Circadian Clocks for all Meal Times: Central and Peripheral Correlates of Anticipation to Multiple Daily Meals in Rats

by

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M.A. (Psychology), Simon Fraser University, 2010

Dissertation Submitted in Partial Fulfillment of the
Requirements for the Degree of
Doctor of Philosophy

in the
Department of Psychology
Faculty of Arts and Social Sciences

SIMON FRASER UNIVERSITY

Fall 2013

Approval

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Degree: **Doctor of Philosophy (Psychology)**

Title of Thesis: ***Circadian Clocks for all Meal Times: Central and Peripheral Correlates of Anticipation to Multiple Daily Meals in Rats***

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Abstract

Rodents anticipate a scheduled daily meal using food-entrained circadian oscillator(s) (FEOs). These oscillators are separate from the light-entrained master circadian pacemaker in the suprachiasmatic nucleus. However, their location in the brain or body remains uncertain, despite studies employing lesion, clock gene mapping, and clock gene knockout techniques, rats and mice can also anticipate two daily meals. Behavioural studies suggest that each bout of anticipation is controlled by a separate FEO, but a single FEO model has not been ruled out. A two-oscillator model predicts that rhythmicity at the tissue level will exhibit bimodality. To determine if anticipation of two daily meals is associated with unimodal or bimodal rhythmicity, we mapped clock gene expression in both the brain (in situ hybridization for *Bmal-1*) and periphery (RT-PCR for *Bmal-1, Rev-erb* α*, Per1* and *Per2*) from rats anticipating one or two daily meals provided in the light period (AM) and/or the dark period (PM). AM feeding, relative to PM feeding, inverted the phase of clock genes rhythms in the adrenal gland and stomach, while the 2-meal schedule was associated with unimodal rhythms with an intermediate phase. Similar results were obtained for plasma levels of the adrenal hormone corticosterone and the gastric hormone ghrelin. Within the brain the results were less clear. AM feeding, compared to PM feeding, shifted *Bmal-1* rhythms in the olfactory bulb and nucleus accumbens but not in other areas previously reported to exhibited shifting of clock gene rhythms by AM restricted feeding. The 2-meal schedule was again associated with intermediate phases in olfactory bulb and nucleus accumbens. In conclusion, assessment of clock gene rhythms in the brain and periphery has provided no definitive evidence for bimodality in rats anticipating two daily meals. Intermediate phases in the two meal group could represent a unique phase of entrainment of one FEO (or population of FEOs), or averaging of two populations of FEOs that are spatially intermingled within the tissues and brain regions examined. Further investigation using a technique that can localize clock gene expression within individual cells will be needed to resolve this issue.

Keywords: circadian*, Bmal-1, Per1, Per2, Rev-erb*α*,* adrenal gland, stomach, corticosterone, ghrelin, food restriction.

This dissertation is dedicated to my family.
I could never have attempted this endeavour
without your love and support.

Acknowledgements

I would like to thank my Senior Supervisor Dr. Ralph Mistlberger for teaching me to “think circadian”. Thank you for your excellent advise and mentorship throughout the years. Throughout graduate school there are many experiences that shake your confidence, times when you stumble and want to give up. Maksim Parfyonov, without your love and support I don’t know that I would have persisted through each of those hick-ups along the way. Thank you for always challenging me both professionally and personally. To my parents, you have always supported me in everyway possible. I am who I am today because of you both, Thank you. Jenny, Stacie, and Sean, each one of you are amazing. In your own ways you have each helped me more than you know. To members of the Mislt berger Lab past and present I am extremely grateful for all of your support and friendship. I would like to thank Andrea Smit, Mateusz Michalik, Teresa Dattolo, Christian Petersen, Curtis Hazelwood, and Ilya Pavlovski for your help on this specific project. Andrea thanks for being my sounding board through it all, it helped me a lot. To Dr’s Ian Webb and Elliott Marchant, I am indebted to you both for your incredible guidance.

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Glossary

After effects	Changes to the parameters of a rhythm that result from previous stimulation. For example in nocturnal animals housed in constant light or diurnal animals housed in constant darkness tau lengthens and shortens respectfully.
Amplitude	The difference between the peak and the trough or mean of an oscillation
Circadian	Refers to a rhythm that is endogenously generated and approximates the solar day (24h)
Circadian Oscillator	An oscillator that maintains nearly but not exactly 24 hour oscillations in the absence of any external cues.
DD	Constant dark
Entrainment	The process of entraining the period and phase of an endogenous rhythm with an environmental cycle
Food anticipatory Activity	The food seeking behaviour and physiology exhibited by animals under daily schedules of restricted food availability
Food-entrainable oscillator	A circadian oscillator that is entrained by timed food access.
Freerun	The expression of an endogenous circadian rhythm in the absence of an entraining stimulus (zeitgeber)
Hourglass Timer	A timer that times a specific interval and then stops. Each subsequent timed interval requires the timer to be reset. This type of timer is therefore not endogenous and dependent on external cues.
LD	Light/Dark, refers to the scheduled daily light dark cycle employed during an experiment. Typically LD is 12:12.
LL	Constant light
Period	The duration of one complete cycle of a rhythm. The period of a circadian rhythm is ~24h.
Phase	Any point in a cycle
Range of Entrainment	The range of variation in a zeitgeber that can entrain an endogenous rhythm
Slave Oscillator	An oscillator that is driven or entrained by another oscillator
Subjective Day	The phase of a circadian cycle in constant conditions during which the animal behaves as if it is day. For a nocturnal rodent, the subjective day is its rest period.
Subjective Night	The phase of a circadian cycle in constant conditions during which the animal behaves as if it is night. For a nocturnal rodent, the subjective night is its active period.

Suprachiasmatic Nucleus	A group of neurons (~18 000 cells) in the ventral hypothalamus directly above the optic chiasm which serves as the master light-entrained circadian pacemaker
Tau	Greek letter denoting the period of a circadian rhythm
Zeitgeber	An environmental stimulus that can entrain a rhythm
ZT (zeitgeber time)	A unit of time that is based on the period of the external cycle (zeitgeber).
ZT0	By convention, this corresponds to lights-on in a LD cycle with a 12h light period.
ZT12	By convention, this corresponds to lights-off in a LD cycle with a 12h light period.

Chapter 1.

Introduction

The behaviour and physiology of all mammals is controlled by cell-autonomous circadian clocks in the brain and most peripheral tissues. These clocks are synchronized to local environmental time by two primary cues, the daily light-dark (LD) cycle, and the timing of food intake. LD cues act on a master pacemaker, the suprachiasmatic nucleus (SCN), while cues related to food intake are thought to act on many other circadian oscillators independently of the SCN. A working hypothesis is that some of these non-SCN oscillators are responsible for generating daily rhythms of food anticipatory activity that animals exhibit prior to predictable daily meals. In natural environments, this circadian mechanism for predicting temporal regularities of food availability is likely critical for survival, especially for opportunistic feeders. In humans, the effects of meal timing on circadian rhythms have not been well characterized, but there is increasing evidence that the timing of food intake affects metabolism and body weight regulation, and that disrupted daily rhythms of food intake, as in shiftwork, may predispose to metabolic disorder and other negative health effects (Green et al., 2008). Almost all of the work on the effects of feeding on circadian rhythms in animals (primarily rats and mice) has utilized a single daily mealtime (typically a few hours of access) as a model for entraining circadian clocks. Animals and humans characteristically eat more than once per day, and when fed twice daily, rodents show behavioural anticipation of both meals, with properties consistent with circadian clock regulation of each bout of anticipation. How peripheral circadian oscillators controlling peripheral organ functions respond to two daily meals has not been well studied. The objective of this thesis is to examine circadian rhythms of clock gene expression in the brain and in two peripheral organs to gain insights into how the circadian system adapts to two daily meals at fixed times of day, to produce circadian rhythms of behavioural food anticipation, and to maintain coordination between peripheral physiology and daily rhythms of food intake. One

ultimate goal is to understand how animals anticipate daily events, such as food availability, that are critical for survival. Working out these mechanisms may also provide insights into how the circadian system in humans may be affected by altered mealtimes, as occurs in shift workers, frequent travelers, in various disease states and in accordance with some social customs (e.g., Ramadan).

1.1. Properties of circadian rhythms

The ability to coordinate behaviour and physiology with the solar day is an advantage conferred by the circadian system. The circadian system in mammals is comprised of a master circadian pacemaker in the suprachiasmatic nucleus (SCN) of the hypothalamus, and local circadian oscillators found in many other brain regions and most peripheral organs and tissues. Circadian oscillators are a property of single cells (the circadian clock machinery is intracellular, i.e., 'cell autonomous'), therefore pacemakers and local oscillators consist of populations of coupled 'clock cells'. For these oscillators to be useful they need to receive one or more entraining stimuli, integrate these stimuli and subsequently generate a rhythmic output. Entrainment is defined as the control of the period (duration of a cycle, represented by the Greek letter τ) and phase (discrete point in a cycle) of one oscillation by another. In the case of circadian rhythms the animals endogenous rhythm comes under the temporal control of an external cycle. The term given to an external cycle that can entrain a rhythm is "zeitgeber", which translates from German as "time giver". It is common to use zeitgeber time (ZT) to indicate time relative to the external cycle that entrains an endogenous rhythm and circadian time (CT) to represent time relative to the endogenous rhythm of the animal. The most prominent zeitgeber for most species is the light-dark (LD) cycle, but non-photic time cues such as locomotor activity, arousal, and food can also entrain circadian rhythms of behaviour and physiology (Mrosovksy and Salmon 1987; Stephan, 1999; Mistlberger, 1994).

