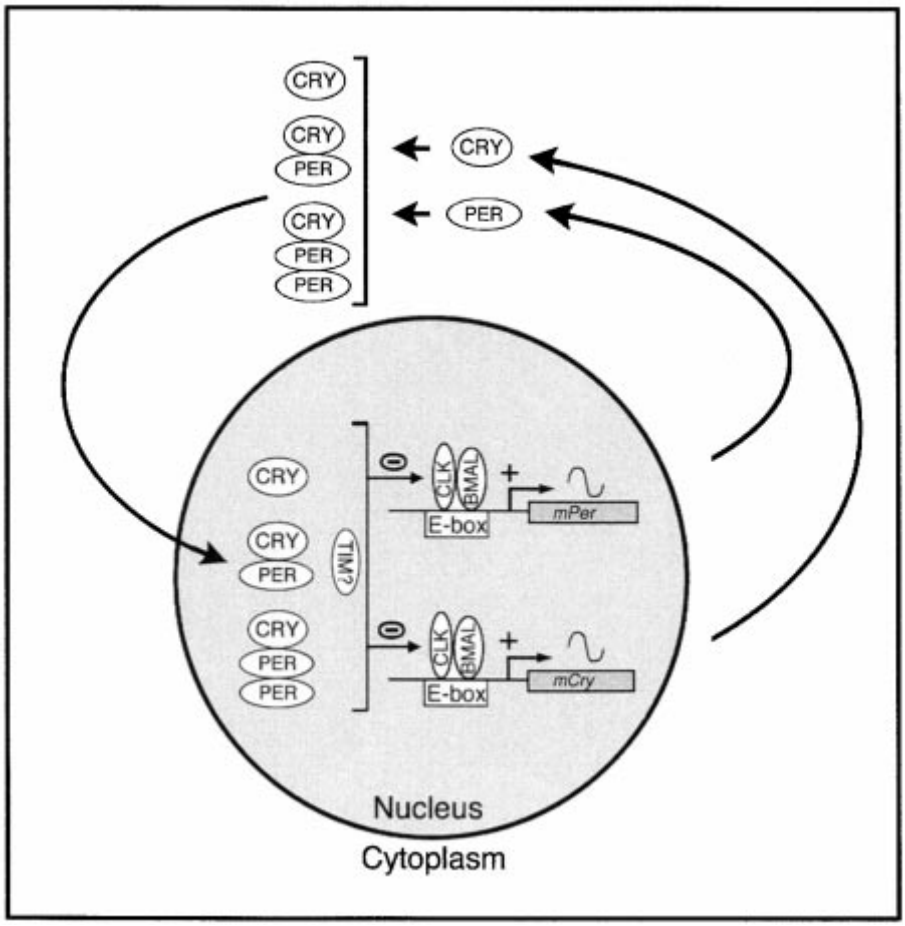

Figure 1

Model of Mammalian Clock Feedback Loop in the SCN incorporating the proposed functions of the mCRY, mPER, and mTIM Proteins. (Kume et al., 1999)

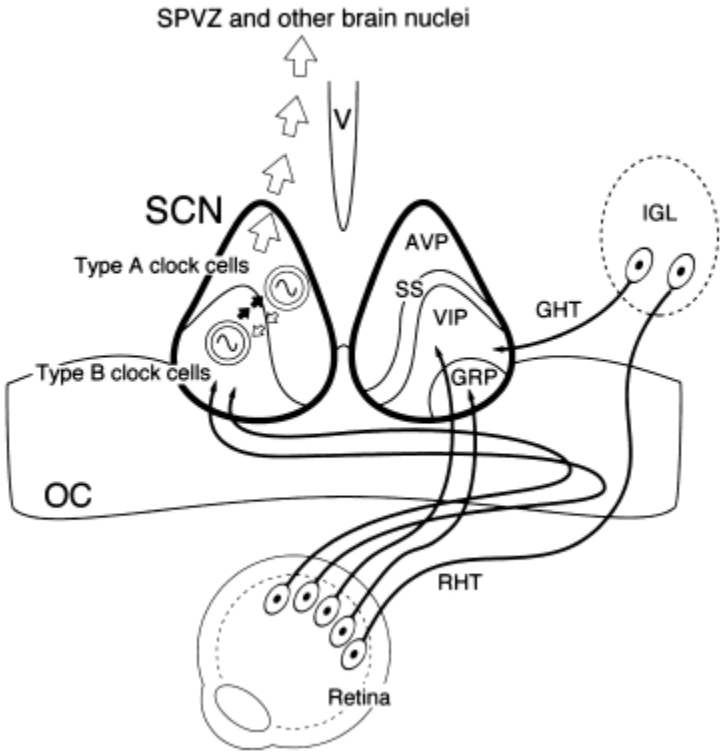

Figure 2

Model of SCN organization. The SCN core receives dense inputs from the visual system and projects to the shell SCN to phase shift the shell's rhythms. (Yan et al., 1999)

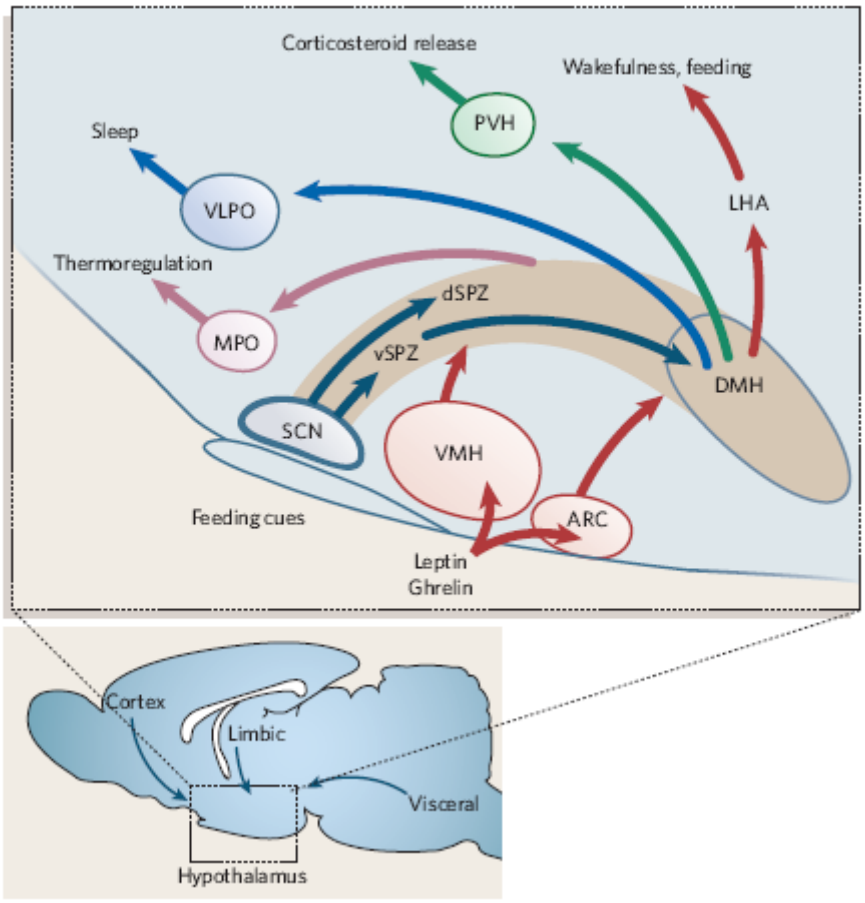

Figure 3

Model of food-entrainable neural circuit.

Chapter 2: Involvement of Serotonin$_{2C}$ Receptors in the Regulation of Circadian Phase Shifts to Light.

Abstract

Many aspects of physiology and behavior are driven by circadian rhythms that are coordinated by the suprachiasmatic nucleus (SCN) and strongly entrained by light stimuli. Multiple lines of evidence implicate the serotonin system in the regulation of circadian rhythms, although the mechanisms underlying this regulation remain unclear. In this study, we investigate the contribution of serotonin$_{2C}$ (5-HT2C) receptors to the regulation of circadian rhythms using 5-HT2C receptor knockout (5-HT2CRKO) mice. These mice exhibit essentially normal patterns of locomotor activity in a standard light/dark schedule, normal circadian rhythms in constant darkness, and impaired phase shifts to an early evening light pulse but normal phase advances to a late night light pulse. In addition, we show that 5-HT2C receptor mRNA is predominantly expressed in the retinorecipient ventral core subdivision of the SCN. Analysis of acute c-*fos*, *mPer1*, and *mPer2* gene expression following an early evening light pulse indicates that impaired light-induced phase shifts in KO mice are not accompanied by perturbed induction of these genes. We propose that 5-HT2C receptors play an important role in the photic resetting of circadian rhythms, by regulating events downstream or independent of the acute SCN response to light.

Introduction

Multiple physiological and behavioral processes exhibit circadian rhythms that oscillate with a period of roughly twenty-four hours (Schibler and Sassone-Corsin, 2002).

These circadian rhythms are coordinated by a master pacemaker that resides in the SCN (Albrecht, 2006). Circadian clocks have well-characterized molecular components, with positive elements (the transcription factors Clock and BMAL) driving the expression of negative elements (*Per* and *Cry*) that suppress Clock and BMAL transcriptional activity. These feedback loops drive self-sustained oscillations of transcriptional activity that in turn drive circadian cycles of biological processes (Albrecht, 2006).

A critical feature of circadian rhythms is the ability to entrain to environmental stimuli, particularly to light. Light stimuli are conveyed to the SCN directly via the retinohypothalamic tract and indirectly via the intergeniculate leaflet (IGL). The SCN responds to light stimuli by activating *Per* and *Cry* expression, thus resetting the endogenous molecular clock and entraining circadian rhythms to fluctuations of light and darkness (Challet, 2007). Photic entrainment of circadian rhythms is assessed by investigating the effects of discrete light pulses on phase shifts of circadian rhythms. Application of light pulses in the early evening cause a phase delay of circadian rhythms, while light pulses applied in the early morning cause a phase advance of circadian rhythms (Daan, 1977).

Multiple lines of evidence support the hypothesis that serotonin systems play a major role in the resetting of circadian rhythms by light. Serotonin neurons from midline raphe nuclei provide major inputs to the SCN; this is demonstrated in hamsters, rats, and mice (Meyer-Bernstein and Morin, 1996). In mice, lesions of serotonergic inputs to the SCN enhance the phase shifting effects of an early evening light pulse (Bradbury et al., 1997). Furthermore, treatment of hamsters with nonselective serotonin antagonists enhances the phase shifting effects of an early evening light pulse (Lall and Harrington, 2006).

However, it should be noted that the effects of some serotonergic compounds on light-induced phase shifts do not always generalize across species (Antle et al., 2003).

Serotonin mediates its effects via at least fourteen different receptor subtypes. The relative contributions of these receptors to the regulation of circadian rhythms remain unclear. 5-HT2C receptors are expressed in both mouse and rat SCN (Molineaux et al., 1989; Roca et al., 1993; Moyer and Kennaway, 1999), although their distribution within this nucleus remain unclear. In rats, non-selective 5-HT2C receptor agonists have been shown to mimic the phase-delaying effects of an early evening light pulse on circadian rhythms of body temperature and melatonin (Kennaway and Moyer, 1998), as well as on c-*fos* and *Per* gene expression in the SCN (Varcoe et al., 2003; Varcoe and Kennaway, 2008). In this study, we investigate the role of 5-HT2C receptors in the regulation of circadian rhythms (1) by analysis of 5-HT2C receptor expression within the SCN, (2) by characterizing circadian rhythm phenotypes in mice that lack 5-HT2C receptors, and (3) by analysis of SCN gene expression in 5-HT2C receptor knockout mice exposed to a phase-delaying light pulse.

Materials and Methods:

Animals. 5-HT2C receptor knockout (KO) mice were generated as described previously (Tecott et al., 1995). Animals used in this study were 12-16 week old male KO mice and wild-type littermates (WT). All experiments performed were in accord with guidelines of the National Institute of Health *Guide for Care and Use of Laboratory Animals*, and approved by the University of California San Francisco Institutional Animal Care and Use Committee.

Behavioral analysis of circadian rhythms. WT and KO mice (*n*=8 per genotype) were placed in photobeam break detection systems (Flexfield, San Diego Instruments) for chronic monitoring of locomotor activity. All animals had *ad libitum* food and water access throughout the duration of the experiment. Animals were maintained on a standard 12 hours light:12 hours dark cycle (LD1) with light (100 lux) on at 7:00 AM, denoted as ZT0. After 4 days of habituation and 8 days of baseline behavior measurements, animals were switched to constant darkness for 14 days (DD1). Because 5-HT2CRKO mice exhibit abnormal responses to contextual novelty (Rocha et al., 2002), we selected the Aschoff Type 2 protocol for determining phase shifts (Aschoff, 1965; Spoelstra et al., 2004; Jud et al., 2005) over the more commonly used Aschoff Type 1 protocol, in order to avoid the potential confounding effects of novelty stress (Hannibal et al., 2008). All animals received sequential light pulses (15 minutes at 100 lux) at ZT16 and ZT22, with 10 days of LD preceding and 10 days of DD following each light pulse.

Analysis of locomotor activity. Photobeam breaks were collected in 10 minute bins. Data were analyzed using Clocklab software (Actimetrics). Activity onsets were determined by an observer blinded to the genotype of the subjects. Phase angle of entrainment was calculated as the difference between the active onset from the first day after the LD1-DD1 transition and the lights off time during the LD schedule. Endogenous period (tau) was calculated by fitting a linear regression line to the activity onsets of DD1. Phase shifts were calculated as the difference between predicted onsets of two separate linear regression lines fitted to the activity onsets before and after the light pulse, corrected for by subtracting the phase shift to the first LD-DD transition without light pulse. When

fitting linear regression lines, the first day after transition to DD was omitted to avoid possible transient effects.

Gene expression analysis. WT and KO mice (*n*=14 per genotype) were transferred to a housing room and allowed to habituate for 5 days. The animals were given *ad libitum* access to food and water and were maintained in a standard LD cycle. On Day 6, mice serving as light-pulse negative controls were sacrificed between ZT16.5 and ZT17 without being exposed to light (*n*=2 per genotype). On Day 7, remaining animals were exposed to a ZT16 light pulse, then sacrificed either at ZT16.5 (*n*=6 per genotype) for analysis of c-*fos* induction, or sacrificed at ZT17 (*n*=6 per genotype) for analysis of *mPer1* and *mPer2* expression.

Tissue preparation. Brains were rapidly dissected under dim red light (Kodak Safelight with GBX2 filter, 15 watts), flash frozen in an isopentane/dry ice bath (-45 degrees Celsius), and stored at -80 degrees Celsius. Coronal brain sections (20 microns) were collected using a cryostat (Leica 1950) and stored at -80 degrees Celsius. For analysis of 5-HT2C receptor gene expression in the SCN, coronal brain sections (14 microns) were collected using a cryostat (Leica 1950).

In situ hybridization. A 220 bp fragment of the mouse GRP cDNA (Open Biosystems) was used to generate the digoxigenin-labeled antisense GRP riboprobe. A 0.5kb fragment of mouse c-*fos* cDNA was used to generate the c-*fos* riboprobe. A 1.6 kb fragment of the mouse 5-HT2C receptor cDNA was used to generate the 5-HT2CR riboprobe. The *mPer1* probe construct was a gift from Dr. Michihiro Mieda (University of Texas Southwestern Medical Center). The *mPer2* probe construct was a gift from Dr. Hitoshi Okamura (Kyoto University). The *mAVP* probe construct was a gift from Dr.

Nirao Shah (University of California San Francisco). Sections were fixed in ice-cold 4% paraformaldehyde for 30 minutes, washed with PBS, acetylated with 0.25% acetic anhydride in 0.1M triethanolamine for 10 minutes, washed with PBS, and then equilibrated with hybridization buffer for 30 minutes. Sections were then hybridized overnight with antisense riboprobe (200 ng/ml) in a humidified chamber at 62 degrees Celsius. Following hybridization, sections were rinsed with 0.1X SSC at 72 degrees Celsius for 1 hour, and incubated with 1:5000 alkaline phosphatase conjugated anti-digoxigenin antibody (Roche) overnight at 4 degrees Celsius. Slides were then incubated in BM Purple overnight, rinsed with 10mM Tris, 1mM EDTA pH 8.0 for 10 minutes, dehydrated in an ascending ethanol series, and coverslipped with Aquamount.

Quantitation of expression. Borders of the SCN were determined by adjacent cresyl violet-stained sections (Paxinos and Franklin, 2001). Expression levels were quantified by densitometry and normalized to background using NIH ImageJ (http://rsb.info.nih.gov/ij/). The background was determined by calculating the average of three 50 x 50 micron sample regions from the same tissue section which contained no detectable staining. Expression= (mean OD_{SCN}-mean $OD_{Background}$)/mean $OD_{Background}$.