The process of entrainment has been explained in two ways, formalized as parametric (Aschoff, 1964) and non-parametric models of entrainment (Pittendrigh 1976). The parametric model posits that zeitgebers entrain circadian rhythms by either speeding up or slowing down the rate of cycling of the endogenous clock. Evidence for

parametric entrainment comes from the observation that τ in nocturnal animals lengthens in constant light, while it shortens in diurnal animals under the same conditions (Aschoff, 1960). Also, τ in constant dark exhibits aftereffects of previous LD cycles; in nocturnal animals, entrainment to LD cycles that have a period (T) longer or shorter than 24h systematically alters τ in subsequent DD, such that τ is initially close to T, before lengthening or shortening toward the species average. This indicates that LD entrainment involves modulation of pacemaker frequency to match τ with T. Interestingly, diet can also have an effect on τ; mice fed a diet where 45% of calories come from fat have a longer tau than mice fed regular chow (Kohsaka et al., 2007). This could be due to an effect of high fat diet on daily activity levels, because activity levels can modulate τ in DD (Yamada et al, 1986).

The non-parametric model of LD entrainment posits that entrainment results from discrete phase shifts as a result of acute exposure to light. This model uses phase response curves to explain how animals compensate for the difference between τ and T of the zeitgeber (Pittendrigh and Daan 1976). A phase response curve is a plot of the size and direction of the phase shifts that occur following exposure to a zeitgeber at different phases of the circadian cycle. The phase response curve to light is bi-directional. Light exposure early in the subjective night will result in phase delays (i.e., the animal become active later the next day), while light exposure late in the subjective night will result in phase advances (the animal becomes active earlier the next day) (Pittendrigh, 1958; DeCoursey et al., 1960). In this way the circadian cycle becomes locked in position by the LD cycle, as slight deviations from the external LD cycle will result in a phase shift that offsets the difference between the animals τ and T (Daan and Pittendrigh, 1976).

Circadian rhythms are said to be endogenous and innate. The primary evidence that these rhythms are endogenous is that they persist in time-free, 'constant' environments. The first reported demonstration of this property came from a study of the sensitive plant *Mimosa pudica* in 1729 by the French scientist J.J. de Mairan. He was able to demonstrate that the rhythmic opening and closing of the plant's leaves persisted in the absence of an LD cycle, indicating that the mechanism underlying this rhythm must be internally generated. In the 20th century, the endogenous nature of these

rhythms was met with some skepticism as they appear to defy a central tenant of biology, namely the Q10 rule. For every 10 degree increase in temperature the rate of reactions within the body should approximately double. If this were true of the circadian clock then altering tissue temperature should change the rate of the circadian clock. The Q10 rule does not apply to the circadian clock, however, as it is temperature compensated. The mechanism for temperature compensation remains to be determined (Dibner et al., 2008).

Another way to demonstrate the endogenous nature of the circadian clock is to localize it in the organism. Early circadian biologists knew that LD cycles could act as a zeitgeber to the circadian system. In mammals, information about light reaches the brain exclusively via projections from the retina. Therefore, it seemed plausible that the circadian clock might lie somewhere within this projection system, and could be located by identifying the specific visual pathways that mediated LD entrainment. In a series of elegant experiments during the 1970's Robert Moore transected visual system pathways at various locations. Transections of the optic nerve anterior to the optic chiasm resulted in free-running rhythms, while transections of the optic tract posterior to the optic chiasm left behavioural rhythms and LD entrainment intact, suggesting that the pathway critical for entrainment exited the optic chiasm. The structure immediately above the optic chiasm, at the base of the brain in the anterior and medial hypothalamus is the SCN. In 1972, it was demonstrated that bilateral lesions of the SCN eliminated circadian rhythms of drinking and locomotor activity (Stephan and Zucker, 1972) and plasma corticosterone (Moore & Eichler, 1972). Evidence from electrophysiological recordings (Gillette and Green, 1982; Welsh et al, 1995), stimulation (Rusak and Groos, 1982), transplant (Lehman et al., 1987; Ralph et al, 1990) and bioluminescence reporter systems (Yamazaki et al., 2000) have established that the SCN are comprised of a population of intrinsically rhythmic, coupled circadian clock cells that function together as a self-sustaining, master light-entrainable circadian pacemaker.

1.2. Clock Genes

In 1988 the discovery of the tau mutant hamster (a hamster with a endogenous period of 20 or 22 hrs in homozygotes and heterozygotes respectively) provided a model

that confirmed the role of the SCN as a genetically determined master circadian pacemaker SCN transplants can re-instate behavioural circadian rhythms in SCN lesioned and therefore arrhythmic animals (Lehman et al., 1987) In animals receiving an SCN transplant the rhythm that is re-instated is that of the donor and not the host. Therefore if you were to lesion the SCN of a wildtype (WT) hamster (with a tau of 24hrs) and then transplant the SCN of a homozygous tau mutant hamster upon recovery, this WT hamster will now display the tau mutant phenotype of 20 hrs (Ralph et al., 1990). This observation not only identified the SCN as the master circadian pacemaker but also confirmed that the period of the rhythm is directly related to the genotype of the SCN neurons.

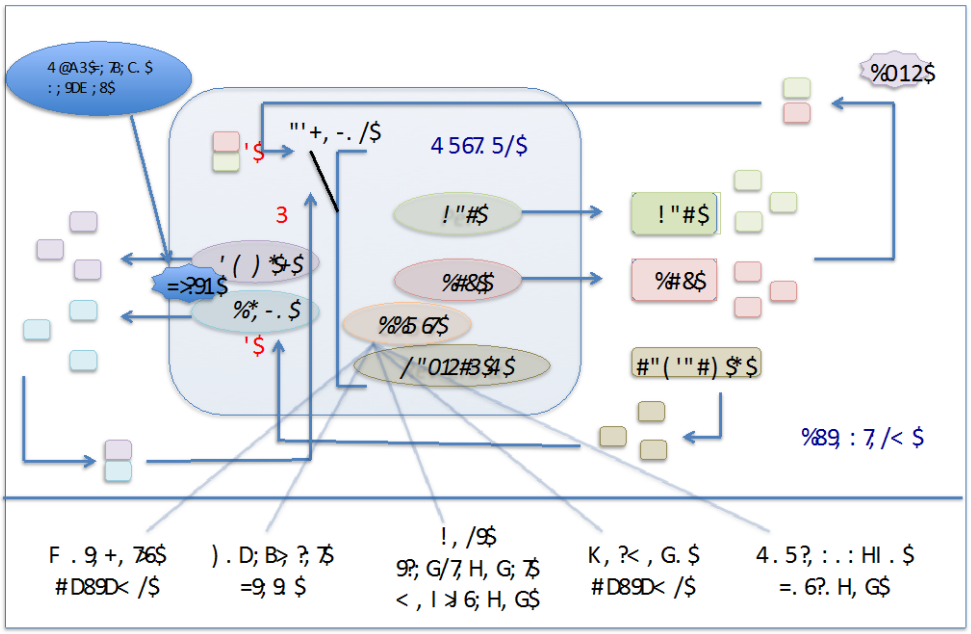
The first circadian clock gene, *Per* (for 'period') was identified in drosophila by Konopka and Benzer in 1971. In 1990 it was reported that *Per* gene expression and PER protein levels exhibited a circadian rhythm, with the peak in *per* gene expression occurring 6h before the peak of its protein product PER. This finding lead to a working model of the circadian clock, the transcription translation feedback loop (TTFL) (Hardin et al., 1990). This model posits that clock genes are transcribed in the nucleus and translated into proteins within the cytoplasm, and that clock proteins accumulate in the cytoplasm until they feedback on the nucleus stopping subsequent transcription of their own genes.

The first mammalian clock gene, *clock* was later identified by Vitaterna et al in 1994. Since then, a complex, TTFL-based circadian clock mechanism has been reasonably well worked out (Fig.1). At the core of the molecular clock are two interconnect feedback loops, one feed-forward and one feed-back. CLOCK and BMAL 1 are the feed-forward loop and form a heterodimeric transcription factor complex that activates the transcription of the *period (Per 1,Per 2, and Per 3)* and *cryptochrome (Cry1 and Cry2)* genes. The protein products of these genes PER1, PER2, and PER3 and CRY1 and CRY2, can form various hetero and homodimers which function as a repressive complex that eventually feeds back on the nucleus interacting directly with the CLOCK/BMAL complex to inhibit the transcription of their own genes (*Per* and *Cry*) (Lowrey and Takahashi, 2004). These two feedback loops are further augmented by other proteins including RORα, REV-ERBα, and casein kinase 1ε (CK1ε). These secondary pathways may be important for the fine tuning of the molecular clock. The

ROR enhancer elements RORα and REV-ERBα drive the rhythmic expression of *Bmal-1* by activating or inhibiting its transcription respectively (Adelmant et al., 2002). CK1ε phosphorylates PER before it can dimerize, thereby introducing critical time delays in the feedback loop to lengthen the cycle toward 24 h (Vielhaber et al., 2000; Lee et al., 2001).

Several links between the core circadian clock and metabolism have been identified. For example, the DNA binding activity of BMAL-1 and CLOCK is influenced by the NAD(P)+/NAD(P)H ratio, which is commonly referenced as a measure of metabolic state (Rutter et al., 2001). SIRT1, an NAD+ dependent deacetylase also modulates circadian clock gene expression. SIRT1 accumulation is needed for the high-magnitude transcription of *Bmal-1, Ror-alpha, Per2 and Cry1*. Additionally SIRT1 promotes the deacetylation of PER2, via binding influences on CLOCK and BMAL1 (Asher et al., 2008). In addition to this, posttranslational modifications are being recognized as important factors in circadian oscillations. Modifications of protein half -life and cellular localization via posttranslational mechanisms are beginning to be recognized as key modulators of the circadian system (Albrecht, 2012). Therefore, a more accurate schematic of the molecular circadian clock consists of transcription, translation and posttranslational modifications. These observations suggest that temporal organization of clock genes and metabolism are bi-directional and further implicate functional roles for temporal regulation of metabolic processes.

Figure 1. Visual representation of the molecular circadian clock (the TTFL).



The dimerization of the protein products of bmal-1 and clock serve as a transcription factor promoting the expression of Per's and Crys. As the protein products (PER and CRY) dimerize and accumulate in the cytoplasm they eventually feedback onto the nucleus suppressing Bmal-1. Rev-erbα is expressed in phase with Per and Cry and is also a repressor of Bmal-1. As levels of per and cry decline Bmal-1 is released from inhibition and the cycle repeats. CK1ε influences the time it takes for Per and Cry dimers to form and feedback on the nucleus, while SIRT1 also acts as an accessory loop influencing the binding of Bmal-1 and Clock and or Npas2. This rhythms within cells exert an affect on many processes within the body including metabolism, gene expression (of circadian clock controlled genes (CCG's)), hormone rhythms, neuropeptide secretion and behavioural state.

1.3. Food as a zeitgeber

The SCN controls the daily rest activity cycle by consolidating rest and activity into two distinct phases. Typically animals engage in their largest feeding bouts during periods of activity. As a consequence the SCN controls the phase when animals eat by controlling when they are actively foraging for food. In nocturnal animals this

corresponds to the dark phase (Rosenwasser et al., 1981) When food access is restricted to a few hours at the same time each day (restricted feeding, RF), rats will eat increasingly large meals at that time. If the RF schedule is maintained for a number of days, daily rhythms of behaviour and physiology are altered. These changes are mediated by food entrainable circadian oscillators (FEO) independent of the SCN.

Under normal LD conditions nocturnal rats are primarily active during the dark phase. In animals that become arrhythmic either as a result of SCN lesions or housing in constant light, circadian rhythmicity is lost and feeding becomes polyphasic. If food is then restricted to 2-3 hours at the same time each day, rats will begin to show a bout of activity in anticipation of the scheduled meal time, thereby re-instating rhythmicity in arrhythmic animals (Boulos et al., 1980; Stephan et al., 1979; Mistlberger 1992; Marchant and Mistlberger, 1997). This food anticipatory activity (FAA) is therefore SCN independent. FAA can also be readily observed in SCN intact animals. If food is available for a few hours only during the light period, the rest-activity rhythm becomes bimodal, with a bout of diurnal FAA and nocturnal activity at lights out. FAA can be measured by running wheel activity (Richter, 1922), general cage activity (Mistlberger and Rechtschaffen, 1984), food bin directed activity (Mistlberger and Rusak, 1988), lever pressing, or drinking (Boulos et al., 1980). FAA exhibits a number of properties that suggest it is the behavioural output of a circadian oscillator. First, FAA persists under constant conditions; if rats that are showing FAA are food deprived for 2 or more days in constant darkness or constant light, the animals will continue to concentrate most of their activity to the hours immediately before and during the usual meal time (Bolles and Moot, 1973; Boulos et al., 1980; Mistlberger, 1992; Stephan et al., 1979a;1979b). Second, if rats are taken off RF and placed back on ad lib food access and are subsequently food deprived a week later, FAA will re-emerge at a phase predicted by the previous meal time (Clarke ad Coleman, 1986; Coleman et al., 1982; Rossenwasser et al., 1984). Third, FAA displays a circadian range of entrainment; animals do not anticipate feeding schedules outside of the circadian range, which is somewhere between 22 and 31 hours (Stephan, 1981). Fourth, if the meal time is shifted, re-entrainment is gradual, again suggesting that a FEO that can only advance or delay a certain amount each day (Stephan, 1984; 1992). These properties distinguish circadian FAA from timing of short intervals in the seconds to minutes range, which exhibit

properties of an hourglass or stopwatch mechanism, as opposed to a self-sustaining oscillator.

1.4. Clock Genes and FAA

The role of clock genes in the generation of FAA rhythms is unclear. It is reasonable to predict that the molecular clock responsible for light-entrainable rhythms generated by the SCN is also responsible for generating food entrained circadian rhythms. Two observations suggest that this may not be true. First, the range of entrainment differs substantially between light entrained rhythms and food entrained rhythms in the same species. In the rat, LD entrainment is not stable to LD cycles beyond 23-25 hrs whereas food entrainment has been seen to feeding cycles ranging from 22-31 hrs (Stephan, 1981). This could reflect differences in the strength of LD and food as zeitgebers for clock genes, or it could reflect a difference in the molecular basis of food and light-entrainable oscillators. Differences could involve additional clock genes or post-translational modifications induced by changes in metabolic state. A second observation is the differential effect of heavy water consumption on light entrained vs. food entrained circadian rhythms. Heavy water consumption shortens the period of behavioural rhythms beyond the range that is light entrainable (Daan and Pittendrigh, 1976; Richter, 1977) yet has no affect on the phase of food-entrained rhythms (Mistlberger et al., 2001).

Aside from these behavioural observations numerous genetic mouse models have been used to investigate the role of clock genes in food entrainment using a loss of function approach (Bunger et al., 2000; Dudley et al., 2003; Pitts et al., 2003; Iijima et al., 2005; Feillet et al., 2006; Storch and Weitz, 2009; Pendergast et al., 2009). These studies are limited in several ways. First, there is the potential for compensatory mechanisms, such as the use of *Bmal2* in the absence of *Bmal-1* (Shi et al., 2010). Second, mice are not the best physiological model for testing the role of circadian clock genes in generating FAA. Mice have a higher basal metabolic rate and a smaller body size relative to rats and do not tolerate well the 48 hr food deprivation tests that are necessary to rule out hourglass or stopwatch mechanisms. Food deprivation tests longer than a day in mice often induce high levels of locomotor activity that persist until food is

provided or the mice collapse in exhaustion (Mistlberger et al., 2009; Storch and Weitz 2009; Hsu et al., 2010). This can make it impossible to determine if mice of a particular mutant genotype have a normal or defective FEO. Third, most clock gene perturbations result in changes in amplitude or duration of FAA (Chellet et al., 2009). However, changes in amplitude or duration of FAA could reflect changes to the FEO, to input pathways, or to pathways downstream from the FEO (Mistlberger, 2009).

Food anticipatory rhythms have been found to be surprisingly resistant to most perturbations of the molecular clock. The only single clock gene knock out that eliminates light entrained circadian rhythms is the *Bmal-1* null mutant (-/-). These animals are behaviourally arrhythmic in DD and show masked rest-activity cycles in response to LD (Bunger et al., 2000). When placed on RF, *Bmal*-1 null mutant (-/-) mice show robust FAA so long as the food restriction is implemented gradually, as they can become torpid in response to RF and are generally a precarious genotype to work with (Storch and Weitz, 2009; Pendergast et al., 2009).

The positive loop of the TTFL is comprised of heterodimers of BMAL and either NPAS2 or CLOCK. Unlike *Bmal-1* KO, single knock outs of either *Npas2* or mutation of *Clock* do not result in behavioural arrhythmicity (Dudley et al., 2003;Vitaterna et al., 1994). *Npas2* knockouts do show FAA when placed on RF, although the number of days required for the emergence of FAA is longer in KO's compared to WT's. In addition they eat less and maintain a lower body weight in comparison to WT's. However, once FAA has emerged the amplitude and duration of this anticipation is virtually indistinguishable between genotypes (Dudley et al., 2003). These findings suggest that either *Npas2* is not required for FAA or that *clock* can compensate for the loss of *Npas2*. *Clock* (-/-) *Npas2 (-/-)* double knockouts would be useful to address this question.