Data analysis. Locomotor activity measurements are expressed as beam breaks. Endogenous period (tau), phase angle of entrainment, and light-induced phase shifts are measured in hours. Student's t-tests were used to determine effect of genotype on behavioral measures of circadian rhythm function, and to determine effect of genotype on light-induced gene expression. Bonferroni correction was used to adjust for multiple comparisons of activity across different portions of the LD cycle. Significance level was set at $p<0.05$.

Results

5-HT2C receptor mRNA is expressed predominantly in the SCN ventral core.

To visualize the distribution of 5-HT2C receptor gene expression in the mouse SCN, adjacent sections were stained for either 5-HT2C receptor, arginine vasopressin (AVP), or gastrin-releasing peptide (GRP) mRNA. We found strong 5-HT2C receptor gene expression in the ventral core region of the SCN, with strong expression in the GRP-expressing regions of SCN and sparse expression in AVP-expressing regions of SCN (Figure 4). Quantitation reveals a nearly three-fold increase in 5-HT2C receptor mRNA density in the GRP versus AVP-expressing region of SCN (p=0.0027).

5-HT2C receptor knockout mice exhibit impaired phase shifts to an early evening light pulse.

We compared locomotor activity rhythms of WT and KO mice in a standard light-dark schedule, under constant darkness conditions, and following light pulse stimuli known to produce phase shifts of circadian rhythms (Figure 5).

In a standard LD schedule, WT and KO mice exhibited a typical nocturnal activity pattern during the dark cycle, with most of their locomotor activity concentrated at the beginning and end of the dark cycle (Figure 6A). There were no phenotypic differences in light cycle activity (WT=3846.1±414.4, KO=5388.3±835.4, p=0.12). By contrast, KO mice exhibited a strong trend towards hyperactivity during the dark cycle (WT=11392.7.1±937.1, KO=15349.8±1794.2, p=0.071). This was due to a marked hyperactivity specifically during the beginning of the dark cycle, as there was a significant effect of genotype for the first two hours but not any other portion of the dark cycle (p=0.01 after Bonferroni correction, Figure 6A). KO mice exhibit similar phase

angle of entrainment to the LD cycle (WT=-0.06±0.14, KO=0.01±0.12, p=0.742, Figure 6B). Under constant darkness, KO free run with a similar period to WT mice (WT=23.8±0.02, KO=23.74±0.02, p>0.05, Figure 6C).

To assess the photic regulation of circadian rhythms, we assessed phase shift responses to pulses at ZT16 and ZT22. We found a significant impairment of the light-induced phase delay response in KO mice (WT=-0.67±0.15, KO=0.02±0.13, p=0.01) but no phenotypic differences in the light-induced phase advance (WT=0.25±0.23, KO=0.48±0.18, p=0.424, Figure 6D).

Acute SCN responses to a phase shifting light pulse are normal in 5-HT2CR knockout mice.

The impact of phase-delaying light pulses on SCN c-*fos*, *Per1*, and *Per2* expression has been well-characterized, and expression of these markers is commonly used to assess the acute response of the SCN to these light stimuli. WT and KO mice exhibited minimal c-*fos* expression in the absence of a light pulse and strong c-*fos* induction thirty minutes after a light pulse, without phenotypic differences (p=0.848, Fig. 3a). Similar findings were observed regarding *mPer1* expression, with minimal expression in the absence of a light pulse and strong induction 60 minutes after the light pulse, without phenotypic differences (p=0.265, Fig. 3b). Similar findings were observed regarding *mPer2* expression, with weak expression in the absence of light pulse and robust induction 60 minutes after the light pulse, without phenotypic differences (p=0.756, Fig. 3c). These data suggest that 5-HT2C receptors do not substantially influence these molecular responses of the SCN to light.

Discussion

The results of the present study demonstrate that 5-HT2CRKO mice exhibit normal patterns of locomotor activity in a standard LD cycle, normal free-running rhythms of locomotor activity in constant darkness, and a selective impairment of phase shifts to an early evening light pulse. By contrast, SCN gene expression studies suggest that 5-HT2CRKO mice exhibit similar acute molecular responses to the light pulse. In addition, 5-HT2C receptor mRNA was found to be abundant in the SCN ventral core region. Taken together, these data indicate that 5-HT2C receptors regulate the photic resetting of circadian rhythms.

The enhanced locomotor activity exhibited by KO mice at dark cycle onset may relate to a generalized hyperactivity phenotype of these mice. Previous studies indicate that these mice exhibit enhanced dopamine neurotransmission and enhanced locomotor responses to arousing stimuli such as exposure to a novel environment and cocaine administration (Rocha et al., 2002). It is possible that this disinhibition of dopamine systems impairs gating of locomotor activity in response to behaviorally arousing stimuli such as dark cycle onset.

KO mice exhibit normal phase advances but abolished phase delays to light stimuli. Mice exhibit a significantly larger phase delay component than phase advance component to phase-shifting light stimuli (Schwartz and Zimmerman, 1990), indicating that in this species, early evening light stimuli provide stronger phase-resetting signals to the circadian pacemaker compared to early morning light stimuli. Therefore, the magnitude of the phase delay reduction in KO indicates an important contribution of the 5-HT2C receptor to the expression of light-induced phase shifts in mice. Our data are consistent with previous studies indicating that pharmacological activation of 5-HT2C

receptors in rats mimics the effect of an early evening light pulse on gene expression in the SCN and on circadian phase shifts of body temperature and melatonin release (Kennaway and Moyer, 1998; Varcoe et al., 2003; Varcoe and Kennaway, 2008). It should be noted that the role of 5-HT2C receptors in circadian rhythm biology may be species specific, as 5-HT2C receptor agonists did not affect light-induced phase advances in hamsters (Gannon and Millan, 2006), and 5-HT2C receptor expression in the hamster SCN has not been described (Morin and Allen, 2006).

Our finding that 5-HT2C receptor KO mice exhibit impaired circadian phase delays to light without a corresponding attenuation of acute SCN c-*fos*, *Per1*, or *Per2* gene expression is notable, given the dearth of reported dissociations between these acute responses and long-term circadian phase shifts. Light-induced expression of c-*fos*, *Per1*, and *Per2* genes has been strongly implicated in the neural mechanisms underlying light-induced phase shifts. Phase-shifting light stimuli produce rapid induction of SCN c-*fos*, *Per1*, and *Per*2 expression, while light stimuli that fail to produce phase shifts also fail to induce SCN c-*fos* or *Per* expression (Kornhauser et al., 1990; Rusak et al., 1990; Shigeyoshi et al., 1997). In addition, multiple studies demonstrate a positive association between phase shift magnitude and magnitude of c-*fos* or *Per* induction in the SCN (Akiyama et al., 1999; Amy et al., 2000; Wakamatsu et al., 2001).

The precise role of these gene products in mediating long-term circadian phase shifts remains unclear, as there are few examples of dissociation between acute SCN gene expression and phase shifts. Such observations can help elucidate events downstream or independent of SCN gene expression that regulate circadian phase shifts. One study indicates that light and melatonin pretreatment can inhibit serotonin-induced phase

advances in hamsters by mechanisms independent of *Per* gene expression (Caldelas et al., 2005). Another report describes exaggerated light-induced phase shifts in mice lacking PACAPR1 receptors but a notable absence of acute c-*fos* and *Per1* induction, and reduced *Per2* induction by a light pulse in these mice (Hannibal et al., 2001). However, in the previous study the authors used an Aschoff Type 1 protocol to determine behavioral phase shifts to light and an Aschoff Type 2 protocol to determine light-induced gene expression. Subsequent studies using the Aschoff Type 2 protocol revealed behavioral phenotypes that were consistent with light-induced gene expression (Hannibal et al., 2008). To our knowledge, the only other reported instance of a dissociation between behavioral phase shifts to light and light-induced gene expression is the recent finding that 5-HT1A receptor knockout mice exhibit exaggerated phase advances to early morning light pulses but attenuated c-*fos* and *Per1* expression to the same light pulse stimulus (Smith et al., 2008). It is intriguing that the two dissociations between light-induced phase shifts and light-induced gene expression involve manipulations of serotonin receptors, as these findings suggest the possibility that serotonin systems participate in events downstream or independent of light-induced SCN gene expression to mediate long-term phase shifts to light. Furthermore, our study raises the possibility that 5-HT2C receptors impact light-induced phase delays through events downstream or independent of acute light-induced SCN gene expression.

One possibility is that 5-HT2C receptors mediate light-induced phase delays by influencing events downstream of acute SCN gene expression. The SCN is divided into two functional subregions: (1) a retinorecipient ventral core region that expresses VIP and GRP, does not exhibit endogenous rhythms of *Per* expression, and rapidly expresses

c-fos and *Per* in response to light (Yan et al., 1999; Karatsoreos et al., 2004) and (2) a non-retinorecipient dorsal shell region that expresses AVP, exhibits endogenous circadian rhythms of *Per* expression, and is not directly responsive to light stimuli (Yan et al., 1999; Schwartz et al., 2000). Tract-tracing studies of intra-SCN projections indicate unidirectional communication from the core to the shell (Leak et al., 1999). These and other data support the hypothesis that the SCN core plays an important role in mediating light-induced phase shifts via a two-step process: first by mediating the acute induction of *Per* genes, and consequently by resetting the phase of the SCN shell via efferent projections to the shell (Leak et al., 1999; Yan and Silver, 2002; Henk et al., 2005). The expression pattern of 5-HT2C receptors in the ventral core of the SCN suggests that these receptors are well-positioned to impact light-induced phase shifts by influencing communication from the core to the shell.

Our findings do not rule out the alternative hypothesis that 5-HT2C receptors impact light-induced phase delays via events independent of SCN acute gene expression. Direct projections from the retina to the raphe have been demonstrated in rats (Shen and Semba, 1994) but have not been found in hamsters (Morin and Allen, 2006), nor assessed in mice. In their 1998 study, Kennaway and Moyer proposed that stimulation of 5-HT2C receptors via retino-raphe projections may influence circadian phase shifts to light (Kennaway and Moyer, 1998), however, this hypothesis has not been tested.

In summary, our findings indicate an important role for 5-HT2C receptors in the photic resetting of circadian rhythms. In addition, analysis of 5-HT2CRKO mice reveals a dissociation between acute light-induced molecular changes in the SCN and long-term

behavioral phase shifts. These results may help shed light on downstream or independent events that mediate circadian phase shifts to light.

Figure Legends

Figure 4: 5-HT2C receptors are expressed mainly in the ventral core subdivision of the SCN. Images were taken of adjacent tissue sections along the rostro-caudal extent of the SCN, depicting the distribution of A. GRP, B. 5-HT2C receptor, and C. AVP mRNA.

Figure 5: Representative double-plotted actograms from WT and KO mice. The light/dark cycle is indicated by white/black bars above, darkness epochs are shown in gray shading. Filled ovals denote active phase onsets. Inverted triangles denote fifteen-minute light pulses at ZT16 and ZT22.

Figure 6: Circadian rhythm characteristics in WT and 5-HT2CRKO mice. A. 24-hour profile of locomotor activity at baseline conditions in a 12:12 light/dark cycle. The light/dark cycle is indicated by the white/black bars at the bottom of the plot. B. Phase angle of entrainment. C. Endogenous period (tau) of circadian rhythms in constant darkness. D. Phase shift to ZT16 light pulse. E. Phase shift to ZT22 light pulse.

Figure 7: Initial molecular response of the SCN to light is similar in WT and KO mice. A. Induction of c-*fos* expression 30 minutes following at ZT16 light pulse. B. Induction of *mPer1* expression 60 minutes following at ZT16 light pulse. C. Induction of *mPer2* expression 60 minutes following at ZT16 light pulse. Light-negative values are represented as the mean of two data points, light-positive values are represented as means±S.E.M.

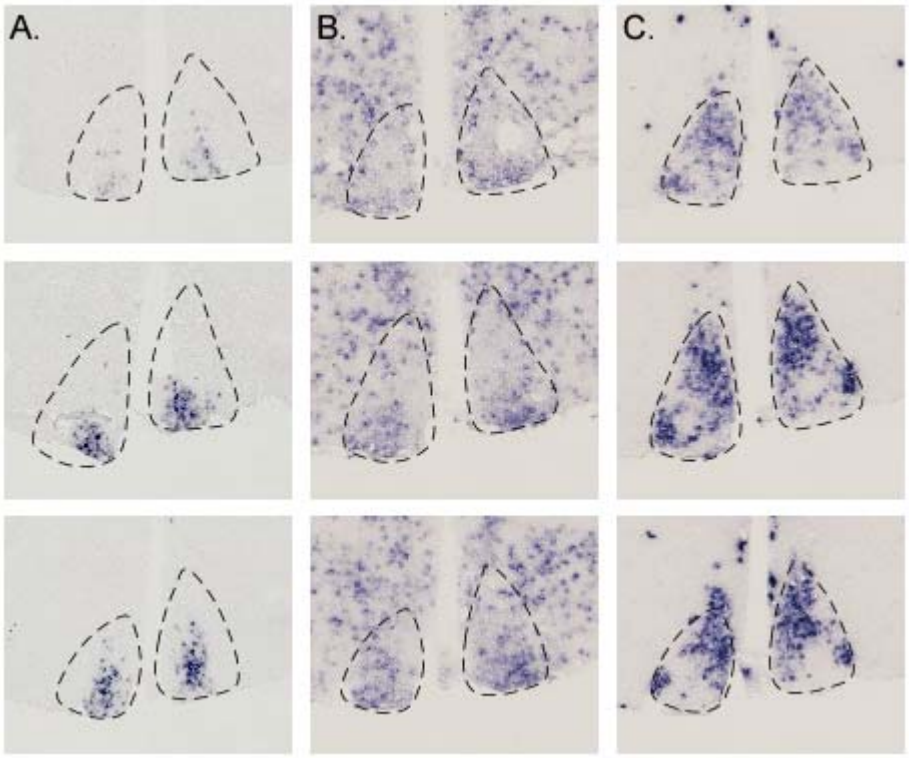

Figure 4

Images were taken of adjacent tissue sections along the rostro-caudal extent of the SCN, depicting the distribution of A. GRP, B. 5-HT2C receptor, and C. AVP mRNA.

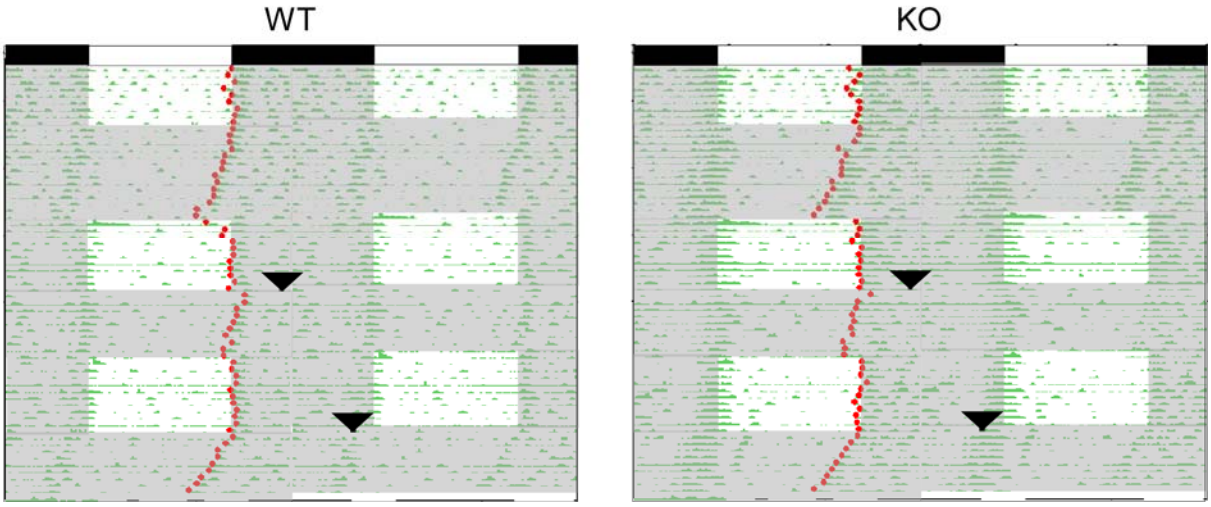

Figure 5

Representative double-plotted actograms from WT and KO mice.