Homozygous clock mutant mice (*Clk/Clk)* display normal behavioural rhythms in LD, but become arrhythmic in DD. *Clk/Clk* mutant mice readily develop FAA under RF. As the RF schedule progresses they may display more FAA than WT controls (Pitts et al., 2003)

The negative limb of the TTFL is mediated by PER and CRY hetero and homodimers. Loss of either *Cry1* or *Cry2* produces alterations in τ. Double knockout of

Cry1 and *Cry2* results in a behaviourally arrhythmic animal (Van der Horst et al., 1999). When these animals are placed on RF FAA does emerge but again the development of FAA is delayed and the amount is reduced and more variable (Iijima et al., 2005). From these results it appears that the *Cry* genes are also not required for FAA. Loss of *Per1* or *Per2* also results in changes to the period of circadian rhythms. Loss of both *Per1* and *Per2* results in behavioural arrhythmicity. FAA differs between *per1/2* double knockouts and *Per2* ^{Brdm1} mutant mice. Storch and Weitz (2009) reported that double KO mice show FAA and therefore concluded that the per genes are not necessary for FAA. The *Per2* ^{Brdm1} mutant mouse has essentially normal circadian rhythms when fed ad libitum. However these mice exhibit no food anticipatory rhythms of behaviour or body temperature to meals scheduled during the usual rest phase of the light-entrainable circadian cycle (Feillet et al., 2006). Whether these mice can anticipate a daily mealtime in the usual active phase has not been assessed The mutated PER protein produced by these animals may prevent compensation by other genes, and thus may be more informative than a null mutation. Mice lacking all three *per* genes, the *Per1-/-, Per2-/-, Per3-/-* mouse appear rhythmic in LD, but locomotor activity appears ultradian in DD. Under RF in LD, FAA emerges, but FAA fails to emerge in DD. However, the triple per KO mice do appear to anticipate shorter T-cycles of food availability (every 21 hrs in this study) in DD. The authors suggest that in the *Per1-/-, Per2-/-, Per3-/-* genotype the period of the FEO is reduced to around 21 hrs (Pendergast et al., 2012).

The persistence of FAA rhythms in the absence of components of the known circadian TTFL loops suggests that there are additional molecular components to the loops that are activated by restricted feeding, or that there is another clock mechanism altogether. The non-transcriptional rhythms of peroxiredoxin discovered in red blood cells that are suggested to phylogenetically pre-date the known light entrained clock genes may be a good place to continue this search (O'Neil et al., 2001; Edgar et al., 2012).

1.5. Peripheral and Extra-SCN Central Clocks

In mammals, the first independent circadian oscillator to be discovered outside of the SCN was the retina. The retina exhibits a circadian rhythm of melatonin secretion

that can oscillate independent of the SCN under constant darkness (Tosini and Menaker, 1995). An SCN independent oscillator has also been identified in the olfactory bulb (Granados-Fuentes et al., 2004a; 2004b). Semiautonomous or slave oscillators (those oscillators that are reliant on an entrainment signal from the SCN or FEOs) have been localized in many brain regions, including the piriform cortex (Honma et al., 1998; Wakamatsu et al., 2001), habenula (Zhao and Rusak, 2005; Guillding et al., 2010), central nucleus of the amygdala (Lamont et al., 2005), basal nucleus of the amygdala, bed nucleus of the stria terminalus (Amir et al., 2004), nucleus accumbens (Angeles-Castellanos et al., 2007), paraventricular nucleus of the hypothalamus (Abe et al. ,2002) and paraventricular nucleus of the thalamus, arcuate nucleus (Kriegsfeld et al., 2003), dorsal and medial raphe nuclei (Abe et al., 2002), hippocampus (Wakamatsu et al., 2001), and cerebellum (Mendoza et al., 2010). For review, see Guilding and Piggins, 2007.

Early indications of circadian oscillations in other organs can be traced back to 1972 when it was reported that rat liver suspension exhibits a circadian rhythm in oxygen consumption (Langer and Rensing, 1972). Since then nearly every organ in the body has been found to exhibit circadian rhythmicity, with the exception of some rapidly differentiating tissues such as the thymus and the testes (Alvarez et al., 2005). These oscillators are defined as slave oscillators, as they are dependent on entrainment signals from the SCN or food for maintained rhythmicity. In other words in culture these clocks will desynchronize in the absence of a zeitgeber.

Dibner et al (2010) suggest 3 possible reasons for why peripheral clocks evolved; 1. for the anticipatory activation of metabolic pathways to optimize food processing, 2. to limit metabolic processes with adverse side effects to time periods when they are needed, and 3. for the temporal sequestration of chemically incompatible reactions within a tissue to different times of day (e.g., the antiphasic relationship of glycogen synthase and glycogen phosphorylase within the liver). If all the rhythmic functions in the periphery required independent timing signals from the SCN there would need to be many such signals, as different tissues and organ functions exhibit differently phased rhythms. The existence of peripheral oscillators simplifies the number of signals that are necessary. In principle, the existence of peripheral oscillators reduces the number of signals to one, since as long as the organ itself remains synchronized to the rest of the

circadian system, all the other rhythmic events within that tissue could be phase aligned to the rhythm set by that one signal (Schibler et al., 2003). However, it appears that different tissues are differentially sensitive to different signals, so there are likely to be multiple signals.

Most cells in the body contain the circadian molecular machinery (Balsalobre et al., 1998). In addition to this, microarray studies suggest that 3 to 10% of the rat genome is rhythmic (Akhtar et al., 2002; Panda et al., 2002; Storch et al., 2002; Hughes et al., 2009). Of the identified rhythmic genes a large number are involved in biosynthetic and metabolic processes, including glycolysis and gluconeogenesis, cholesterol and lipid metabolism, oxidative phosphorylation and detoxification pathways (Panda et al., 2002). The rhythmic expression of these genes varies by tissue, and this tissue specific variation suggests a functional role allowing each tissue to carry out its unique function (McCarthy et al., 2007). The signal that induces this rhythmicity in the periphery is unclear. Observations from SCN lesioned animals that subsequently received SCN transplants suggest that there must be multiple entrainment signals to peripheral clocks, as SCN transplant was sufficient to restore rhythms in the liver and kidney, but not in the heart, spleen or adrenal (Guo et al, 2006). It is possible that rhythms in the liver and kidney are restored secondary to recovery of daily rhythms of eating and drinking. In fact, food intake does appear to be a powerful zeitgeber for peripheral clocks, as RF entrains peripheral clocks to meal time despite the presence of the LD cycle and an SCN that is oscillating in antiphase to the peripheral tissues (Damiola et al., 2000). The correlates of feeding that act as zeitgebers remain unclear, but candidates include eating-induced thermogenesis, hormones such as glucocorticoids, ghrelin and insulin, and ingested nutrients such as glucose. An elegant study by Sassone-Corsi's group showed that mouse embryonic fibroblasts that were implanted subdermally could be entrained by feeding. This provides clear evidence that peripheral tissues can be entrained by a systemic signal, such as a blood borne factor or body temperature (Pando et al., 2002).

1.6. Hormones

Hormones may be involved in the entrainment of peripheral and central neural clocks. The secretion of many hormones is tightly linked to feeding and fasting rhythms. In animals on RF, the phase of hormone secretion becomes entrained to meal time instead of the LD cycle. The endocrine cells of the gastrointestinal tract express about 30 genes that encode peptide hormones that can be posttranslationally modified into about 100 bioactive peptides (Rehfeld, 1998). These include ghrelin, leptin, cck, glp-1, npy, secretin, gastrin, insulin, and melanocortin. It is of note that many of these gastrointestinal peptide hormones are also produced within the hypothalamus and receptors for many of them are widely distributed throughout the central nervous system (Kojima et al., 1999; Cowley et al., 2003; Nicholson et al., 1983).

Daily rhythms in hormone secretion may act as input signals to food entrained oscillators responsible for FAA, or they may directly drive rhythms of FAA. If they directly drive FAA, then the hormonal rhythm must be shifted by restricted feeding schedules, and must persist during total food deprivation tests. To interpret food shifted hormonal rhythms it is important to differentiate between entrained circadian release of hormones and hour glass mechanisms that can produce daily variations without involvement of a circadian clock (Mistlberger, 2009). In the case of an entrained circadian rhythm, the phase of hormone secretion needs to be examined on a day of total food deprivation in the absence of any temporal cues. If the hormone continues to peak at the same time of day despite the omission of a meal then it is not generated by a correlate of eating itself and is likely to be endogenously generated. The key is what happens to the secretion of the hormone when the next scheduled mealtime approaches after a meal was omitted. If the hormone rhythm is generated by a circadian clock, it will rise (or fall) at the expected mealtime, whereas if it is generated by an hourglass clock, it will remain constantly low or high, as no resetting stimulus has occurred.

An instructive example is provided by analysis of the hormone ghrelin, Peripheral ghrelin can stimulate activity and food intake. Ghrelin is secreted by oxyntic gland cells in the stomach, which express circadian clock gene rhythms entrainable by daily feeding schedules (Le Sauter et al, 2009). Plasma ghrelin rises in anticipation of a predictable mealtime in various species. This makes a strong circumstantial case for ghrelin as a

driver or entrainer of FEOs responsible for behavioural FAA. However, if a meal is omitted, active ghrelin levels drop and remain low, whereas the daily rhythm of FAA continues to cycle for 4-5 days in rats. Also, ghrelin KO mice can anticipate daily meals (LeSauter et al., 2009; Blum et al., 2009; Szentirmai et al., 2010; Gunapala et al., 2011). Ghrelin is secreted by FEOs in the stomach, but production of the hormone has properties of hourglass regulation, and it is not required for FAA rhythms.

1.7. The effects of restricted feeding on central oscillators

One approach to identifying the FEO has been to examine FAA in rats and mice with lesions to discrete brain regions. To date, no discrete brain lesion has eliminated FAA. The areas tested by the lesion approach include the ventromedial hypothalamus and lateral hypothalamus (Mistlberger and Rechtschaffen 1984), paraventricular nucleus of the hypothalamus (Mistlberger and Rusak, 1988), dorsomedial hypothalamus (Landry et al, 2006, 2007, 2011; Moriya et al, 2009; Acosta-Gavin et al 2011), hippocampus and nucleus accumbens (Mistlberger and Mumby, 1992), neocortex (Mistlberger 1994), arcuate nucleus (Mistlberger and Antle, 1999), parabrachial nucleus (Davidson 2000), area postrema (Davidson et al., 2001), olfactory bulbs (Davidson et al., 2001), tuberomammiliary nucleus (Landry et al,), PVN of the thalamus (Landry et al., 2007), and infralimbic cortex (Racabarren et al 2005). Taken together, a distributed network of FEO's may explain why discrete lesions have not been effective at identifying the elusive FEO.

Another approach is to examine changes in the rhythms of clock gene expression in extra SCN structures. Under RF, rhythmicity of clock gene expression may be induced in regions that were not previously rhythmic under LD, presumably via cues associated with feeding or metabolic state. In other words, there may be regions within the brain or periphery that are exclusively entrained by an output of the FEO(s) during RF. Alternatively, clock gene expression that was entrained by the LD cycle may shift when the animal is put on an RF schedule. This second possibility has been observed in many different brain regions in animals on RF during the midsubjective day. Studies of clock gene expression within the rodent brain under RF have produced results that vary

depending on the gene (e.g., *Per 1* vs. *Per 2*), the protein products (e.g., PER1 but not PER2 is located to a region), the species (rats vs. mice), and the lab.

Feillet and colleagues (2008) reported rhythmic oscillations that differed across brain regions in mice. Some regions were PER1 expressing only, some were PER2 expressing only, and some were both PER1 and PER2 expressing. PER1 expression was observed in the anterior piriform cortex, dorsal medial hypothalamus, and dorsal striatum. Rhythmic oscillations of PER2 were observed in the ventral medial hypothalamus, paraventricular nucleus of the hypothalamus, and basal nucleus of the amygdala. PER1 and PER2 expression was rhythmic in the hippocampus, central nucleus of the amygdala, paraventricular nucleus of the thalamus, and the arcuate nucleus. In mice maintained on restricted feeding schedules providing only 66% of normal daily caloric intake, PER1 was advanced by 3h in the anterior piriform cortex. and the amplitude was increased without a phase change in both the dorsal medial hypothalamus and the dorsal striatum. In structures expressing PER2, 9 h and 7 h phase advances were observed in the paraventricular nucleus of the hypothalamus and ventral medial hypothalamus, respectively, while the basal nucleus of the amygdala exhibited a bimodal rhythm. In structures expressing both PER1 and PER2 restricted feeding increased the amplitude of PER1 without altering its phase in the arcuate nucleus, paraventricular nucleus of the thalamus, and central nucleus of the amygdala. PER2 expression was advanced by 5h in the arcuate nucleus, and by 6h in the paraventricular nucleus of the thalamus and the central nucleus of the amygdala. Taken together, PER1 amplitude tends to be increased without changes in phase, while PER2 appears to be advanced by around 6 h by restricted feeding except in the hippocampus.

Angeles-Castellanos and colleagues (2007) examined both C-FOS, a correlate of neuronal activation, and PER1 proteins in response to RF in limbic forebrain, amygdala, thalamus and hippocampus of rats using immunocytochemistry. C-FOS was found to increase prior to mealtime in all structures examined with the exception of the hippocampus. In the ventral striatum the amplitude of PER1 increased and the peak was advanced by 6 h from ZT18 to ZT12 (where ZT0 is lights-on by convention in a 12:12 LD cycle). This same pattern was also observed in the hippocampus, while in the lateral septum the peak was shifted from ZT18 to ZT12, but the amplitude of the PER1 rhythm was decreased rather than increased. They did not observe statistically significant

rhythms in the amygdala under ad lib conditions. Under RF the amount of PER1 in the amygdala increased but rhythmicity was not induced. In the bed nucleus of the stria terminalus, the amplitude of PER1 was altered but there was no significant difference in the phase of the PER1 oscillation between RF and ad-lib fed animals. In the paraventricular nucleus of the thalamus RF advanced the peak of the oscillation to ZT6 and increased the amplitude. The prefrontal cortex exhibited no change in phase but was increased in amplitude. The authors comment that these findings are indicative of activation within the limbic system suggesting a high level of behavioural arousal and motivation which is necessary for foraging.

Amir and colleagues have investigated the rhythmic expression of PER2 within the amygdala and associated limbic structures in rats. They have demonstrated that the PER2 rhythm differs between regions of the amygdala, with the oval nucleus of the bed nucleus of the stria terminalis and the central nucleus of the amygdala being phased similarly to the SCN while the basal nucleus of the amygdala and also the hippocampal dentate gyrus of the hippocampus are in antiphase. The rhythm in the bed nucleus of the stria terminalus and central nucleus of the amygdala is eliminated by lesions of the SCN, whereas unilateral lesions of the SCN result in ipsilateral elimination of the rhythm in the central nucleus of the amygdala and bed nucleus of the stria terminalus without affecting the contralateral side. This indicates that oscillations within these regions are dependent on the SCN. Furthermore, adrenalectomy eliminates PER2 rhythms in the central nucleus of the amygdala and bed nucleus of the stria terminalus without altering the rhythm in the basal nucleus of the amygdala or dentate gyrus of the hippocampus. PER2 rhythms in the bed nucleus of the stria terminalus and the central nucleus of the amygdala can be rescued in adrenalectomized animals by administering corticosterone in the rats' drinking water, but not by steady release pellets (Seagall et al 2006). This route of administration results in a circadian pattern of corticosterone levels since there is a circadian rhythm of water intake. Therefore it appears that corticosterone replacement must be rhythmic to re-instate PER2 rhythms in the central nucleus of the amygdala and bed nucleus of the stria terminalus.

Under restricted feeding, PER2 rhythms shift in the central nucleus of the amygdala, the bed nucleus of the stria terminalus the basal nucleus of the amygdala and the dentate gyrus of the hippocampus, but not in the SCN, therefore under RF the PER2

rhythms in the bed nucleus of the stria terminalus and the central nucleus of the amygdala can dissociated from the rhythm in SCN (Verwey et al., 2007, Waddington-Lamont et al., 2007). Studies examining the effect of restricted access to a palatable meal on an ad lib background (palatable treat, PT) vs. restricted access to a palatable treat in the absence of ad lib chow (restricted treat, RT) suggest that the amygdala is responsive to signals conveying alleviation of a negative energy balance and not the rewarding properties of palatable foods. This is indicated by differential synchronization of the central nucleus of the amygdala, bed nucleus of the stria terminalus and the basal nucleus of the amygdala on the two feeding paradigms: PT or RT, the central nucleus of the amygdala, bed nucleus of the stria terminalus and basal nucleus of the amygdala is uncoupled from the SCN when under restricted feeding, but not under restricted palatable meal access. In this case, it is important to note that palatable food entrainment on an ad-lib background does not induce the bimodal corticosterone rhythm that is seen under RF. Also, it is not associated with changes in C-FOS expression in hypothalamic regions or peripheral oscillators, but is with changes within the nucleus accumbens. Whether this is due to the lack of an entraining signal from anticipatory corticosterone release is not completely understood, but would be a testable hypothesis.

Minana-Solis and colleagues (2009) examined *Per1, Per2* and *Bmal-1* within the hypothalamus of rats on RF from ZT6 to 8 and ad lib feeding schedules. The aim of the study was to assess whether RF affects clock gene expression in a similar way throughout the hypothalamus. Within the SCN, all three genes displayed a circadian pattern. Aside from a 3h phase advance in *Per1,* RF had little impact on clock gene expression in this area. In the arcuate nucleus under ad lib conditions, *Per1* and *Per2* were arrhythmic. The authors state that there is a significant rhythm in *Bmal-1* expression with a peak at ZT 18. However in examining the data, the nocturnal *Bmal-1* rhythm appears bimodal and the peak at ZT18 is based solely on measurements taken at one time point. Usually, rhythms in clock gene profiles tend to be gradual and not spiked.