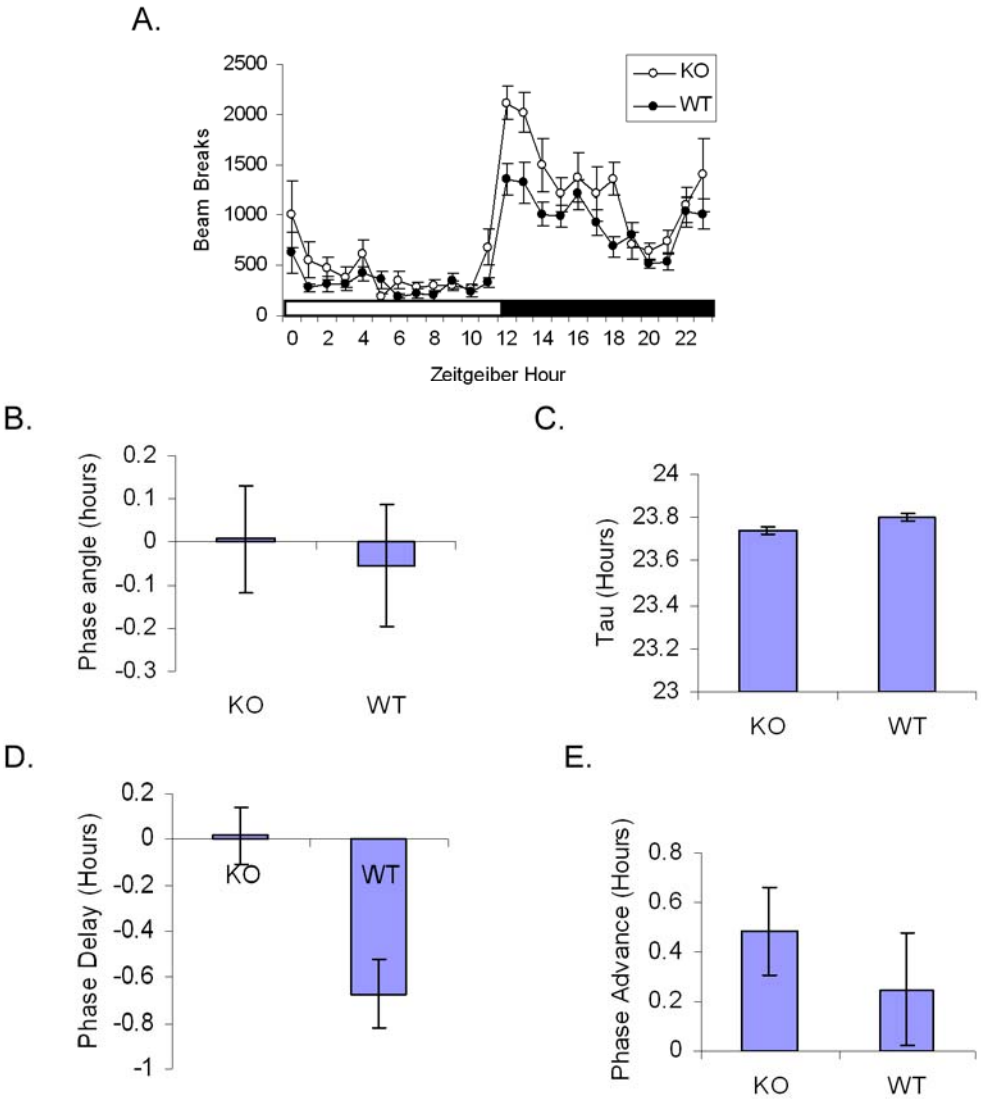

Figure 6

Circadian rhythm characteristics in WT and 5-HT2CRKO mice.

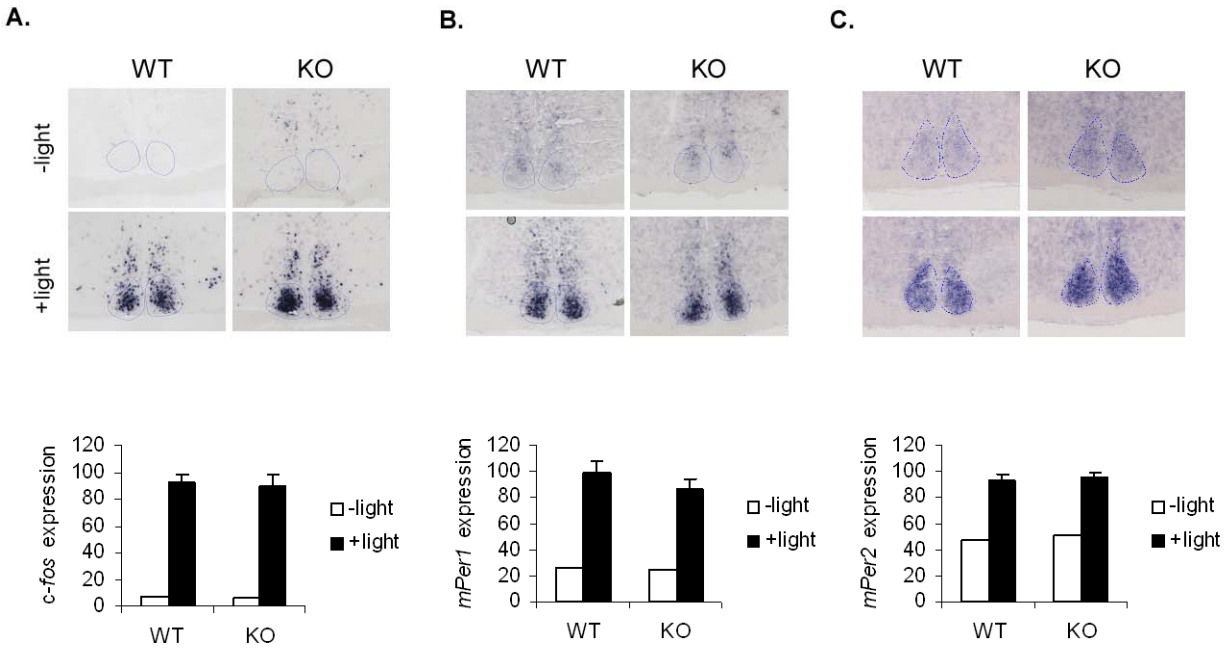

Figure 7

Initial molecular response of the SCN to light is similar in WT and KO mice. A. Induction of c-*fos* expression 30 minutes following at ZT16 light pulse. B. Induction of *mPer1* expression 60 minutes following at ZT16 light pulse. C. Induction of *mPer2* expression 60 minutes following at ZT16 light pulse.

Chapter 3: Enhanced food anticipatory activity is associated with enhanced activation of extrahypothalamic neural substrates in serotonin$_{2C}$ receptor knockout mice.

Abstract

The ability to entrain circadian rhythms to food availability is important for survival. Food-entrained circadian rhythms are characterized by an anticipatory increase in locomotor activity prior to the meal (food-anticipatory activity). However, the molecular components and neural circuitry underlying the regulation of food-entrainable circadian rhythms remain unclear. Here we show that serotonin$_{2C}$ receptor (5-HT2CR) knockout mice subjected to a daytime restricted feeding schedule exhibit enhanced anticipatory locomotor activity compared to wild-type littermates, without phenotypic effects of restricted feeding on food consumption, body weight loss, or blood glucose levels. Moreover, we show that the enhanced food anticipatory activity in 5-HT2CR knockout mice develops independent of external light cues and persists after two days of food deprivation. Restricted feeding induces similar c-*fos* expression in hypothalamic nuclei of wild-type and mutant animals, but enhanced expression in several extrahypothalamic regions of knockout mice relative to wild-type subjects. These data support the hypothesis that 5-HT2C receptors gate food anticipatory activity through mechanisms involving extrahypothalamic neural substrates.

Introduction

Multiple aspects of physiology and behavior exhibit circadian rhythmicity (Schibler and Sassone-Corsin, 2002). These circadian rhythms are driven by an endogenous molecular clock that originates in the suprachiasmatic nucleus (SCN) and

entrains to light/dark cues from the environment (Albrecht, 2006). However, multiple species have also evolved mechanisms for the entrainment of circadian rhythms to other environmental cues important for survival, such as food availability. Restricted feeding schedules, in which food availability is fixed to a particular time of day, have been shown to robustly induce rhythms of locomotor food anticipatory activity (FAA), body temperature, and circulating hormone release in anticipation of the daily scheduled meal (Mistlberger, 1994; Stephan, 2002; Feillet et al., 2006a).

Multiple lines of evidence indicate that these anticipatory rhythms are governed by a separate, SCN-independent food-entrainable oscillator (FEO) (Stephan, 1983; Marchant and Mistlberger, 1997). Several studies demonstrate that FAA can develop in the absence of external lighting schedules (Mistlberger, 1993; Lax et al., 1999; Abe et al., 2007). In addition, FAA entrains only to feeding cycles of roughly 24 hours, but not to 19-hour or 29-hour cycles, indicating that FAA is dependent on an internal circadian oscillator (Boulos et al., 1980; Mistlberger, 1994). These studies have been used to support the hypothesis that FAA represents the behavioral output of food-entrained circadian rhythms. However, an alternative hypothesis is that FAA is driven by cycles of energy depletion and restoration imposed by the daily feeding schedule, and is triggered when energy depletion reaches a certain threshold prior to feeding time ("hourglass hypothesis") (Mistlberger, 1994). However, FAA has been shown to persist after two or more days of total food deprivation (Boulos et al., 1980), indicating that FAA is not driven by hourglass mechanisms but instead reflects the output of a true food-entrainable circadian oscillator.

Recent studies have begun to uncover regional and molecular components of the FEO. Lesion and c-*fos* expression studies implicate several brain regions as potential substrates of the FEO, including the dorsomedial hypothalamic nucleus (DMH) (Angeles-Castellanos et al., 2004; Gooley et al., 2006), lateral hypothalamic nucleus (LHA) (Mieda et al., 2004; Kaur et al., 2008), and the nucleus accumbens (NAc) (Mendoza et al., 2005; Angeles-Castellanos et al., 2007). However, the neural mechanisms underlying food-entrainable rhythms remain unclear (Mistlberger, 1994; Stephan, 2002).

5-HT2C receptors are G_q-coupled receptors that are widely expressed through the brain and responsive to serotonin (Tecott et al., 1995). 5-HT2C receptors are strongly expressed in the DMH, LHA, and NAc (Tecott lab, unpublished observations), and thus are well-positioned to influence food anticipatory activity. In addition, non-selective 5-HT2C receptor agonists reduce starvation-induced hyperactivity, a phenomenon in which animals develop excessive locomotor activity in response to severely restricted food availability, particularly in the two hours leading up to the scheduled feeding (Wilckens et al., 1992; Pirke et al., 1993). In the present study, we investigate the role of 5-HT2C receptors in the regulation of food-anticipatory activity and its underlying neural circuitry.

Materials and Methods:

Animals. 5-HT2C receptor knockout mutant mice were generated as described previously (Tecott et. al, 1995). Mice used for experiments in this study are 12-16 week old male mutant mice and their wild-type littermates. All experiments performed were according to guidelines of the National Institute of Health *Guide for Care and Use of*

Laboratory Animals, and approved by the University of California San Francisco Committee on Animal Research.

Restricted Feeding protocol

Experiment 1: All mice were housed in a 12 hour light:12 hour dark (LD) cycle with lights on at 7 AM (ZT0). Wild-type (WT) and 5-HT2C receptor knockout (KO) mice were individually housed in photobeam break detection cages (PAS Flexfield, San Diego Instruments) for 10-12 days with *ad libitum* food (Purina PicoLab 5058) and water access, with food weighed every day at ZT4 and ZT8 (the same times used for restricted feeding, such that all animals are uniformly disturbed at the same times throughout the experiment). Restricted animals (n=13 WT, n=11 KO) were then subjected to 4 hours of daytime restricted feeding (ZT4-ZT8) for 14 days, while control animals (n=13 WT, n=11 KO) remained on an *ad libitum* feeding schedule. Main behavioral measurements were daily food intake, daily locomotor activity, and food-anticipatory activity (activity during the two hours prior to food availability). Body weight measurements were taken at the end of the baseline period and at the end of the study.

Experiment 2: To determine whether food anticipatory activity occurred independent of oscillations in energy status imposed by the restricted feeding schedule, WT (n=16) and KO (n=16) animals were subjected to the same restricted feeding protocol as above, with two additional days of total food deprivation following the 14 days of restricted feeding. Behavioral measurements and body weight measurements were taken as above.

Experiment 3: To determine whether food anticipatory activity occurred independent of light cues, WT (n=16) and KO (n=16) animals were subjected to the same

restricted feeding protocol as in Experiment 1, except that the housing room was switched to dim red light at ZT12 the day prior to restricted feeding and maintained in dim red light for the duration of the restricted feeding phase of the experiment. Main behavioral measurements and body weight measurements were obtained as described above.

Tissue preparation. For c-*fos* gene expression studies, mice were subjected to the same restricted feeding paradigm as in Experiment 1. Mice (*n*=4-8 per group) were sacrificed by decapitation at ZT4 and ZT13 on day 14 of restricted feeding. Brains were rapidly dissected and flash frozen in an isopentane/dry ice bath (-45 degrees Celcius) and stored at -80 degrees Celcius. Mice sacrificed at ZT13 were sacrificed under dim red light conditions (Kodak Safelight with GBX-2 filter, 15W). Coronal brain sections (20 microns) were collected using a cryostat (Leica 1950) and stored at -80 degrees.

In situ hybridization. A 0.5kb fragment of mouse c-*fos* cDNA was used to generate the digoxigenin-labeled antisense ribroprobe. Sections were fixed in ice-cold 4% paraformaldehyde for 30 minutes, rinsed with PBS, acetylated with .25% acetic anhydride in 0.1M triethanolamine for 10 minute, rinsed with PBS, and then equilibrated with hybridization buffer for 30 minutes. Sections were then hybridized overnight with antisense riboprobe (200 ng/ml) in a humidified chamber at 62 degrees Celsius. Following hybridization, sections were rinsed with 0.1X SSC at 72 degrees Celsius for 1 hour, and incubated with 1:5000 alkaline phosphatase conjugated anti-digoxigenin antibody (Roche) overnight at 4 degrees Celsius. Slides were then incubated in BM Purple overnight, rinsed in 10mM Tris, 1mM EDTA pH 8.0 for 10 minutes, dehydrated in an ascending ethanol series, and coverslipped with Aquamount.

Quantitation of c-fos expression. Outlines for regions of interest (ROI) were determined by adjacent cresyl violet-stained sections (Paxinos and Franklin, 2001). Expression values were quantified by densitometry and normalized to background as determined by calculating the average of three 50 x 50 micron sample regions from the same tissue section which contained no detectable staining. Expression= (mean OD_{SCN}-mean $OD_{Background}$)/mean $OD_{Background}$.

Data analysis. Locomotor activity measurements were averaged over the baseline period, the first week of restricted feeding, and the second week of restricted feeding. For behavioral measurements, repeated measures two-way ANOVA was used to determine the effects of genotype, feeding condition, and experimental period on daily locomotor activity and food anticipatory activity. Two-way ANOVA was used to determine the effects of genotype and feeding condition on daily food intake, body weight, and blood glucose. c-*fos* gene expression values for each data set were normalized to the average WT-*ad libitum* value. Regression analysis was used to determine the correlation between c-*fos* gene expression with locomotor activity in the hour leading up to the sacrifice time, also normalized to the average WT-*ad libitum* group value. c-*fos* expression data sets were tested for normality of distribution and equality of error variances. Data sets that met these criteria were analyzed using two-way ANOVA. Data sets that did not meet these criteria were analyzed using the Mann-Whitney U test, with the following comparisons: WT versus KO, Restricted versus Ad Lib, Restricted KO vs Restricted WT. Significance level was set at $p<0.05$.

Results

KO mice exhibit enhanced food-anticipatory activity.

To determine whether 5-HT2C receptors modulate food-entrainable circadian rhythms, we compared food-anticipatory activity between wild-type and 5-HT2CR knockout mice in response to a daytime restricted feeding schedule (Figure 8). Under baseline conditions, all experimental groups exhibited a spike in locomotor activity at ZT4, denoting behavioral activation in response to the daily food exchange (Figure 9A). However, baseline locomotor activity at ZT3 and at all other time points in the light cycle were minimal for all groups (Figure 9A). By the first week of restricted feeding, both restricted WT and KO mice exhibited a food-anticipatory rise in locomotor activity in the two hours prior to feeding time (Figure 9B). By the second week of restricted feeding, the restricted KO mice demonstrated robust enhancement of food anticipatory activity compared to WT (repeated measures ANOVA: effect of experiment phase, p=0.002, experiment phase x genotype, p=0.004, experiment phase x feeding condition, p=<0.001, experiment phase x genotype x feeding condition, p=0.001, Figure 9C). It is intriguing that while restricted WT mice displayed stable levels of food-anticipatory activity in the first and second week of restricted feeding, KO mice exhibited a significant increase in their anticipatory activity from the first to the second week of restricted feeding, almost doubling their anticipatory activity in that time period (Figure 9D).

5-HT2CR knockout mice are hyperactive at dark cycle onset and exhibit enhanced locomotor responses to contextual novelty (Rocha et al., 2002). To test the hypothesis that enhanced FAA of KO mice arises from a non-specific hyperactivity response to the restricted feeding paradigm, we analyzed locomotor activity occurring in

the first hour of the dark cycle, comparing baseline measurements with the second week of restricted feeding (Figure 21A). If KO animals respond to the restricted feeding schedule with general hyperactivity, we would also expect to see this enhanced locomotor activity at dark cycle onset. We found that locomotor activity decreased with experiment phase for all four experimental groups, with a significant genotype x feeding condition interaction (repeated measures ANOVA: effect of experiment phase, $p<0.001$, experiment phase x genotype, $p=0.168$, experiment phase x feeding condition, $p=0.26$, experiment phase x genotype x feeding condition, $p=0.011$). Post-hoc analysis revealed that this interaction was driven by the *ad libitum*-fed WT and KO animals, and that restricted feeding impacted dark cycle onset activity similarly in WT and KO mice (repeated measures ANOVA for restricted groups: effect of experiment phase, $p=0.024$, experiment phase x genotype, $p=0.504$. Repeated Measures ANOVA for *ad libitum* fed groups: effect of experiment phase, $p<0.001$, experiment phase x genotype, $p<0.001$).