In the dorsal medial hypothalamus under ad lib conditions, *Per1* and *Bmal-1* were found to be arrhythmic, while *Per2* showed an increase throughout the dark phase with a peak at ZT18. Under RF *Per1* showed a gradual increase preceding meal time, while the *Per2* rhythm was drastically dampened relative to ad lib, and *Bmal-1* remained

arrhythmic with a bimodal pattern in the dark phase. In the paraventricular nucleus of the hypothalamus, *Per1* was rhythmic with a gradual peak at ZT 15, *Per2* was arrhythmic, and *Bmal-1* was reported to have a clear diurnal rhythm, although inspection of the waveform again shows a bimodal pattern with a peak at ZT3 and then another at ZT21. In the RF condition, the amplitude of the *Per1* oscillation was decreased and the expression was more variable with multiple peaks in the dark phase. A gradual peak in *Per2* at ZT12 was induced by RF, while *Bmal-1* is flat in the RF condition. Finally, in the ventral medial nucleus a peak in *Per1, Per2,* and *Bmal-1* is reported at ZT3, 12 and 18, respectively. In all three cases, the peaks are attributable to high values at each of those time points and not the usual gradual peaks typically observed in clock gene profiles. In the paraventricular nucleus under RF, the waveforms of *Per2* and *Bmal-1* become flat, *Per1* showed a slight increase in amplitude, and also a bimodal pattern in the dark phase.

From these clock gene data observed in SCN, arcuate nucleus, dorsal medial hypothalamus, paraventricular nucleus of the hypothalamus, and ventral medial nucleus of the hypothalamus, the authors conclude that clock gene expression was not uniformly impacted by RF, and that RF tended to result in a shift of induction of rhythmicity in *Per1* and *Per2* mRNA close to meal time. The authors do not address how rhythms in one clock gene are possible in the absence of rhythms in others. The molecular circadian clock effectively produces a 24h rhythm based on the interlocking feed forward and feedback loops of clock genes, so it is difficult to imagine how for example, *Per1* expression could oscillate without the feed-forward effects of rhythmic *Bmal-1*. The fact that they did not observe rhythmicity in all three genes in any structure may suggest that the clock gene rhythms observed were evoked by feeding related stimuli, and were not representative of a self-sustaining circadian clock in these cells. Alternatively, there may be yet to be identified-food entrained genes that substitute for known circadian clock genes.

In addition to limbic and hypothalamic structures, *Per1, Per2, Rev-erbα*, and *Dbp* expression have been reported in the cerebellum of mice, and these rhythms are altered by RF. All four genes are phase advanced under RF, with the peak of *Per1* and *Per2* advancing by 9h, and *Rev-erbα* and *Dbp* advanced by 3 and 6 h, respectively. These oscillations were also observed in cerebellar tissue cultured from per1:luciferase rats.

Under RF the cultured cerebellums showed a 7h phase advance. These findings suggest that a circadian oscillator that can be influenced by RF may exist in the cerebellum.

Wakamatsu and colleges (2001) have examined *Per1 and Per2* expression using in situ hybridization in the hippocampus, piriform cortex, striatum, paraventricular nucleus of the hypothalamus, cerebral cortex, and the SCN of mice. With the exception of the SCN it appears based on the two time points that they sampled (ZT7 and ZT15), that RF inverted the pattern of expression of both *Per1* and *Per2*. In a second experiment, they examined *Per1* and *Per2* at 6 time points in the cerebral cortex and SCN. In the case of the SCN, RF had no impact on the amplitude or phase of the oscillation of either *Per1* or *Per2.* In the cerebral cortex they demonstrated a rhythm under ad lib conditions and the phase of the rhythm was advanced in both genes.

1.8. The effects of restricted feeding on peripheral oscillators

Rhythmicity in peripheral tissues is thought to serve several functional roles. Dibner and colleagues (2010) have suggested 3 putative roles for circadian rhythmicity in peripheral tissues. First, to activate metabolic pathways implicated in food processing in anticipation of consumption. Second, to limit metabolic processes with harmful side effects to only the times that they are required. Third, to sequester intracellular reactions which are chemically incompatible to different times of day. Given the importance of coordinating peripheral organ systems with the daily rhythm of food intake, it is not surprising that feeding time is a powerful zeitgeber for setting the phase of rhythmic gene expression in nearly all peripheral tissues, including the adrenal gland, stomach, liver, kidney and heart (Damiola et al., 2000, Stokkan et al., 2001).The daily rhythm of food intake is controlled by the SCN pacemaker. Although the SCN regulates neural and endocrine outputs to peripheral organs, It is reasonable to predict that the SCN entrains peripheral tissues indirectly by controlling the rest activity cycle of animals and therefore the phases at which they eat. Evidence that entrainment of peripheral clocks by the SCN is indirect and that instead the dominant zeitgeber for peripheral clocks is feeding time comes from two findings concerning the impact of RF on clock gene expression in the

SCN, and the phasing of clock genes in the periphery of SCN lesioned animals. Despite the induction of a large mid-day activity bout, preprandial increases in body temperature, glucocorticoids and other feeding related hormones, by RF during the mid subjective day the phase of clock gene expression in the SCN is unchanged (Damiola et al., 2000; Stokkan et al., 2001; Hara et al., 2001). The phase of peripheral oscillators can also be set using RF in arrhythmic SCN lesioned animals (Hara et al., 2001). The stimuli that entrain these oscillators remain to be determined.

1.8.1. Peripheral clock resetting: role of glucocorticoids

Generally the SCN is resistant to phase changes induced by restricted feeding. It is therefore a valid strategy to narrow down potential food entrainment stimuli based on whether or not appropriate receptors are expressed in the SCN. One of the first candidates to be examined were glucocorticoids as the glucocorticoid receptor is not found within the SCN, but is found in many peripheral tissues and brain regions..

Balsalobre and colleagues (2000) demonstrated a phase response curve for shifting of daily rhythms of DBP and RE VERBα in the liver of rats injected with dexamethasone at different phases. Importantly, the shape of this phase response curve is drastically different from the phase response curve to light within the SCN. The phase response curve to light in the SCN has 3 discernable phases, a dead zone during the subjective day when the SCN is not sensitive to light, a phase delay zone early in the subjective night and a phase advance zone late in the subjective night. The phase response curve to dexamethasone in the liver shows only two phases, a phase delay zone that appears to extend from mid subjective day to late subjective night, and a smaller phase advance zone from the late subjective night to mid subjective day. This phase response curve has two interesting features. First, the phase delay zone appears much larger than the phase advance zone. This is also thought to be true for the FEO driving FAA, because rhythms of FAA are more likely to respond to a shift of mealtime by phase delaying than phase advancing (Stephan, 1982). Second, aside from the acute phase where phase delays transition to phase advances there is no appreciable dead zone in the liver. This may be of functional significance as in the absence of a dead zone, the liver would always be sensitive to zeitgebers perhaps facilitating re-entrainment. Work done with mice lacking hepatic GR receptors ($GR^{AlfpCre}$) indicate that

glucocorticoids cannot be the sole signals regulating the phase of peripheral tissues as entrainment of *Per1, Per2, Per3, Cry1 and reverb* in $GR^{AlfpCre}$ is the same as in WT mice. This indicates that GR are not required for the entrainment of the liver (Balsalobre et al., 2000).

Recall that the phase of peripheral clocks is not completely explained by glucocorticoid signalling, as $GR^{AlfpCre}$ mice showed clock gene expression in the liver that was similar to WT controls on RF. Based on this finding it isn't overly surprising that peripheral tissues are entrained by RF in animals without adrenal glands and therefore no glucocorticoid signalling (LeMinh et al., 2001). In rats fed ad lib the phase of clock gene expression appears to be in phase with the SCN. When food is restricted to the mid subjective day the phase of these clock genes shift and align with food availability. Interestingly, the rate of this shift is markedly slower in animals with adrenals vs. adrenalectomized animals. This suggests that glucocorticoids have an inhibitory affect on the rate at which peripheral oscillators shift. Additionally, the rate at which peripheral clocks shift to a mid subjective meal varies by tissue and the extent to which glucocorticoids impede this shift varies by tissue. (Damiola et al., 2000; Stokkan et al., 2001; Davidson et al., 2003; LeMinh et al., 2001). Taken together, these results indicate that there must be other resetting signals for peripheral clocks.