Restricted feeding has a similar impact on measures related to energy balance in WT and KO mice.

To determine whether the restricted feeding paradigm has a differential impact on measures related to energy balance in WT and KO mice, we assessed the impact of restricted feeding on daily food intake, body weight reduction, and blood glucose levels. Baseline measures of daily food intake were not significantly different between WT and KO mice (univariate ANOVA: effect of genotype, $p=0.568$, effect of feeding condition, $p=0.912$, genotype x feeding condition interaction, $p=0.758$, Figure 10A). On the first day of restricted feeding, there was a significant decline in food intake for restricted mice of both genotypes, with restricted KO showing a significantly greater decrease in food

intake compared to WT (univariate ANOVA: effect of genotype, p=0.107, effect of feeding condition, p<0.001, genotype x feeding condition interaction, p=0.003, Figure 10A). However, both restricted WT and KO mice recovered their food intake at similar rates and to similar absolute levels by the second week of restricted feeding (repeated measures ANOVA: effect of experiment phase, p<0.001, experiment phase x genotype, p=0.566, experiment phase x feeding condition, p=0.001, experiment phase x genotype x feeding condition, p=0.870, Figure 10A).

Body weight measurements taken at the end of the baseline experimental period and the end of the restricted feeding period were used to calculate the percent change in body weight. KO mice subjected to *ad libitum* feeding conditions gained slightly more weight than WT counterparts, but restricted KO mice lost a similar percentage of body mass as their WT counterparts (univariate ANOVA: effect of genotype, p=0.474, effect of feeding condition, p<0.001, genotype x feeding condition, p=0.302, Figure 10B.) Restricted feeding also induced similar reduction of blood glucose in WT and KO mice (univariate ANOVA: effect of genotype, p=0.216, effect of feeding condition, p=0.002, genotype x feeding condition, p=0.234, Figure 10C.)

Enhanced food-anticipatory activity of KO mice persists after two days of food deprivation.

It is generally agreed that FAA arises as the behavioral output of a food-entrainable circadian clock; however, an alternative hypothesis states that FAA may be driven by cycles of energy depletion and restoration due to the imposed feeding schedule ("hourglass hypothesis"). To address the hypothesis that enhanced FAA of KO mice reflects an hourglass mechanism, we subjected WT and KO mice to a restricted feeding

paradigm with two additional days of food deprivation following the 14 days of restricted feeding (Figure 11). In these conditions, FAA developed in a similar manner as in a standard light-dark cycle, with significant enhancement in restricted KO compared to WT during the second week of restricted feeding (repeated measures ANOVA: effect of experiment phase, $p<0.001$, experiment phase x genotype, $p=0.005$, experiment phase x feeding condition, $p<0.001$, experiment phase x genotype x feeding condition, $p=0.038$, Figure 12A-12D). This level of FAA persisted on the second day of restricted feeding (repeated measures ANOVA: effect of experiment phase, $p<0.001$, experiment phase x genotype, $p=0.004$, experiment phase x feeding condition, $p<0.001$, experiment phase x genotype x feeding condition, $p=0.056$, Figure 12E).

Enhanced food-anticipatory activity of KO mice does not require light cues.

Many studies have shown that FAA occurs in the absence of external temporal cues such as light/dark schedules. To determine whether the enhanced FAA phenotype demonstrated by KO mice is independent of light cues, we compared levels of FAA in WT and KO mice in animals subjected to a restricted feeding schedule in constant dim red light (Figure 13). In these conditions, FAA developed in a similar manner as in a standard light/dark schedule, with significantly enhanced FAA in KO mice compared to WT by the second week of restricted feeding (repeated measures ANOVA: effect of experiment phase, $p<0.001$, experiment phase x genotype, $p=0.013$, experiment phase x feeding condition, $p<0.001$, experiment phase x genotype x feeding condition, $p=0.009$, Figure 14A-D).

Restricted feeding induces a similar pattern of c-*fos* gene expression in hypothalamic nuclei of WT and KO mice.

To assess neural correlates of food-anticipatory activity in WT and KO mice, we analyzed c-*fos* gene expression in animals subjected to a restricted feeding schedule and sacrificed at ZT4 (*n*=7-8 per group). We found minimal c-*fos* gene expression in the DMH of *ad libitum*-fed animals but strong induction in restricted animals, with no significant differences between the genotypes (univariate ANOVA: effect of genotype, p=0.178, effect of restriction, p<0.001, genotype x feeding condition interaction, p=0.170, Figure 15A-E). Moreover, linear regression analysis revealed a significant correlation between DMH *c-fos* expression and FAA (R^2=0.65, p<0.001, Figure 15F.) Similar findings were observed for c-*fos* expression in the LHA. We found minimal c-*fos* gene expression in *ad libitum*-fed controls, and strong induction in restricted animals, without phenotypic differences (univariate ANOVA: effect of genotype, p=0.17, effect of feeding condition, p=0.009, genotype x feeding condition interaction, p=0.427, Figure 15A-D, 15G). Moreover, there was a significant correlation between LHA c-*fos* expression and FAA (R^2=0.61, p<0.001, Figure 15H.)

The arcuate nucleus and SCN are proposed to provide afferent signals to the putative food entrainment circuit related to energy status information and circadian clock information (Saper et al., 2005c). We found modest c-*fos* gene expression in the SCN, with no effect of feeding condition or genotype (univariate ANOVA: effect of genotype, p=0.605, effect of feeding condition, p=0.741, genotype x feeding condition interaction, p=0.664, Figure 16). Similarly, we found modest c-*fos* expression in the arcuate nucleus with no effect of feeding condition or genotype (univariate ANOVA: effect of genotype, p=0.320, effect of feeding condition, p=0.620, genotype x feeding condition interaction, p=0.113, Figure 17). Linear regression analysis revealed a modest correlation between

SCN c-*fos* expression and FAA (R^2=0.13, p=0.044, Figure 16F), and no correlation between arcuate c-*fos* expression and FAA (R^2=0.06, p=0.203, Figure 17F).

Phenotypic differences in c-*fos* induction by restricted feeding in extrahypothalamic regions.

In addition to analysis of hypothalamic c-*fos* expression, a survey of extrahypothalamic sites was conducted for regions that demonstrated c-*fos* induction by restricted feeding. Four regions demonstrated strong c-*fos* induction but without phenotypic effects (see supplementary table 1). In addition, three regions exhibited c-*fos* induction by restricted feeding with enhanced induction in restricted KO mice relative to WT: the nucleus accumbens (NAc), the ventral posterior area of the thalamus (VPT), and the sensory barrel cortex. In the NAc, restricted feeding induced c-*fos* expression modestly in WT mice, with enhanced induction in KO mice (Mann Whitney U: effect of genotype p=0.055, effect of feeding condition p=0.002, KO-Restricted>WT-Restricted, p=0.003, Figure 18A-E). Moreover, there was a significant correlation between NAc c-*fos* expression and FAA (R^2=0.60, p<0.001, Figure 18F). In the VPT, restricted feeding induced c-*fos* expression modestly in WT mice, with enhanced induction in KO mice (univariate ANOVA: effect of genotype p<0.05, effect of feeding condition p<0.001, genotype x feeding condition interaction p=0.002, Figure 19A-E). Moreover, there was a significant correlation between VPT c-*fos* expression and FAA (R^2=0.67, p<0.001, Figure 19F). In the sensory barrel cortex, restricted feeding strongly induced c-*fos* expression in both genotypes, with a strong trend towards enhanced induction in restricted KO mice (Mann-Whitney U: effect of genotype p=0.728, effect of feeding condition p=0.001, KO-Restricted>WT-Restricted, p=0.07, Figure 20A-E). Moreover,

there was a significant correlation between barrel cortex c-*fos* expression and FAA (R^2=0.61, p<0.001, Figure 20F).

Neural correlates of FAA do not correlate with non-specific locomotor activity.

To determine whether neural activation in the above regions correlate specifically with FAA or reflect general locomotor activity, we assessed ZT13 c-*fos* expression in the DMH, LHA, NAc, and barrel cortex. ZT13 c-*fos* expression provides a useful neural correlate of locomotor activity that occurs at dark cycle onset—which is a time point at which mice exhibit a significant increase in locomotor activity unrelated to food anticipation, and during which KO mice are significantly hyperactive compared to WT. We found no significant correlation for any of the regions tested (DMH correlation: R^2=0.051, *p*=0.338, Figure 21C; LHA correlation: R^2=0.109, *p*=0.156, Figure 21D; barrel cortex correlation: R^2=0.085, *p*=0.211, Figure 21E; NAc correlation: R^2=0.019, *p*=0.567, Figure 21F).

Discussion:

We find that mice lacking 5-HT2C receptors exhibit enhanced FAA relative to WT animals in response to a temporally restricted feeding schedule. This enhanced FAA phenotype is not accompanied by alterations in c-*fos* induction by restricted feeding in the DMH or LHA, but is associated with enhanced c-*fos* induction in a number of extrahypothalamic regions. These findings indicate that 5-HT2C receptors selectively gate food-anticipatory activity through mechanisms involving extrahypothalamic neural substrates.

The enhanced FAA phenotype of KO mice relative to WT is notable, in light of the limited number of studies investigating serotonergic regulation of FAA. In one study,

investigators assessed the impact of fluvoxamine (an SSRI) administration on food-anticipatory and post-feeding locomotor activity, and found that fluvoxamine had no effect on FAA but reduced post-feeding locomotor activity (Yokoyama et al., 2007). Another study demonstrates that impaired FAA in old rats may be restored to levels comparable to young animals by treatment with non-selective 5-HT2 receptor antagonists (Shibata et al., 1995), which is consistent with our findings indicating that these receptors may negatively regulate FAA.

5-HT2CRKO mice exhibit enhanced locomotor responses to a number of stimuli, such as dark cycle onset, novelty, and cocaine administration (Rocha et al., 2002). This raises the question of whether the enhanced FAA phenotype of these mice reflects a non-specific locomotor response to the restricted feeding paradigm. Our data reveals enhanced locomotor activity of restricted KO mice only during the FAA time window and not at dark cycle onset, another time of high locomotor activity that is independent of food anticipation. These results indicate that the enhanced locomotor activity response of restricted KO mice is specific to FAA. In addition, KO mice exhibit FAA in both LD and constant dim red light conditions, which argue against the possibility that these animals are using the light onset cue as a predictor of food availability. Furthermore, the enhanced FAA phenotype of KO mice persists after two days of food deprivation, arguing against the hypothesis that FAA in these mice is driven by an hourglass mechanism. Taken together, these data support the notion that the FAA phenotype in KO mice, as in other rodents, reflects a true behavioral manifestation of a food-driven circadian oscillator.

The DMH and LHA are regions previously implicated to play a central role in the regulation of food anticipatory activity. The DMH receives indirect projections from the suprachiasmatic nucleus (SCN), and also receives projections from hypothalamic nuclei involved in processing hunger and satiety-related signals (Saper et al., 2005a). In addition, the DMH projects to regions critically involved in the regulation of wakefulness, locomotor activity, and feeding (Saper et al., 2005a). Lesion studies have yielded mixed results on the role of the DMH in the regulation of FAA (Gooley et al., 2006; Landry et al., 2006). More recently, it was reported that restoration of the clock gene BMAL selectively in the DMH restores food entrainable circadian rhythms but not light-entrainable rhythms (Fuller et al., 2008). Based on these studies, the DMH has been proposed as a component of the FEO; however, due to conflicting results its role in the regulation of FAA remains an open question. The LHA has been proposed to mediate the expression of FAA, based on findings that this region exhibits c-*fos* induction in response to daily restricted feeding and coincident with the timing of FAA (Angeles-Castellanos et al., 2004), and mice with genetic ablations of LHA orexin neurons exhibit reduced FAA. Our finding of similar c-*fos* induction in the DMH and LHA indicates that the enhanced FAA phenotype exhibited by KO mice cannot be attributed to enhanced activation of these nuclei.

Studies investigating the neural substrates underlying food entrainment have largely focused on hypothalamic nuclei, particularly the DMH and LHA. However, the contribution of extrahypothalamic regions to the regulation of food entrainment has been less thoroughly examined. Restricted feeding paradigms that induce FAA have been shown to induce c-*fos* in a number of corticolimbic structures, such as the nucleus

accumbens (NAc), amygdala, prefrontal cortex, and septal area, and to shift *Per1* rhythms in the accumbens (Angeles-Castellanos et al., 2007). The NAc in particular has been proposed as a crucial link that integrates homeostatic signaling, motivational state, and behavioral expression (Mogenson et al., 1980; Berthoud, 2004). In our study, c-*fos* expression in the NAc is induced by restricted feeding, enhanced in restricted KO animals, and is significantly correlated with FAA but not with dark cycle onset locomotion. These results raise the possibility that enhanced FAA of KO animals may be attributed to increased activation of the NAc. The NAc receives direct and indirect projections from LHA orexin neurons (Peyron et al., 1998; Fadel and Deutch, 2002; Vittoz et al., 2008). Indeed, orexin increases VTA-mediated dopamine neurotransmission in the nucleus accumbens, and direct orexin A administration into the nucleus accumbens induces both feeding and locomotor activity (Thorpe and Kotz, 2005). Taken together, the above findings suggest the possibility that the NAc acts as an LHA output effector to mediate expression of FAA.

Our finding of enhanced activation of the NAc in restricted KO mice relative to WT is consistent with the known inhibitory role of 5-HT2C receptors on NAc output. 5-HT2C receptors are expressed on GABAergic neurons in the accumbens (Eberle-Wang et al., 1997). Moreover, pharmacological studies reveal that constitutively active 5-HT2C receptors in the nucleus accumbens exert a tonic inhibitory influence on accumbens dopamine neurotransmission (De Deurwaerdere et al., 2004; Navailles et al., 2006). In addition, 5-HT2CRKO mice exhibit enhanced behavioral responses to novelty and cocaine administration, an effect that has been attributed to enhanced dopamine neurotransmission in the NAc (Rocha et al., 2002). These data support the hypothesis

that 5-HT2C receptors tonically inhibit NAc output by promoting the activity of local interneurons. It is possible that this tonic inhibition also gates the sensitivity of the NAc to signals from the LHA orexin system.

The enhanced activity of the VPT and barrel cortex of restricted 5-HT2CR knockout mice is an unexpected result. Neither region receives afferents from the DMH or LHA, or has been reported to express c-*fos* in response to restricted feeding. In addition, neither region expresses 5-HT2C receptors during development nor in adulthood, therefore, the mechanisms by which VPT and barrel cortex are influenced by food entrainment or by 5-HT2C receptor-mediated signaling are unclear. The VPT and barrel cortex represent the thalamic and cortical relay of whisker-mediated sensation in rats and mice, both of which rely on tactile sensation from their whiskers to navigate the environment (Petersen, 2007). It is possible that FAA causes secondary activation of these regions due to the increased sensory input associated with environment navigation. However, our results demonstrate a highly significant correlation between barrel cortex c-*fos* expression levels and FAA, but no correlation with general locomotor activity occurring at dark cycle onset. These findings argue against the notion that activation of the sensory barrel system is solely a secondary consequence of locomotor activity. It is also possible that the enhanced activation of the sensory barrel system as well as the NAc itself may provide stronger entraining signals that ultimately result in enhanced FAA. NPAS2 is a bHLH-PAS domain transcription factor that is functionally similar to the circadian CLOCK protein (Reick et al., 2001) and expressed in forebrain (Zhou et al., 1997). Beta-galactosidase staining in brain sections from NPAS2-lacZ mice revealed particularly strong expression in the barrel cortex, VPT, and NAc (Garcia et al., 2000).

Furthermore, it was discovered that mutant mice lacking functional NPAS2 exhibit acutely impaired FAA which recovers by the second week of restricted feeding (Dudley et al., 2003). These studies have been used to suggest the possibility that under restricted feeding schedules, NPAS2 acts in forebrain loci to entrain an SCN-independent clock using sensory arousal as the principle Zeitgeber (Dudley et al., 2003).

In summary, our findings suggest a novel role for 5-HT2C receptors in the regulation of food-anticipatory activity. Furthermore, c-*fos* gene expression studies in food-entrained WT and KO mice suggest a role for extrahypothalamic substrates in the regulation of FAA, and implicate 5-HT2C receptors in the modulation of these processes. These results may help shed light on the neural mechanisms underlying the regulation of food anticipatory activity.

Figure Legends

Figure 8: Representative double-plotted actograms of wild-type (WT) and 5-HT2CRKO mice (KO) under *ad libitum* feeding conditions or a restricted feeding schedule. The light/dark cycle is indicated by white/black bars above, the restricted feeding schedule is indicated by gray shading.