1.8.2. Peripheral clock resetting: role of temperature

A second putative zeitgeber for entraining peripheral clocks during restricted feeding is the rhythm of core body temperature. During RF core body temperature rises in anticipation of meal time, and rises further when food is ingested. Increases in temperature have been demonstrated to affect circadian gene expression *in vivo* and *in vitro* (Brown et al., 2002). Clock gene rhythms within the SCN are temperature compensated and therefore not sensitive to changes in core body temperature. Interestingly, the temperature resistance of the SCN is a property of the SCN network and not individual SCN neurons. Evidence for this comes from the use of tetrodotoxin to block action potentials and therefore paracrine signalling between neurons. In culture the SCN is not sensitive to changes in temperature. However, if tetrodotoxin is applied to the SCN in culture it becomes sensitive to changes in temperature, indicating that paracrine signalling between neurons of the SCN is needed for temperature compensation.

Specifically, it appears that GABAergic signalling between the dorsal and ventral components of the SCN are responsible for temperature compensation. Warm pulses can induce transcription of heat shock factors. It has been demonstrated that heat shock factor 1 (HSF1) in addition to other components of the heat shock response can influence circadian gene expression. In culture, application of KNK37, a potent antagonist of the heat shock pathway, can block temperature induced phase shifts in peripheral tissues such as the lung (Buhr et al., 2010). To date the affect of RF on rhythmic profiles of heat shock factors has not been studied. It is difficult to parse changes in body temperature from feeding as processing food increases body temperature. However there is evidence that caloric restriction increases *Hsp70* in the gut (Ehrenfried et al., 1996). Therefore heat shock factors may be a viable candidate for setting the phase of peripheral tissues.

1.8.3. Peripheral clock resetting: role of cellular redox state

Food has also been suggested to influence phasing of peripheral clocks via the NAD(P)+/ NAD(P)H ratio. The feed forward loop of the molecular clock comes from the binding of BMAL1 to CLOCK or NPAS2, forming a heterodimeric transcription factor complex influencing the transcription of the *Per* and *Cry* genes. CLOCK or NPAS2 cannot bind to BMAL1 if reduced nicotinamide adenine dinucleotides NADH and NADPH are not present. The oxidized versions of these electron carriers NAD+ and NADP+ inhibit the interaction of CLOCK or NPAS2 with BMAL. The NAD(P)+/NAD(P)H ratio is affected by the metabolic state of the cell. For instance, the oxidization of NADH to NAD+ accompanies the production of lactate that is synthesized in response to high glucose levels in the absence of high oxygen levels. Therefore during RF changes to the NAD(P)+/NAD(P)H ratio may alter binding of BMAL1 to CLOCK or NPAS2. Changes in the binding of BMAL1 with either CLOCK or NPAS2 would thereby alter the phase where they act as a transcription factor for the *per* and *cry genes*. This could result in alterations in entrainment processes to light (if clock gene expression within the SCN is sensitive to this ratio), as phase advances and phase delays are dependent on alterations of the *per* genes during either their rising or falling phases. Further it could alter the phasing of processes within peripheral tissues. This could be a mechanism for

promoting phase alignment between or within tissues to maximize efficiency of metabolic processes.

1.9. Multiple Daily Meals

Rats are opportunistic feeders. In the wild, they feed throughout the active phase at multiple times, and would be expected to find food at multiple times of day. This suggests that it would be advantageous if rats could anticipate multiple daily mealtimes. A small number of studies have investigated the number of meals per day that rats will anticipate. In 1973 Booles and Moot demonstrated that rats will anticipate two daily meals separated by 6 hours or more in the light or the dark phase. If the meals are separated by less than 6 hours stable FAA is not observed. Entrainment to 3 meals per day has not been demonstrated using running wheels (Stephan 1989), but there is some evidence of anticipation to this schedule when looking at food bin directed behaviour in rats (Mistlberger lab, unpublished observations) and mice (Luby et al, 2012)). Anticipation of multiple meals has been explained using concepts of interval timing theory and circadian oscillator entrainment.

Interval timing theorists posit the use of an interval timer to anticipate more than 3 meals per day (Crystal, 2009). Interval timers could be set by either light dark transitions or a feeding bout, but these timers do require a re-setting stimulus, therefore elimination of this stimulus should also result in the elimination of the timed behaviour. However, it has been clearly demonstrated that FAA to multiple meals persists through several cycles of total food deprivation in constant lighting conditions.

Circadian biologists hypothesize that entrained oscillators are used to anticipate multiple meals. This model can explain the persistence of FAA to multiple meals in the absence of a resetting stimulus. As described previously circadian rhythms are endogenous and do not require external cues for their behavioural expression. Therefore persistence of FAA under food deprivation is explained as the behavioural output of entrained circadian oscillator(s).

Anticipation of two daily meals using circadian oscillators could be explained by 3 different mechanisms. Firstly, the bouts of anticipation could be the output of two distinct

oscillators (or populations of oscillators). Evidence for this type of organization in the circadian system comes from the phenomenon of splitting, which refers to dissociation of free-running circadian rhythms into 2 rhythmic components that may recouple in antiphase (typical in Syrian hamsters in constant light; Pittendrigh and Daan,1976b) or that may free-run independently for many months (e.g., in tree shrews in constant dark; Hoffmann, 1969; Meijer et al, 1990). Split activity rhythms can be explained by a dissociation of the left and right SCN (de la Iglesia at al., 2000). A similar two-oscillator model could explain FAA to two daily meals but evidence for anticipation of 3 or more meals would then indicate the need for a potentially infinite number of clocks. This would raise the problem of how multiple clocks could entrain to separate meals, without being destabilized by the other meals and other clocks,

A second model would be a continuously consulted clock. Each meal would be marked by a phase that can be discriminated using one entrained circadian oscillator. This kind of model would allow for the anticipation of many different meal times. Honeybees are able to communicate location of food sources at particular times of day with other bees. They are able to communicate the direction to a food source based on the angle of the sun and then the distance to this source based on the number of waggles of their thorax. This sun compass navigation requires the use of an entrained circadian oscillator that can be consulted at any time like a wristwatch (Frisch, 1974). This model also would require only 1 entrained circadian oscillator such as the SCN. However, multiple meal entrainment in SCN ablated animals rules this structure out.

A third possibility is a hybrid model involving an entrained circadian oscillator and an interval timer. In this case, anticipation to one meal would be driven by an entrained FEO while the other would used an interval timer set by either the other meal or a light dark transition.

Evidence from multiple daily meal experiments demonstrates that anticipation to two daily meals persists during food omission tests (Bolles and Moot, 1973) and in constant lighting conditions (Edmonds and Alder, 1977). During food deprivation tests, there was a peak of activity at both mealtimes (Bolles and Moot, 1973). However, in that study, LD cues were available to support interval timing. Stephan (Stephan 1983, 1989a, 1989) later showed that rats can anticipate two daily meals with different cycle lengths,

e.g., one meal every 24h and a second meal every 24.5h. This constitutes powerful evidence for a 2 oscillator model. However, during food deprivation tests the rats exhibited only a single bout of activity extending from one mealtime to the next. This leaves open the possibility that an interval timer was responsible for the second bout of anticipation

Precise onsets of FAA are needed to help determine a role of interval timers in the anticipation of 2 daily meals and these types of experiments were conducted in our laboratory (Mistlberger et al., 2012). Using single and multiple meal omission tests, constant darkness, and meal shifting manipulations, we found no evidence for the use of interval timers in multiple meal anticipation. In these experiments, omission of meal 1 did not greatly affect the anticipation to meal two (the onset being only slightly delayed). In constant darkness, omission of meal 1 and 2 still resulted in anticipation to both meals with no significant change in the timing of their onset, demonstrating that light dark transitions that could potentially serve as cues to interval timers were not being employed to anticipate either meal. Advancing the first meal by 3 hours did not alter the timing of meal 2, but did acutely phase advance the onset of FAA to the first meal on the subsequent day. This suggests that the timing of meal 1 can be acutely phase shifted without altering the onset of meal two. This argues against the two bouts of FAA being the output of the same clock since if this where the case meal 2 would have to move in parallel with meal 1.

From these behavioural observations, it is unlikely that multiple meal anticipation involves interval timers. The two-oscillator model and the continuously consulted clock models remain a possibility, although the two-oscillator model has the most support from behavioural experiments. Likely, the validation of either the two oscillator model or the continuously consulted clock model will require the identification of FEO's or an FEO respectively.

1.10. Objective of the present research

The preceding literature review reveals several major problems remaining to be solved in the neurobiological analysis of food-entrained rhythmicity in mammals. First, despite multiple lesion and clock gene mapping studies the location of FEOs necessary for FAA remains elusive. Second, the role of clock genes in FAA remains unclear. Third, anticipation of two daily meals can be explained by multiple and single oscillator models. Studies to confirm that rats and mice can anticipate 3 or more meals are lacking. Very little data exists on physiological and cellular correlates of 2-meal anticipation; we do not know if clock gene rhythms in the brain or periphery of rats anticipating two daily meals are unimodal (consistent with a single food-entrainable continuously consulted clock model) or bi-modal (consistent with a 2-oscillator model).