Figure 9: 24-hour activity profile averaged over A. baseline *ad libitum* feeding conditions for WT (squares) and KO (triangles) mice assigned to either *ad libitum* (white) or *restricted feeding* (black) groups. Grey shading indicates the timing of the restricted feeding schedule (except in baseline conditions in which all animals are under *ad libitum* feeding conditions). B. 24-hour activity profile averaged over the first week of restricted feeding. C. 24-hour activity profile averaged over the second week of

restricted feeding. D. Locomotor activity during the two hours preceding food availability. All values represent means ±S.E.M.

Figure 10: Restricted feeding induces similar changes in food intake, body weight, and blood glucose for both WT and 5-HT2CRKO mice. A. Daily food intake of WT or KO mice assigned to either *ad libitum* (white) or restricted feeding (black) groups. B. Percent change in baseline body weight after fourteen days of restricted feeding. C. Blood glucose levels measured at the end of fourteen days of restricted feeding. Values represent means ±S.E.M.

Figure 11: Enhanced FAA in 5-HT2CRKO mice persists after two days of food deprivation. Representative double-plotted actograms of wild-type (WT) and 5-HT2CRKO mice (KO) under *ad libitum* feeding conditions or a restricted feeding schedule. The light/dark cycle is indicated by white/black bars above and by grey shading, the restricted feeding schedule is indicated by rectangles. The start of total food deprivation is indicated by inverted triangles.

Figure 12: Enhanced FAA in 5-HT2CRKO mice persists after two days of food deprivation. 24-hour activity profiles of WT (squares) or KO (triangles) assigned to *ad libitum* (white symbols) or restricted feeding (black) groups, averaged over A. baseline, B. first week of restricted feeding, C. second week of restricted feeding, D. day one of total food deprivation. E. day two of total food deprivation. The light/dark cycle is indicated by white/black bars above, the restricted feeding schedule is indicated by gray shading (except in baseline conditions in which all animals are under *ad libitum* feeding conditions). Values represent means ±S.E.M.

Figure 13: Enhanced FAA in 5-HT2CRKO mice is independent of light cues. Representative double-plotted actograms of wild-type (WT) and 5-HT2CRKO mice (KO) under *ad libitum* feeding conditions or a restricted feeding schedule. The light/dark cycle is indicated by white/black bars above and by grey shading, the restricted feeding schedule is indicated by rectangles.

Figure 14: Enhanced FAA in 5-HT2CRKO mice is independent of light cues. A. 24-hour activity profile averaged over five days of baseline *ad libitum* feeding conditions for WT (squares) and KO (triangles) mice assigned to either *ad libitum* (white) or *restricted feeding* (black) groups. Grey shading indicates the timing of the restricted feeding schedule (except in baseline conditions in which all animals are under ad libitum feeding conditions). B. 24-hour activity profile averaged over the first week of restricted feeding. C. 24-hour activity profile averaged over the second week of restricted feeding. D. Locomotor activity during the two hours hour preceding food availability. All values represent means ±S.E.M.

Figure 15: Restricted feeding induces similar c-*fos* expression in the DMH and LHA at ZT4. A-D. Representative photomicrographs depicting ZT4 c-*fos* mRNA in hypothalamus of A. WT-Ad Lib B. KO-Ad Lib C. WT-Restricted D. KO-Restricted mice. E. Quantitation of ZT4 DMH c-*fos* mRNA. Values represent means ±S.E.M. Black bars denoted *ad libitum* feeding conditions, white bars denote restricted feeding conditions. F. Correlation of locomotor activity to DMH c-*fos* gene expression. Data points represent individual animals. G. Quantitation of ZT4 LHA *c-fos* mRNA. H. Correlation of locomotor activity to LHA c-*fos* gene expression.

Figure 16: No effect of restricted feeding or genotype on ZT4 SCN c-*fos* expression. A-D. Representative photomicrographs depicting ZT4 c-*fos* mRNA in the SCN of A. WT-Ad Lib B. KO-Ad Lib C. WT-Restricted D. KO-Restricted mice. E. Quantitation of ZT4 SCN c-*fos* mRNA. Values represent means ±S.E.M. Black bars denoted *ad libitum* feeding conditions, white bars denote restricted feeding conditions. F. Correlation of locomotor activity to SCN c-*fos* gene expression. Data points represent individual animals.

Figure 17: No significant effect of restricted feeding or genotype on ZT4 c-*fos* expression in the arcuate. A-D. Representative photomicrographs depicting ZT4 c-*fos* mRNA in the arcuate of A. WT-Ad Lib B. KO-Ad Lib C. WT-Restricted D. KO-Restricted mice. E. Quantitation of ZT4 arcuate c-*fos* mRNA. Values represent means ±S.E.M. Black bars denoted *ad libitum* feeding conditions, white bars denote restricted feeding conditions. F. Correlation of locomotor activity to arcuate c-*fos* gene expression. Data points represent individual animals.

Figure 18: Restricted feeding induces c-*fos* expression in the nucleus accumbens, with enhanced induction in restricted KO mice. A-D. Representative photomicrographs depicting ZT4 c-*fos* mRNA in the NAc of A. WT-Ad Lib B. KO-Ad Lib C. WT-Restricted D. KO-Restricted mice. E. Quantitation of ZT4 NAc c-*fos* mRNA. Values represent means ±S.E.M. Black bars denoted *ad libitum* feeding conditions, white bars denote restricted feeding conditions. F. Correlation of locomotor activity to NAc c-*fos* gene expression. Data points represent individual animals.

Figure 19: Restricted feeding induces c-*fos* expression in the VPT, with enhanced induction in restricted KO mice. A-D. Representative photomicrographs depicting ZT4

c-*fos* mRNA in the VPT of A. WT-Ad Lib B. KO-Ad Lib C. WT-Restricted D. KO-Restricted mice. E. Quantitation of ZT4 VPT c-*fos* mRNA. Values represent means ±S.E.M. Black bars denoted *ad libitum* feeding conditions, white bars denote restricted feeding conditions. F. Correlation of locomotor activity to V.P. thalamus c-*fos* gene expression. Data points represent individual animals.

Figure 20: Restricted feeding induces c-*fos* expression in the barrel cortex, with enhanced induction in restricted KO mice. A-D. Representative photomicrographs depicting ZT4 c-*fos* mRNA in the barrel cortex of A. WT-Ad Lib B. KO-Ad Lib C. WT-Restricted D. KO-Restricted mice. E. Quantitation of ZT4 barrel cortex c-*fos* mRNA. Values represent means ±S.E.M. Black bars denoted *ad libitum* feeding conditions, white bars denote restricted feeding conditions. F. Correlation of locomotor activity to barrel cortex c-*fos* gene expression. Data points represent individual animals.

Figure 21: Neither the enhanced FAA phenotype nor neural correlates of FAA are attributable to non-specific locomotor activity. A. Comparison of locomotor activity that occurs between ZT2-4, averaged over baseline and the second week of restricted feeding for WT (squares) or KO (triangles) assigned to *ad libitum* (white symbols) or restricted feeding (black) groups. B. Comparison of locomotor activity that occurs between ZT12-13. C-F. Correlations between ZT12-13 locomotor activity and ZT13 c-*fos* expression for C. DMH, D. LHA, E. Barrel Cortex, and F. NAc.

Table 1: ZT4 c-*fos* expression in extrahypothalamic regions, without phenotypic effects.

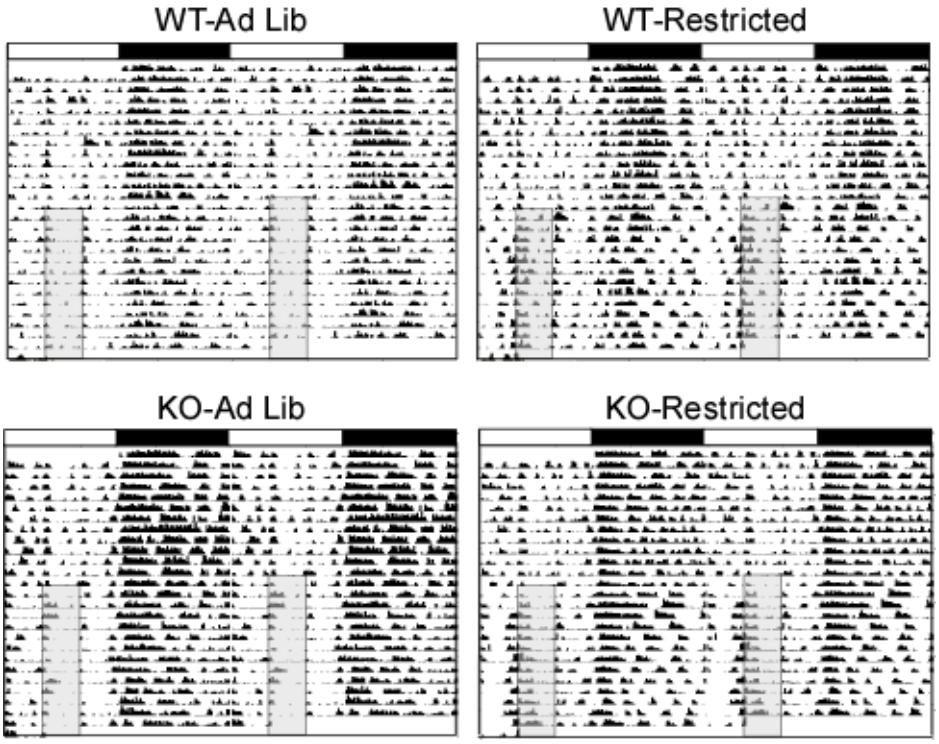

Figure 8

Representative double-plotted actograms of wild-type (WT) and 5-HT2CRKO mice (KO) under *ad libitum* feeding conditions or a restricted feeding schedule. Representative double-plotted actograms of wild-type (WT) and 5-HT2CRKO mice (KO) under *ad libitum* feeding conditions or a restricted feeding schedule. The light/dark cycle is indicated by white/black bars above, the restricted feeding schedule is indicated by gray shading.

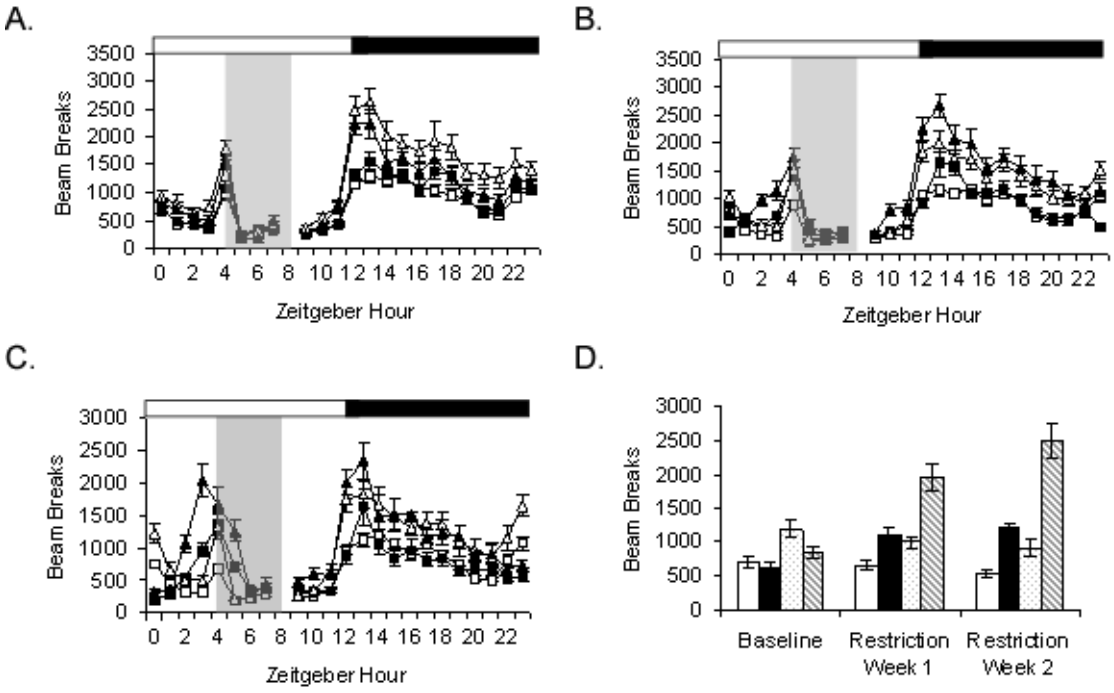

Figure 9

24-hour activity profile of WT (squares) and KO (triangles) mice assigned to either *ad libitum* (white) or *restricted feeding* (black) groups. Grey shading indicates the timing of the restricted feeding schedule (except in baseline conditions in which all animals are under *ad libitum* feeding conditions). A. baseline. B. first week of restricted feeding. C. second week of restricted feeding. D. Locomotor activity during the two hours preceding food availability. All values represent means ±S.E.M.

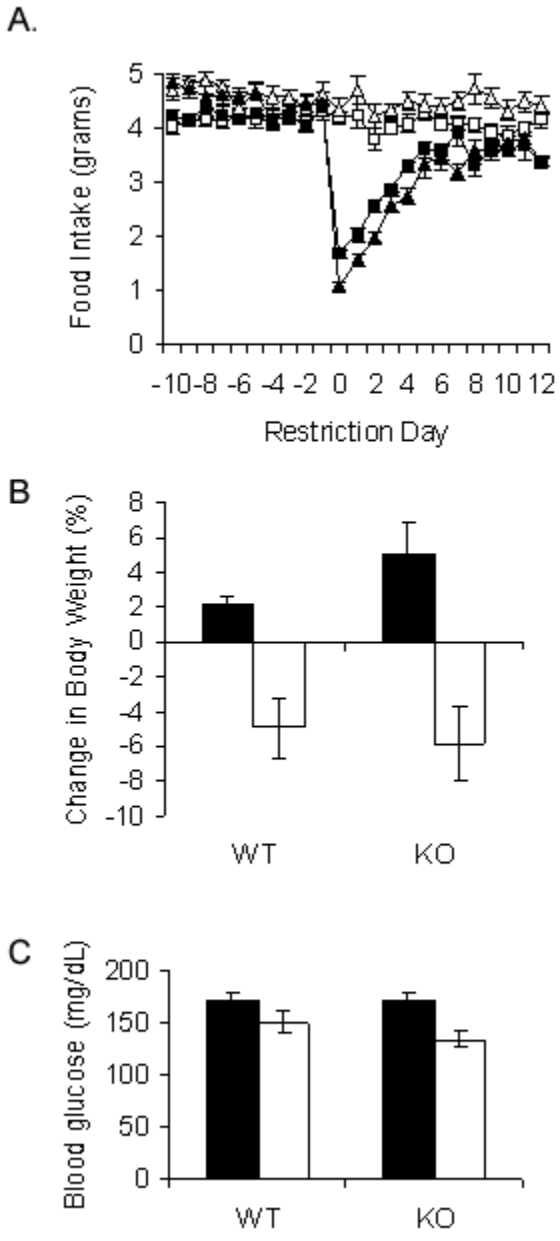

Figure 10

Effect of restricted feeding on A. Daily food intake B. body weight C. Blood glucose levels. Black bars denote *ad libitum* fed animals, white bars denote restricted animals.

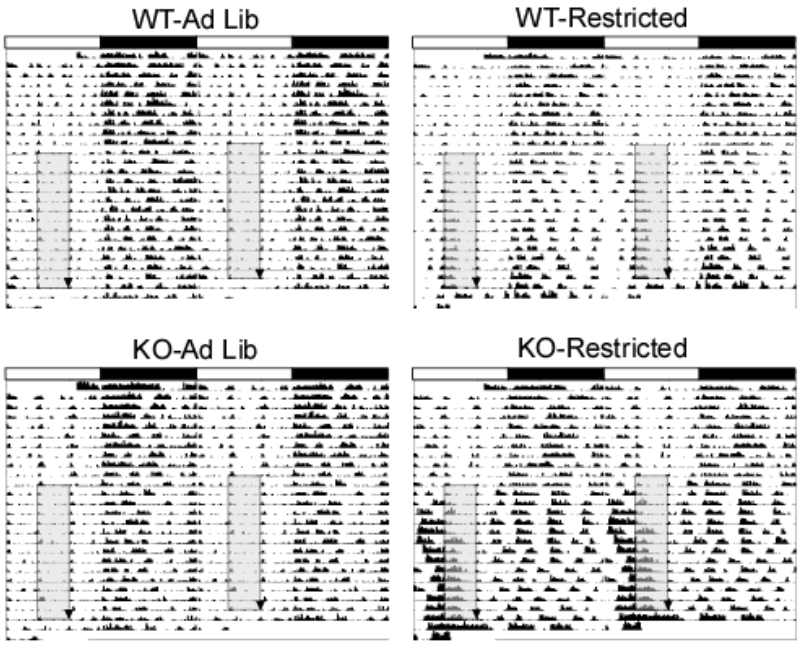

Figure 11

Enhanced FAA in 5-HT2CRKO mice persists after two days of food deprivation. Representative double-plotted actograms of wild-type (WT) and 5-HT2CRKO mice (KO) under *ad libitum* feeding conditions or a restricted feeding schedule. The start of total food deprivation is indicated by inverted triangles.

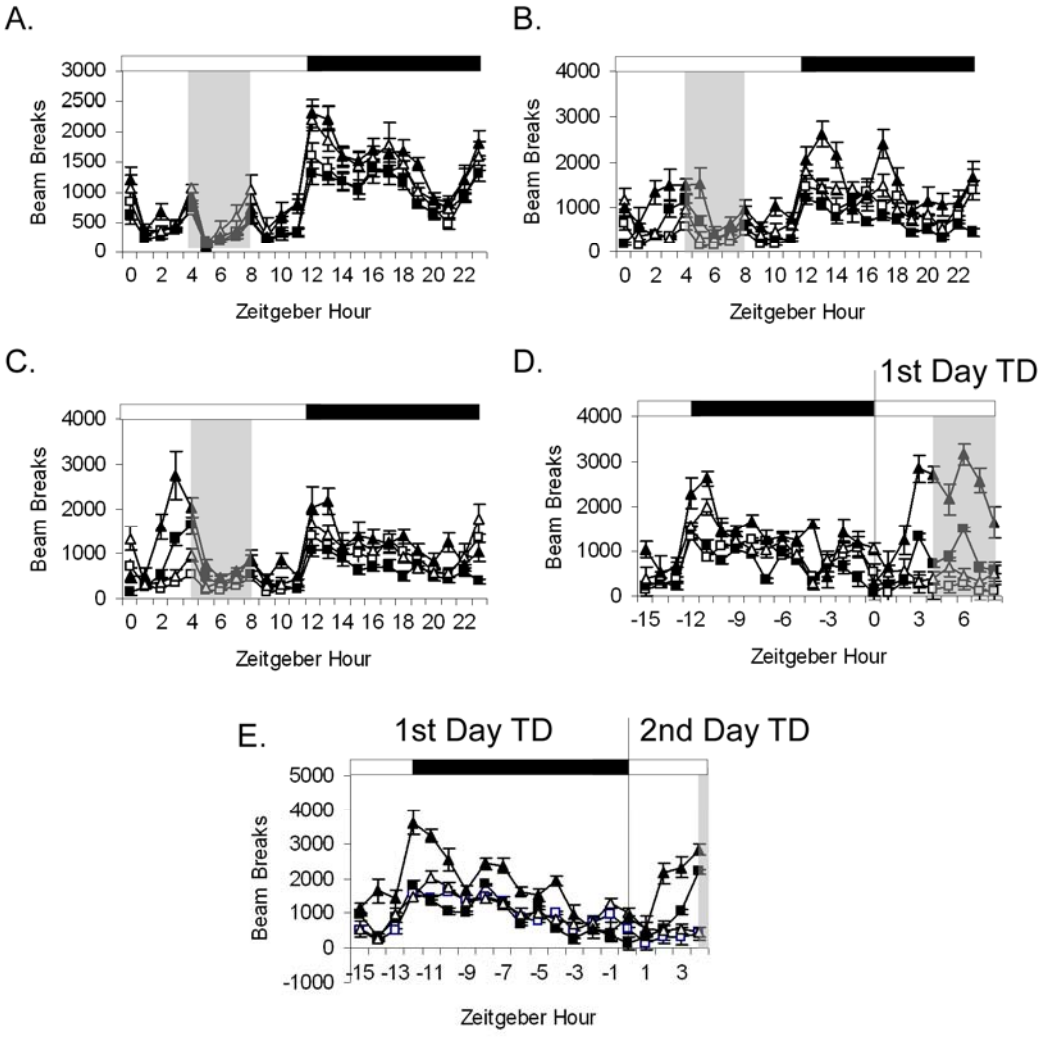

Figure 12

Enhanced FAA of 5-HT2CRKO mice persists after two days of food deprivation. A. baseline. B. first week of restricted feeding. C. second week of restricted feeding. D. First day of total food deprivation. E. Second day of total food deprivation.

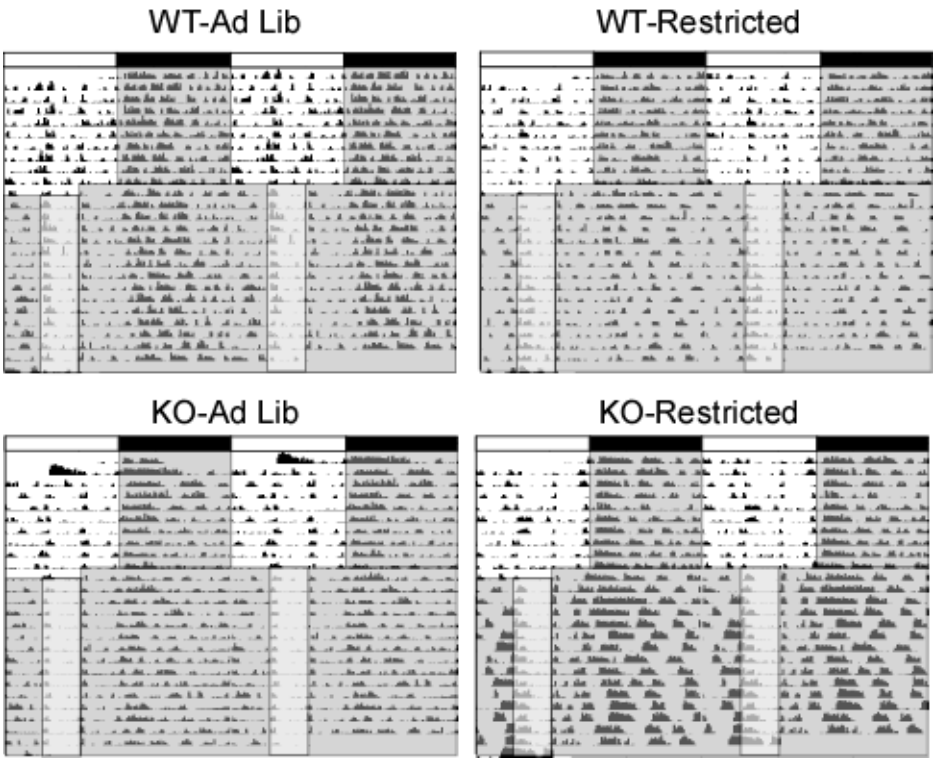

Figure 13

Enhanced FAA in 5-HT2CRKO mice is independent of light cues. Representative double-plotted actograms of wild-type (WT) and 5-HT2CRKO mice (KO) under *ad libitum* feeding conditions or a restricted feeding schedule.

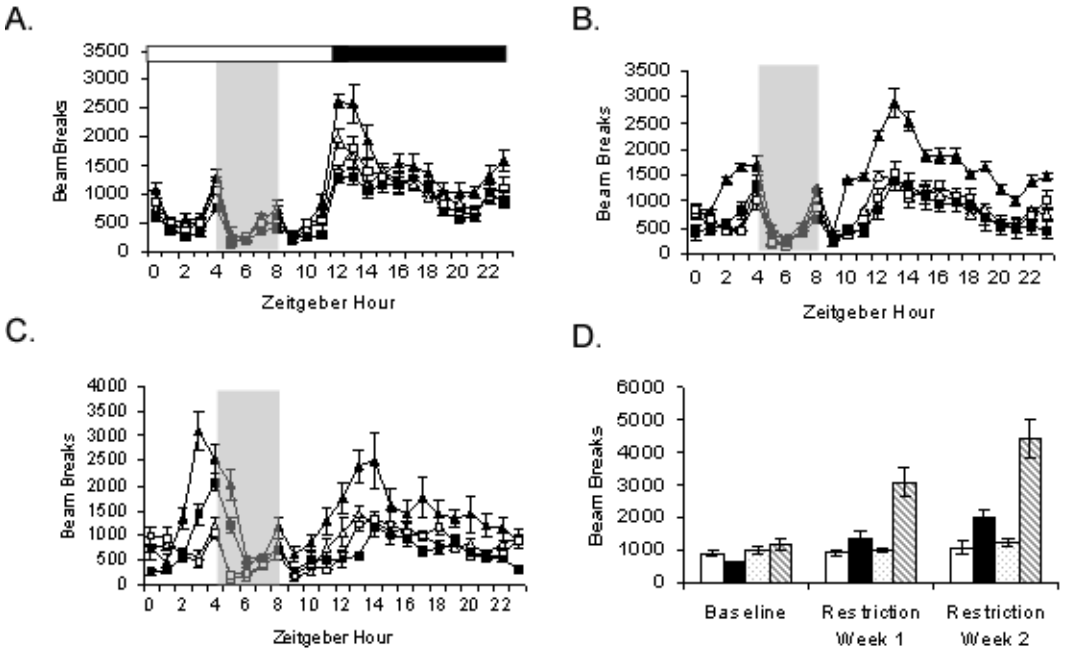

Figure 14

Enhanced FAA in 5-HT2CRKO mice is independent of light cues. 24-hour activity profile of WT (squares) and KO (triangles) mice assigned to either *ad libitum* (white) or *restricted feeding* (black) groups. Grey shading indicates the timing of the restricted feeding schedule (except in baseline conditions in which all animals are under *ad libitum* feeding conditions). A. baseline. B. first week of restricted feeding. C. second week of restricted feeding. D. Locomotor activity during the two hours preceding food availability. All values represent means ±S.E.M.

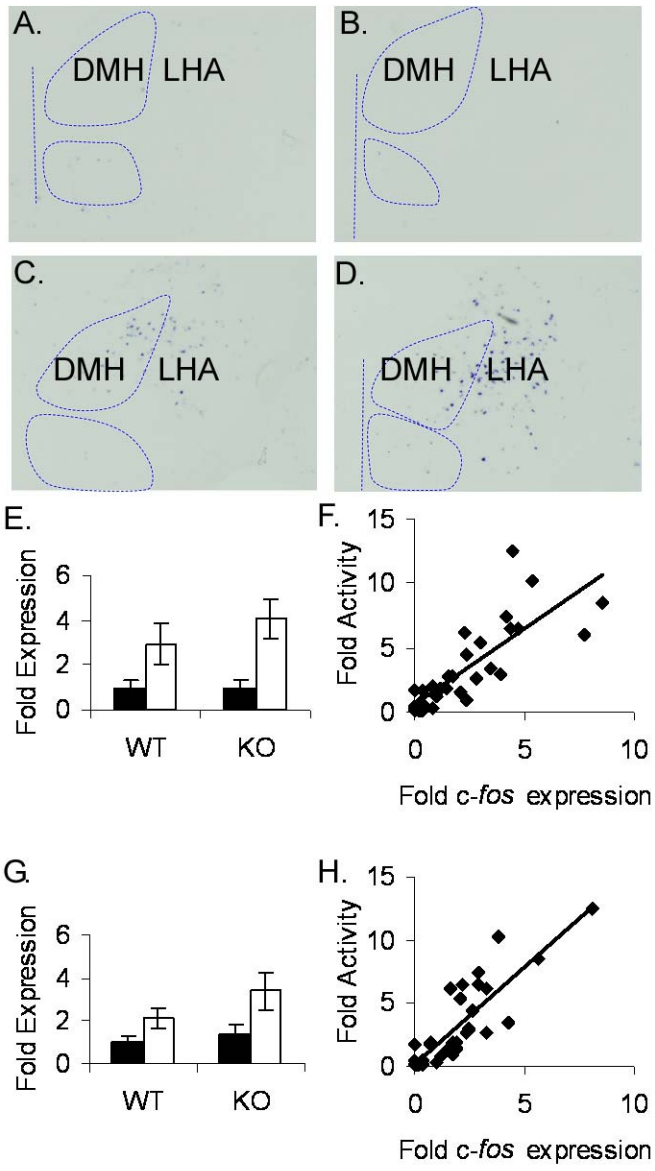

Figure 15

Representative photomicrographs depicting ZT4 c-*fos* mRNA in hypothalamus of A. WT-Ad Lib B. KO-Ad Lib C. WT-Restricted D. KO-Restricted mice. E. Quantitation of ZT4 DMH c-*fos* mRNA. F. Correlation of locomotor activity to DMH c-*fos* gene expression. G. LHA c-*fos* expression. H. Correlation of locomotor activity to LHA c-*fos* expression.

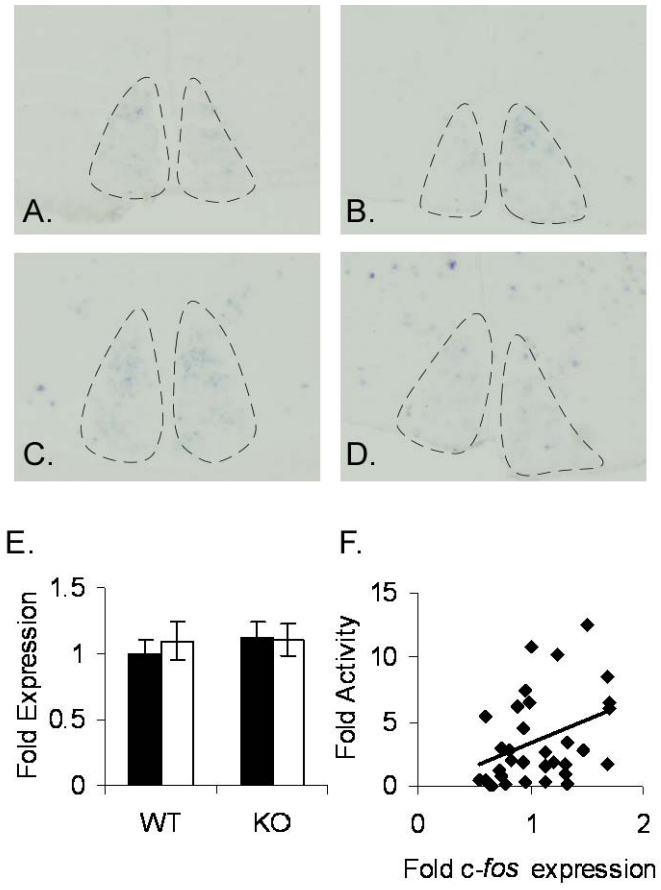

Figure 16

Representative photomicrographs depicting ZT4 c-*fos* mRNA in SCN of A. WT-Ad Lib B. KO-Ad Lib C. WT-Restricted D. KO-Restricted mice. E. Quantitation of ZT4 SCN c-*fos* mRNA. F. Correlation of locomotor activity to SCN c-*fos* gene expression.

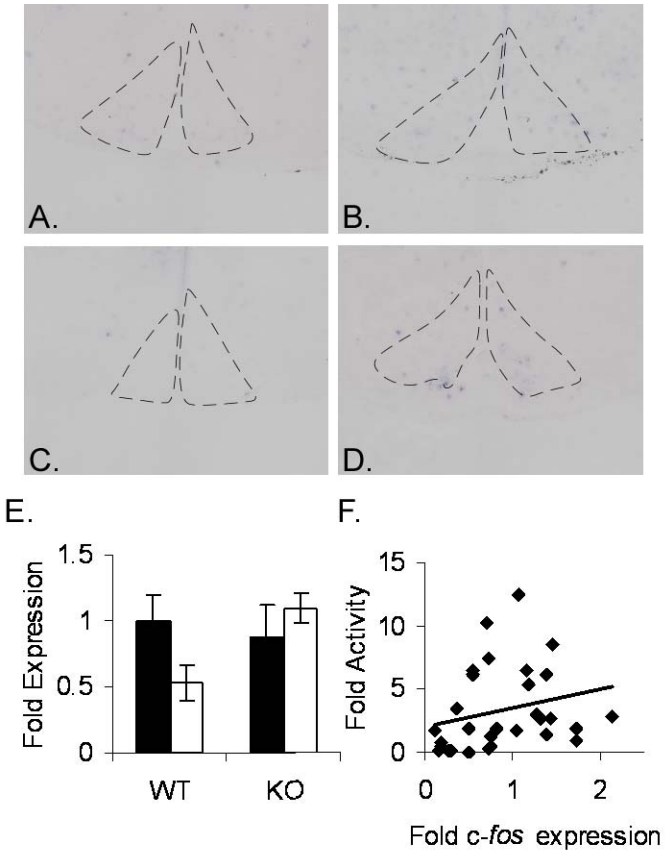

Figure 17

Representative photomicrographs depicting ZT4 c-*fos* mRNA in ARC of A. WT-Ad Lib B. KO-Ad Lib C. WT-Restricted D. KO-Restricted mice. E. Quantitation of ZT4 ARC c-*fos* mRNA. F. Correlation of locomotor activity to ARC c-*fos* gene expression.

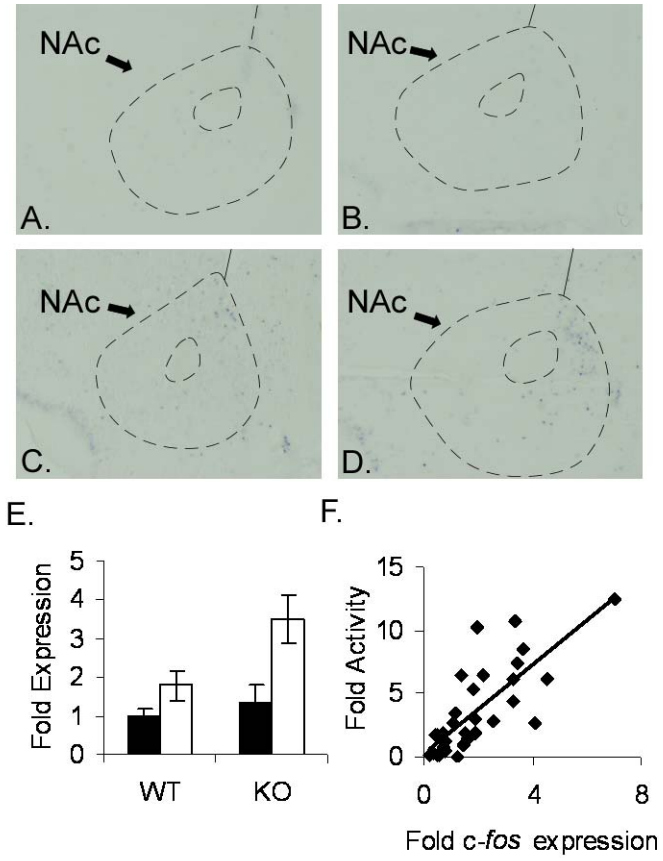

Figure 18

Representative photomicrographs depicting ZT4 c-*fos* mRNA in NAc of A. WT-Ad Lib B. KO-Ad Lib C. WT-Restricted D. KO-Restricted mice. E. Quantitation of ZT4 NAc c-*fos* mRNA. F. Correlation of locomotor activity to NAc c-*fos* gene expression.

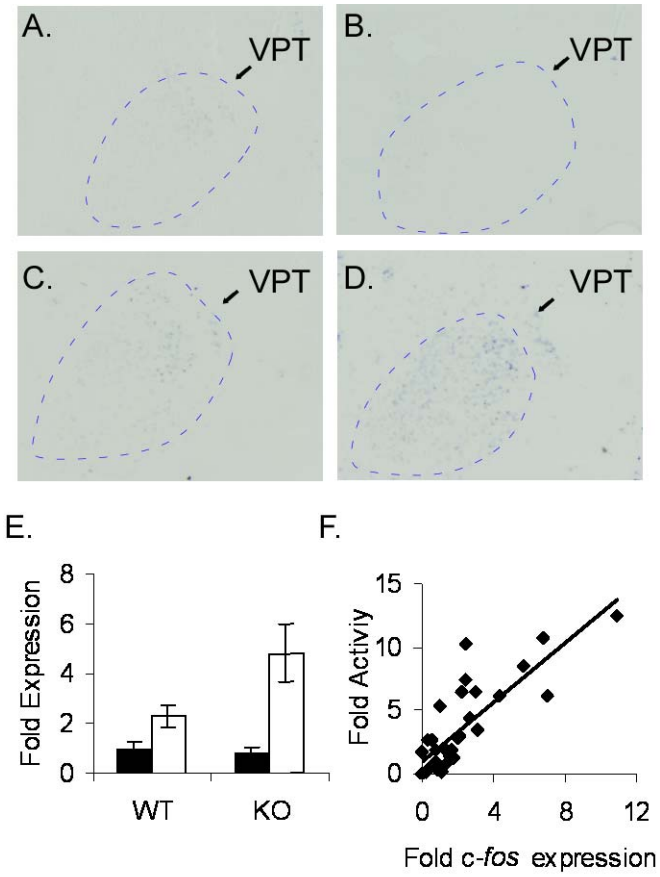

Figure 19

Representative photomicrographs depicting ZT4 c-*fos* mRNA in VPT of A. WT-Ad Lib B. KO-Ad Lib C. WT-Restricted D. KO-Restricted mice. E. Quantitation of ZT4 VPT c-*fos* mRNA. F. Correlation of locomotor activity to VPT c-*fos* gene expression.

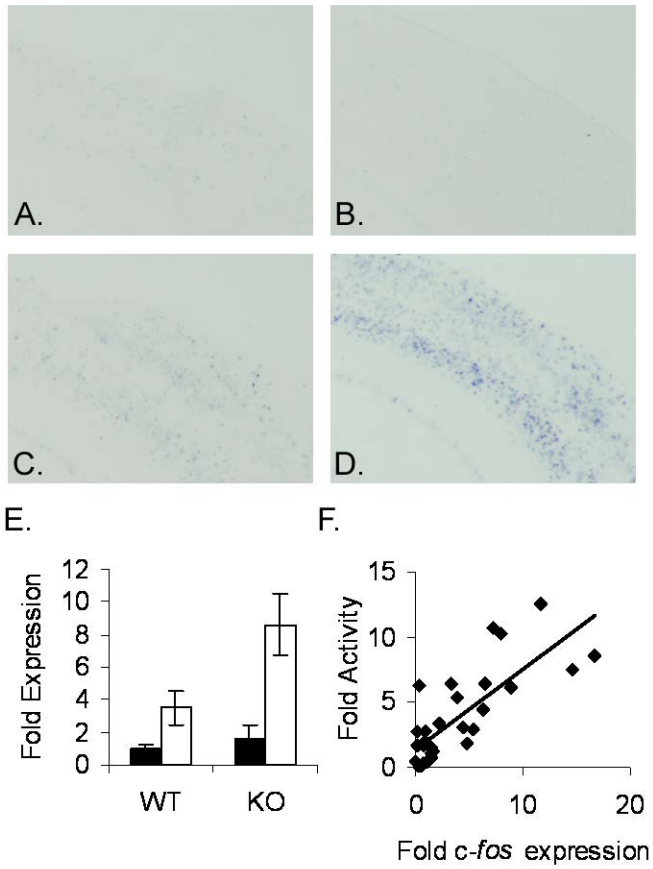

Figure 20

Representative photomicrographs depicting ZT4 c-*fos* mRNA in barrel cortex of A. WT-Ad Lib B. KO-Ad Lib C. WT-Restricted D. KO-Restricted mice. E. Quantitation of ZT4 barrel cortex c-*fos* mRNA. F. Correlation of locomotor activity to barrel cortex c-*fos* gene expression.

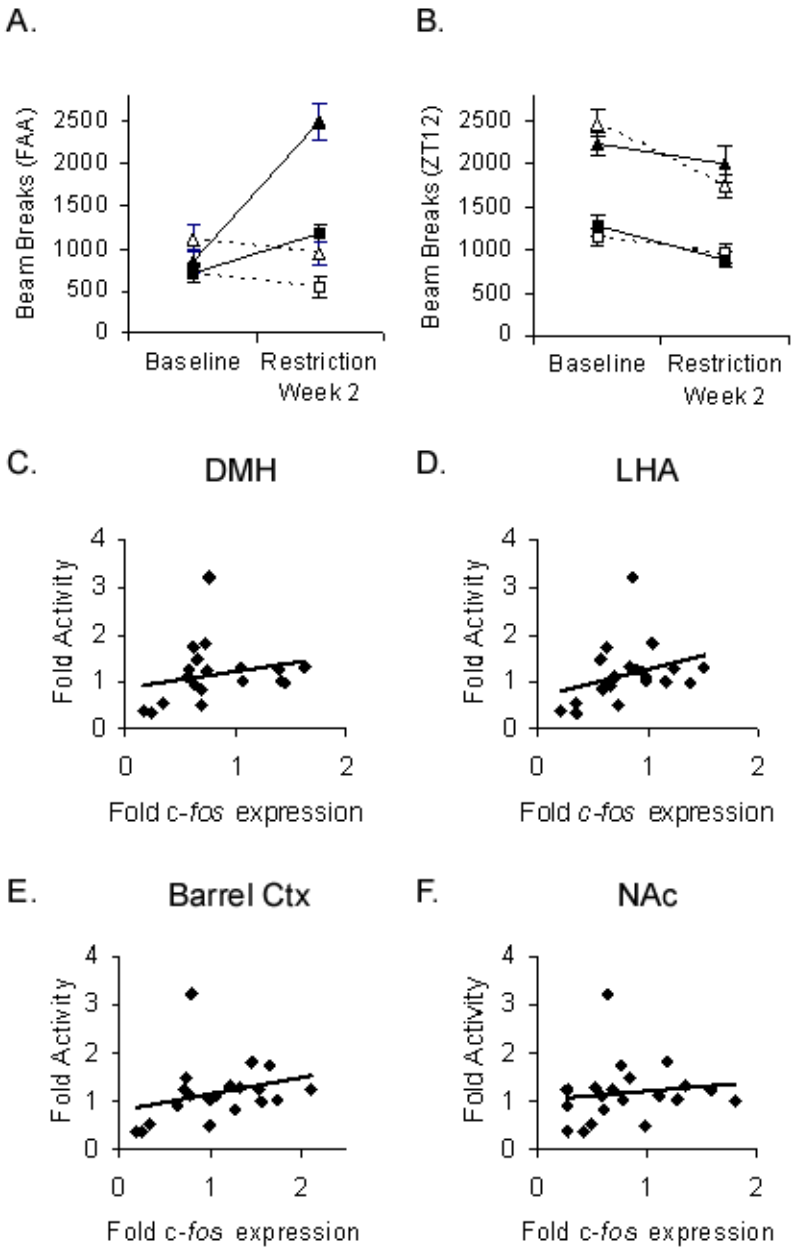

Figure 21

A. Comparison of FAA averaged over baseline and the second week of restricted feeding for WT (squares) or KO (triangles) assigned to *ad libitum* (white symbols) or restricted feeding (black) groups. B. Comparison of locomotor activity that occurs between ZT12-13. C-F. Correlations between ZT12-13 locomotor activity and ZT13 c-*fos* expression

Table 1: ZT4 c-*fos* expression in extrahypothalamic regions, without phenotypic effects.

	Ad Lib		Restricted		*p* value		
	WT	**KO**	**WT**	**KO**	**Geno**	**Feeding condition**	**Geno x feeding condition**
Retrosplenial Ctx	1.07±0.51	0.37±0.26	4.07±0.69	4.94±0.92	0.671	<0.001	0.561
Parietal Ctx	0.80±0.40	0.58±0.21	6.16±2.62	6.34±0.86	0.749	0.004	0.868
Piriform Ctx	2.65±1.20	2.78±1.08	6.59±1.87	7.08±1.70	0.839	0.018	0.906
Striatum	0.02±0.02	0.26±0.26	0.60±0.23	0.51±0.32	0.762	0.104	0.500

Chapter 4: Summary and Integration

Contribution of 5-HT2C receptors to the regulation of behavioral phase shifts to light

Chapter 2 provides a description of SCN-related circadian rhythm phenotypes in mutant mice that lack functional 5-HT2C receptors. These mutant mice exhibit normal patterns of locomotor activity in a standard LD cycle, normal free-running rhythms of locomotor activity in constant darkness, and normal induction of c-*fos* and *Per* genes in response to a phase-delaying light pulse. However, these animals exhibit marked impairment of light-induced behavioral phase delays, indicating an important contribution of the 5-HT2C receptor to the regulation of circadian phase shifts of locomotor activity to light.

Dissociation between light-induced gene expression and behavioral phase shifts to light

The acute response of the SCN to light is well-documented, and is characterized by the acute expression of c-*fos*, *Per1*, and *Per2* mRNA. c-*fos* mRNA peaks 30 minutes after entraining light pulses and rapidly degrades thereafter, while *Per1* and *Per2* mRNA peak 60-120 minutes after a light pulse (Yan and Silver, 2002). Our study is notable in that we report a manipulation that grossly impairs behavioral phase shifts to light without a corresponding impairment in light-induced SCN c-*fos* or *Per* expression. There are few reported dissociations between light-induced behavioral shifts and light-induced SCN gene expression, such studies may help elucidate downstream or independent mechanisms underlying light entrainment. 5-HT1A receptor knockout mice exhibit exaggerated phase advances to early morning light pulses but attenuated c-*fos* and *Per1*

expression to the same light pulse stimulus (Smith et al., 2008). Our findings, in combination with the 5-HT1A receptor KO observation described above, suggest the possibility that serotonin systems regulate long-term circadian phase shifts to light via events downstream or independent of light-induced SCN gene expression.

Compartmentalization of light-induced gene expression within SCN subdivisions

Based on the neuroanatomical organization of the SCN and other studies, people have proposed a two-component model of light entrainment, in which rapid resetting of core oscillators is communicated via efferent connections to the shell, causing an eventual phase shift of shell oscillators. Findings from several laboratories further suggest that SCN shell *Per1* and *Per2* expression mediate circadian phase advances and phase delays to light, respectively. For instance, one study demonstrated SCN core expression of *Per* genes in response to both phase-shifting and non-shifting light pulses. By contrast, light-induced *mPer2* SCN shell expression is associated with phase-delaying light pulses (Dardente et al., 2002; Yan and Silver, 2002), and *mPer1* SCN shell expression is associated with phase-advancing light pulses (Yan and Silver, 2002). In addition, SCN shell *Per* expression lags SCN core expression by roughly 60 minutes (Yan and Silver, 2002), which is consistent with the two-component model. Our gene expression results described in chapter 2 do not distinguish core and shell expression. However, in a preliminary analysis of core versus shell gene expression, we found light-induced expression in both subdivisions but to a lesser extent in the shell versus the core. Moreover, there were no phenotypic differences between WT and KO mice in either core or shell SCN gene expression. One likely possibility is that the time point chosen for our study was suboptimal for determining gene expression in the shell, as we sampled gene

expression prior to the reported peak time for shell expression. A more thorough analysis of gene expression at later time points would be required to more accurately determine whether 5-HT2C receptors impact light-induced gene expression in the shell.

Light-induced SCN protein expression following mRNA induction

The rapid induction of c-*fos*, *Per1*, and *Per2* mRNA is followed by a slower increase in FOS, PER1, and PER2 protein levels. In one study, a 15-minute light pulse applied at ZT14 induced FOS-immunoreactivity 1-2 hours after exposure, followed by increased PER1 immunoreactivity 9 hours later (Field et al., 2000). In a time-course analysis of FOS immunoreactivity following a CT16 light pulse in mice, FOS peaked 1-2 hours after exposure and was greatly reduced 4 hours after the light stimulus (Colwell and Foster, 1992). In another study, it was demonstrated that phase-delaying light pulses induced PER2 protein expression in both the core and shell 4 hours following the light pulse. By contrast, phase advancing light pulses induced PER1 protein expression in both core and shell 10 hours following the light stimulus (Yan and Silver, 2004). These studies demonstrate that PER1 and PER2 protein expression in the shell predicts light-induced phase advances and phase delays, respectively. Our study did not include an assessment of FOS or PER protein; therefore, it is possible that despite the lack of phenotypic differences in light-induced mRNA expression, there may yet be phenotypic differences in protein levels. However, it should be noted that to our knowledge, there have been no reports of dissociation between *Per* mRNA and lag protein levels.

What is the long-term SCN response to phase-resetting light stimuli?

The acute expression of *Per* genes appears to be necessary for behavioral phase shifts, as acute reduction of *mPer1* mRNA by antisense oligonucleotides significantly

impaired behavioral phase shifts to light (Akiyama et al., 1999). However, the dynamics of *Per* mRNA and protein expression beyond 2-10 hours following a light stimulus has not been well-characterized. To our knowledge, there is only one study that monitors *Per* gene expression >10 hours following phase-resetting light stimuli. This study demonstrates that rapid *Per1* induction by light was followed by a two hour phase delay in the subsequent molecular rhythm of *Per1* expression the following day, and the magnitude of the *Per1* phase shift is comparable to the magnitude of the eventual behavioral phase shift (Shigeyoshi et al., 1997). Furthermore, *mPer1* expression 60 minutes following the light pulse was assessed in both core and shell, and found mainly in the core (Shigeyoshi et al., 1997). These findings are consistent with the two-component model of light entrainment, in which core oscillators induce the eventual phase shift of shell oscillators to mediate long-term behavioral phase shifts. However, it should be noted that the assessment of *mPer1* expression the day following the light pulse did not distinguish between core and shell expression. Furthermore, multiple studies implicate a greater contribution of *Per2* to the regulation of light-induced phase delays compared to *Per1* (see above), and the dynamics of *Per2* expression beyond the first several hours following a phase-delaying light pulse has not been reported in the literature. Therefore, the mechanisms by which acute expression of these genes translate into long-term phase shifts of SCN pacemaker cells are poorly understood. A summary of SCN c-*fos*, *Per1*, and *Per2* dynamics in response to a phase-delaying light pulse is shown in Table 2.

What are the mechanisms underlying communication between SCN subdivisions?

The mechanisms by which core and shell oscillators are coupled are also not well-understood. It was determined that surgical knife cuts separating the dorsal from ventral disrupted cell synchronization in the dorsal SCN but did not impact synchronicity in the ventral SCN, indicating that inputs from the ventral SCN are necessary for synchronization of the dorsal SCN (Yamaguchi et al., 2003). But what is the signal that conveys communication from the core to the shell? One study revealed rapid phase resetting of ventral SCN to shifted light/dark schedules and slow resetting of the dorsal SCN, dependent on GABAergic signaling from the ventral region (Albus et al., 2005), suggesting a possible role for GABAergic neurotransmission in the regulation of core-to-shell communication.

The ventral core neuropeptides may also contribute to core-to-shell communication. In dispersed SCN cultured cells, VIP application induced phase shifts of AVP release in a manner similar to light pulses, with early evening VIP application inducing phase delays and early morning application inducing phase advances (Watanabe et al., 2000). In addition, it was found that SCN administration of the ventral core neuropeptides VIP and GRP mimicked the effects of a phase-delaying light pulse on behavioral rhythms and SCN electrophysiological activity (Albers et al., 1991). In another study, GRP SCN administration at CT16 mimicked the effect of a phase-delaying light pulse on behavioral rhythms and induced both *Per1* and *Per2* gene expression in the dorsal SCN. GRP-receptor deficient mice exhibited blunted behavioral phase shifts and blunted acute SCN gene expression responses to light pulses and GRP administration (Aida et al., 2002).

Synthesis and future directions

Taken together, our finding of impaired behavioral phase shifts to light, the absence of impaired acute SCN gene expression, and the expression of 5-HT2C receptors mainly in the retinorecipient core region of the SCN, have led us to hypothesize that 5-HT2C receptors facilitate light-induced behavioral phase shifts by influencing the coupling of light-sensitive core oscillators with light-insensitive shell oscillators. To formally test this hypothesis, it would be useful to conduct a multi-day time-course analysis of c-*fos*, *Per1*, and *Per2* gene expression studies in WT and KO animals subjected to a ZT16 light pulse, in order to assess the long-term dynamics of SCN gene expression in response to light. Furthermore, it would be useful to include neuropeptide marker analysis in this study to delineate core versus shell gene expression. In addition, to test whether 5-HT2C receptors within the SCN regulate circadian phase shifts to light, it would be useful to either eliminate 5-HT2C receptors in the SCN of WT mice (using conditional knockout approaches) or to selectively restore 5-HT2C receptors to the SCN of 5-HT2CR KO animals (using conditional rescue approaches).

Enhanced food-anticipatory activity in 5-HT2C receptor knockout mice

We also present data in Chapter 3 that reveals an enhancement of food-anticipatory activity (FAA) in 5-HT2C receptor knockout mice. Furthermore, our c-*fos* expression results demonstrate that the enhanced FAA of KO mice is correlated with enhanced activation of a subset of extrahypothalamic regions (the NAc, VPT, and barrel cortex) but that activation of the DMH and LHA, two hypothalamic regions strongly implicated in the regulation of FAA, is similar in restricted WT and KO mice. These results indicate a role for 5-HT2C receptors in the negative regulation of FAA, and strongly suggest that 5-HT2C-mediated regulation of FAA occurs at the level of

extrahypothalamic regions. However, our study leaves several questions open to future investigation. One important remaining question is: do 5-HT2C receptors regulate FAA via an FEO-efferent mechanism or do 5-HT2C receptors regulate FAA by regulation of the FEO? To address this question, it would be helpful to summarize the current understanding of the molecular and neural mechanisms of the FEO.

What is the role of clock genes in the regulation of food entrainment?

To help shed light on the molecular and neural basis of the food-entrainable oscillator (FEO), several studies have surveyed the impact of daytime restricted feeding schedules on the circadian oscillations of circadian clock genes in extra-SCN loci. A two-hour restricted feeding schedule shifted the peak of *Per1* expression from ZT18 to ZT12 in various corticolimbic structures, including the nucleus accumbens, hippocampus, lateral septum, and prefrontal cortex (Angeles-Castellanos et al., 2007). In addition, three-hour restricted feeding schedules were shown to phase shift rhythms of PER2 protein expression in the amygdala and dentate gyrus, with PER2 peaks occurring 12 hours after the scheduled feeding (Waddington Lamont et al., 2007). An unbiased survey of brain regions for restricted-feeding induced changes in rhythms of *Per1/Per2* gene expression revealed phase shifts of *Per1/Per2* rhythms in the striatum and cerebral cortex, and *de novo* rhythms of *Per* expression in three regions: the posterior DMH, area postrema, and nucleus of the solitary tract (NTS). The peak of *Per1/Per2* expression in these regions occurred at ZT7, coincident with daily food access. These rhythms persisted after two days of food deprivation in the DMH but not NTS or area postrema. Furthermore, refeeding after an initial fast caused the rapid induction of *Per1/Per2* expression in the posterior DMH (Mieda et al., 2006), a response that is similar to the

acute expression of SCN *Per* genes following an phase-shifting light pulse. The authors proposed that the *de novo* DMH *Per1/Per2* rhythm imposed by restricted feeding represents one of the molecular substrates of the FEO. This result, in combination with the targeted DMH lesion studies and BMAL rescue studies by Cliff Saper's lab, lends credence to the hypothesis that the DMH represents a key regulator of food entrainment, possibly by acting as the FEO (Gooley et al., 2006; Fuller et al., 2008).

The forebrain CLOCK paralog NPAS2 may also be involved in the molecular FEO, as *npas2* knockout mice exhibit significant impairments in FAA development in response to a restricted feeding schedule (Dudley et al., 2003). In a recent study, cortical *bmal* and *npas2* expression in WT mice exhibited shifted peaks that were coincident with the food-anticipatory time window (Sutton et al., 2008).

To address the question of whether 5-HT2C receptors regulate FAA at the level of the FEO, it would be useful to assess daily oscillations of the expression of circadian clock genes in WT and KO mice entrained to a restricted feeding schedule. Clock genes of particular interest would be *Per2* and *npas2*; regions of particular interest would be the DMH (proposed as the putative FEO) and regions demonstrating enhanced c-*fos* expression in restricted KO (NAc, VPT, barrel cortex). In addition, if shifted or *de novo* rhythms are induced by restricted feeding in multiple loci, it would be interesting to conduct a time-course assessment of the development and/or phase shift dynamics of these rhythms in WT and KO mice, by assessing circadian oscillations on the first day of restricted feeding and every few days afterwards. This experiment may provide information on whether the FEO acts as a distributed network of oscillators that entrains

at the same time or whether some oscillators may be driven by a subset of primary FEO's.

What is the impact of 5-HT2C receptors on food entrainment of other physiological rhythms?

In our study described in chapter 3, we assessed FAA as the main behavioral output of food entrainment, but did not assess other rhythms in WT and KO mice in response to daytime restricted feeding. In addition to inducing FAA, daytime restricted feeding schedules have been shown to induce anticipatory rhythms of body temperature and corticosterone secretion (Boulos and Terman, 1980). Furthermore, anticipatory corticosterone rhythms exhibit circadian limits of entrainment to scheduled feeding (Honma et al., 1984). Under a daytime restricted feeding schedule, peripheral oscillations of clock gene expression (such as lung, liver, heart, kidney, and pancreas) entrain to the restricted feeding schedule, while SCN rhythms (such as *Per1/Per2*) remained phase locked to the LD cycle (Damiola et al., 2000; Stokkan et al., 2001). Moreover, *Per1*-driven luciferase rhythms of the liver but not lung persisted after two days of food deprivation (Damiola et al., 2000; Stokkan et al., 2001). These results were used to support the hypothesis that restricted feeding resets the phase of peripheral oscillators independent of the SCN.

However, the relationship between peripheral food-entrained rhythms and the FEO remain a mystery. DMH lesions and DMH-targeted BMAL rescue (Gooley et al., 2006; Fuller et al., 2008) impact both anticipatory locomotor rhythms and anticipatory rises in core body temperature. Furthermore, mice that lack functional *Per2* do not

develop FAA or anticipatory body temperature rhythms in response to restricted feeding (Feillet et al., 2006b).

There are also examples of manipulations that reveal dissociations between behavioral and peripheral rhythms. Orexin knockout mice exhibit reduced FAA but normal anticipatory body temperature rhythms in response to restricted feeding (Kaur et al., 2008). Similarly, mice with genetic ablations of orexin neurons exhibited impaired FAA but normal phase shifts of liver clock gene expression in response to restricted feeding (Mieda et al., 2004). Taken together, these results suggest the possibility that an FEO (or network of distributed FEOs) in the CNS may regulate both behavioral and peripheral food-entrained rhythms, but that manipulations efferent to the FEO can impact behavioral and peripheral rhythms independently. Therefore, analysis of peripheral rhythms in 5-HT2CRKO mice (body temperature, corticosterone, or peripheral clock gene rhythms) may also help shed light on the question of whether these receptors regulate FAA at the level of the FEO or by acting on an FEO-efferent pathway.

What is the evolutionary advantage of negative regulation of FAA?

Another intriguing question raised by our study is: what would be the evolutionary advantage of mechanisms for the negative regulation of FAA? In our investigation of FAA in 5-HT2CRKO mice, we made the intriguing observation that while WT mice hold steady levels of FAA from the first to second week of restricted feeding, KO mice continue to increase their FAA even into the second week of restricted feeing. It is possible that if the restricted feeding schedule continued beyond two weeks, the FAA of KO would continue to rise at the expense of energy balance, eventually becoming so excessive that it leads to enhanced weight loss. This phenomenon would

closely resemble rodent models of anorexia nervosa known as “activity-based anorexia” or “starvation-induced hyperactivity”, in which locomotor activity to a severely restricted feeding paradigm becomes so intense that the animal loses body mass as a result of the excess energy expenditure from hyperactivity (Routtenberg and Kuznesof, 1967). The intriguing possibility that 5-HT2C receptors may act to prevent starvation-induced hyperactivity in times of limited food availability is supported by findings that 5-HT2C receptor agonists repress starvation-induced hyperactivity in rats (Wilckens et al., 1992; Pirke et al., 1993), and may have implications for the treatment of eating disorders.

Interaction between light entrainment and food entrainment systems for regulation of behavior

In addition to 5-HT2C receptor knockout mice, there are only a handful of reported gene manipulations that enhance FAA. Both mutations are in genes that are known regulators of SCN-entrainable circadian rhythms. The *clock* gene is a key regulator of the SCN-controlled circadian oscillator. Investigators found that *clock* mutant mice exhibited enhanced FAA in both LD and DD conditions (Pitts et al., 2003). Prokinectin 2 (PK2) is a CLOCK-controlled gene that encodes a diffusible protein, and has been proposed as the output effector of the SCN that synchronizes central and peripheral oscillators. PK2 knockout mice were found to demonstrate reduced rhythmicity of locomotor activity and a number of other physiological parameters in a standard LD cycle; however, they exhibit enhanced acquisition of food-anticipatory activity in response to a daytime restricted feeding schedule (Li et al., 2006). These results suggest the possibility that the FEO and LEO compete for control of behavior, and that in situations of reduced LEO functionality, the FEO may exert a stronger influence

on overt behavioral rhythms, resulting in enhanced FAA. One question that remains unresolved in the circadian field is: what are the mechanisms by which the FEO and LEO compete for behavior?

It is notable that 5-HT2CR knockout mice also exhibit deficits in light-entrained circadian rhythms and enhanced food-anticipatory activity. One hypothesis that ties together the two circadian entrainment phenotypes of KO mice is that 5-HT2C receptors may gate the relative access of the FEO and LEO to neural loci that control behavior, and activation of 5-HT2C receptors bias the "gate" towards the LEO for control of behavior, facilitating the expression of light-induced phase shifts and reducing the magnitude of FAA (Figure 22A). Let us now consider what would happen in the absence of 5-HT2C receptors (Figure 22B). Under normal *ad libitum* feeding conditions, the FEO and LEO are synchronized to each other. However, a phase-shifting light pulse would normally induce phase shifts of the LEO while the FEO remains in its original phase. In the absence of 5-HT2C receptors, the gate is biased towards the FEO, and the behavioral rhythm would be more phase-locked with the original rhythm, causing a reduction in the magnitude of the light-induced phase shift. Under a daytime restricted feeding schedule, the FEO synchronizes to the scheduled feeding while the LEO remains in its original phase. In the absence of 5-HT2C receptors, the gate is biased towards the FEO for control of overt behavioral rhythms, causing an enhancement in the magnitude of food-anticipatory activity. 5-HT2CR knockout mice may represent a useful genetic model to study the interaction between light-entrainable and food-entrainable circadian rhythms; further analysis of these mice may yield interesting insights into the mechanisms by which circadian rhythm entrainment influences the temporal dynamics of behavior.

Figure Legends

Table 2: Summary of c-*fos*, *Per1*, and *Per2* mRNA/protein dynamics in SCN subdivisions.

Figure 22: Hypothetical model of 5-HT2CR mediated regulation of circadian-entrained behaviors. A. 5-HT2CRs act on "gate" cells that control the relative access of the LEO (white oscillator) and FEO (gray oscillator) for control of behavior. These receptors bias the gate to allow greater control of the LEO on behavior, which would facilitate the expression of light-induced phase shifts, and reduce the magnitude of FAA. B. In the absence of 5-HT2CRs, the gate is biased to allow greater control of the FEO on behavior, reducing the expression of light-induced phase shifts and increasing the magnitude of FAA.

Table 2 Summary of c-*fos*, *Per1*, and *Per2* mRNA/protein dynamics in SCN subdivisions.

Time after LP	0.5 hours	1 hour	2 hours	4 hours	24 hours
SCN core	↑ c-*fos*	↓ c-*fos* ↑ *Per1* ↑ *Per2*	↑ *Per1*(PER1) ↑ *Per2*(PER2)	↓ *Per1*(PER1) ↓ *Per2*(PER2)	
SCN shell			↑ *Per2* ↑ PER2	↓ *Per2* ↑ PER2	

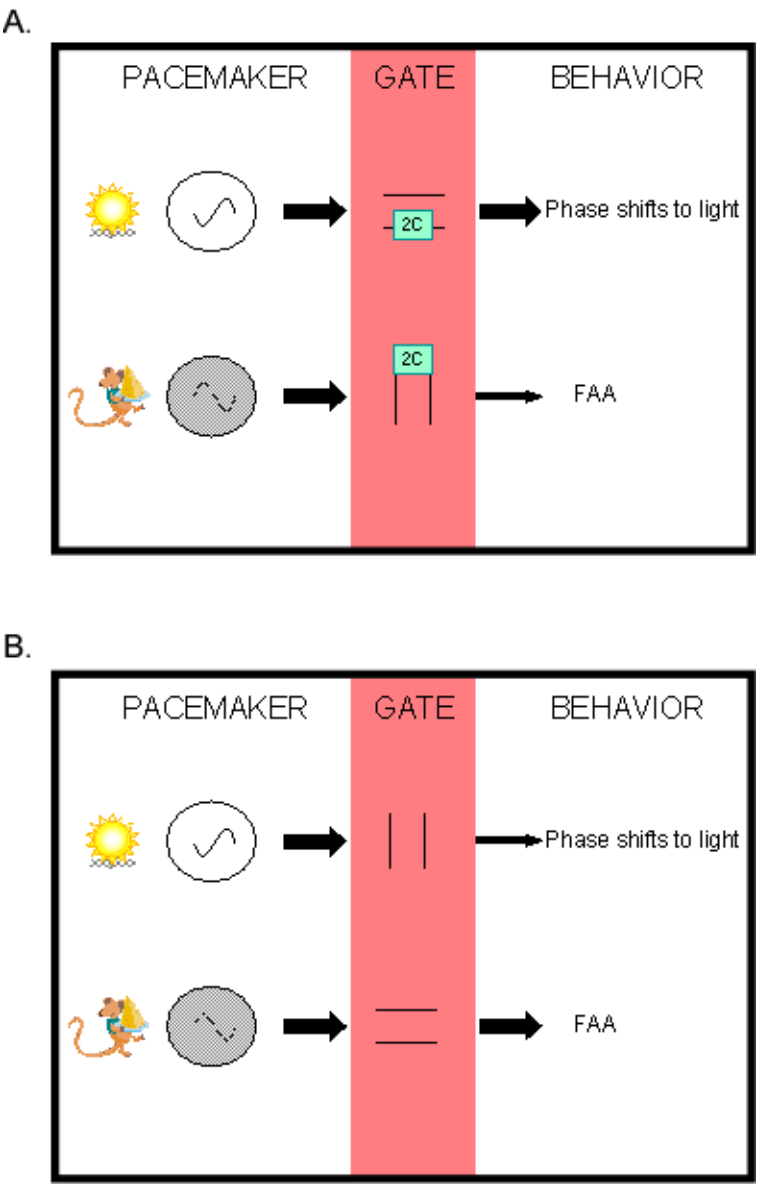

Figure 22

Hypothetical model of 5-HT2CR mediated regulation of circadian-entrained behaviors. A. 5-HT2CRs act on "gate" cells that control the relative access of the LEO (white oscillator) and FEO (gray oscillator) for control of behavior. B. In the absence of 5-HT2CRs, the gate is biased to allow greater control of the FEO on behavior, reducing the expression of light-induced phase shifts and increasing the magnitude of FAA.

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