The objective of this thesis was to evaluate the timing of daily rhythms of metabolic hormones and clock gene rhythms in the periphery and in the brain of rats entrained to 1 or 2 daily meals. Three groups of animals were exposed to different feeding schedules, a daytime (AM) meal (ZT4), a nighttime (PM) meal (ZT16), and a two-meal schedule consisting of AM and PM meals (ZT4 and 16). Brains, blood and peripheral tissues were harvested every 4 hours (ZT0, 4, 8, 12, 16 and 20) to assess clock gene rhythms in various regions of the brain, stomach and adrenal gland. Blood samples were used to assess rhythms in gastric ghrelin and adrenal corticosterone. This experimental design will provide information relevant to the following questions:

1. Where in the brain are daily rhythms of clock genes expressed and in which of these areas is the rhythm rephased by mealtime? These data add to previous studies of clock gene expression in rats and mice anticipating one daily meal.

2. In animals fed two daily meals, are clock gene rhythms in the brain unimodal or bimodal, or does this vary by region? These are unique data as no prior study has quantified clock gene expression in the brain of a rat or mouse anticipating two daily meals.

3. Does the daily rhythm of hormone secretion (ghrelin and corticosterone) match the daily rhythm of FAA in rats anticipating one or two daily meals? If the hormone plays a role in driving FAA or entraining FEOs that drive FAA, then there should be a peak associated with each bout of FAA.

4. Does the daily rhythm of hormone secretion (ghrelin and corticosterone) match the daily rhythm of clock gene expression in the source gland (stomach and adrenal

gland, respectively)? If there are two peaks of ghrelin and corticosterone, are the clock gene rhythms also bimodal?

Chapter 2.

Methods

2.1. Animals and Procedure

Young male Sprague Dawley rats (N=108, 150g) were housed in standard polypropylene rat cages with overhead motion sensors in cabinets with a 12:12 LD cycle (~30lux). Motion sensors were monitored with the Clocklab data acquisition system (Actimetrics, Wilmette, IL, USA). The rats were allowed 12 days to entrain to the LD cycle, during which time standard rat chow (Bio-Serve, Frenchtown, NJ, USA) was provided ad libitum and their average daily intake was recorded. On day 13 each rat was assigned to one of three restricted feeding conditions:

1. condition 1 (AM feeding) 65% (13.0 $\pm$ 0.5g) of their average total daily intake (TDI) was provided from ZT4-6, food was removed at ZT6 until it was evident that all of the was being consumed within meal within 2 hours.

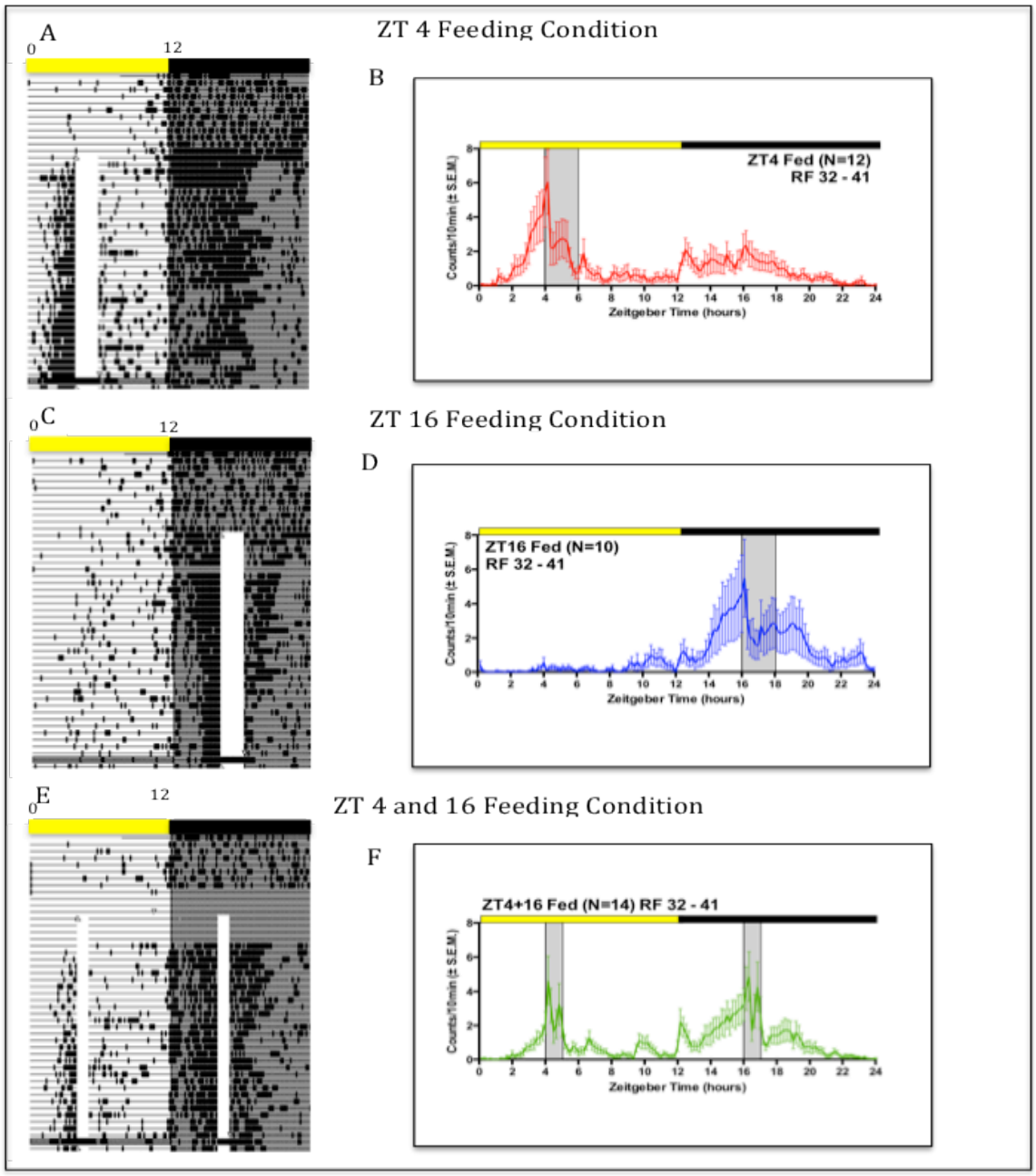
2. condition 2 (PM feeding) 65% of their TDI was provided from ZT 16-18, food was removed at ZT18 until it was evident that all of the meal was being consumed within 2 hours.

3. condition 3 (AM and PM feeding) 32.5% of their TDI was provided from ZT4-5 and ZT16-17, food was removed at both ZT5 and ZT17, until it was evident that all of the meal was being consumed within the 1 hr meal times.

On RF day 33 the rats in each of the conditions were food deprived in DD. During this DD probe day it was demonstrated that FAA persisted in the absence of LD and feeding cues. On days 34-35 the 3 RF schedules were re-instated. On RF day 36 the meal was omitted for the PM condition. On RF day 37 the AM meals were omitted for both the AM and the two meal fed rats. Due to the timing of the meals it was not possible to have equivalent FD durations between conditions. In our design we ensured that all animals did not receive their last meal, resulting in the PM group undergoing 12 additional hours of food deprivation relative to the other conditions.

Beginning at ZT0 on day 37 of RF rats were sacrificed by CO2 in groups of 6 (2 per feeding condition) at 4 hour intervals (ZT0, 4,8,12,16, and 20). Blood samples for ghrelin and corticosterone assays were collected by cardiac puncture, brains were rapidly removed and flash frozen in methyl butane on dry ice, and the stomach and adrenals were then removed and frozen on dry ice.

Figure 2. Representative single plotted actograms and average wave forms from rats in each of the three feeding conditions.



A. Actogram from the ZT 4 feeding condition. The feeding window is denoted by the white rectangle in the light phase. The dark phase is denoted by grey shading. B. Average wave form (± sem) of activity as recorded by overhead motion sensor. The feeding window is denoted by a gray bar, LD is denoted by the yellow (lights on) and black (lights off) bars at the top of the figure. C. Actogram from the ZT 16 feeding condition. The feeding window is denoted by the white rectangle in the dark phase. D. Average wave form (± sem) of activity as recorded by overhead motion sensor from the ZT 16 feeding condition. E. Actogram from the ZT 4 and 16 feeding condition. The feeding windows are denoted by the two white rectangles in the light and the dark phase. F. Average wave form (± sem) of activity as recorded by overhead motion sensor from the ZT 4 and 16 feeding condition.

2.2. Brain Preparation

Upon decapitation, brains were rapidly removed and placed into methyl butane kept between -30 to -35° for 5 minutes. The brains were then transferred to and covered with powdered dry ice for 5 minutes. A thin layer of embedding matrix was then applied to the ventral surface of the brain. The brains were then wrapped in autoclaved tin foil and placed in a -80° C freezer until sectioned. Brains were sectioned at 15µm using a freezing cryostat (Thermo scientific). The sections were divided into 8 series onto vectabond treated slides, made according to the manufactures instructions. Slides were kept at -20°C during sectioning, and then transferred to dry ice and placed back in the 80° C freezer until processing using in situ hybridization.

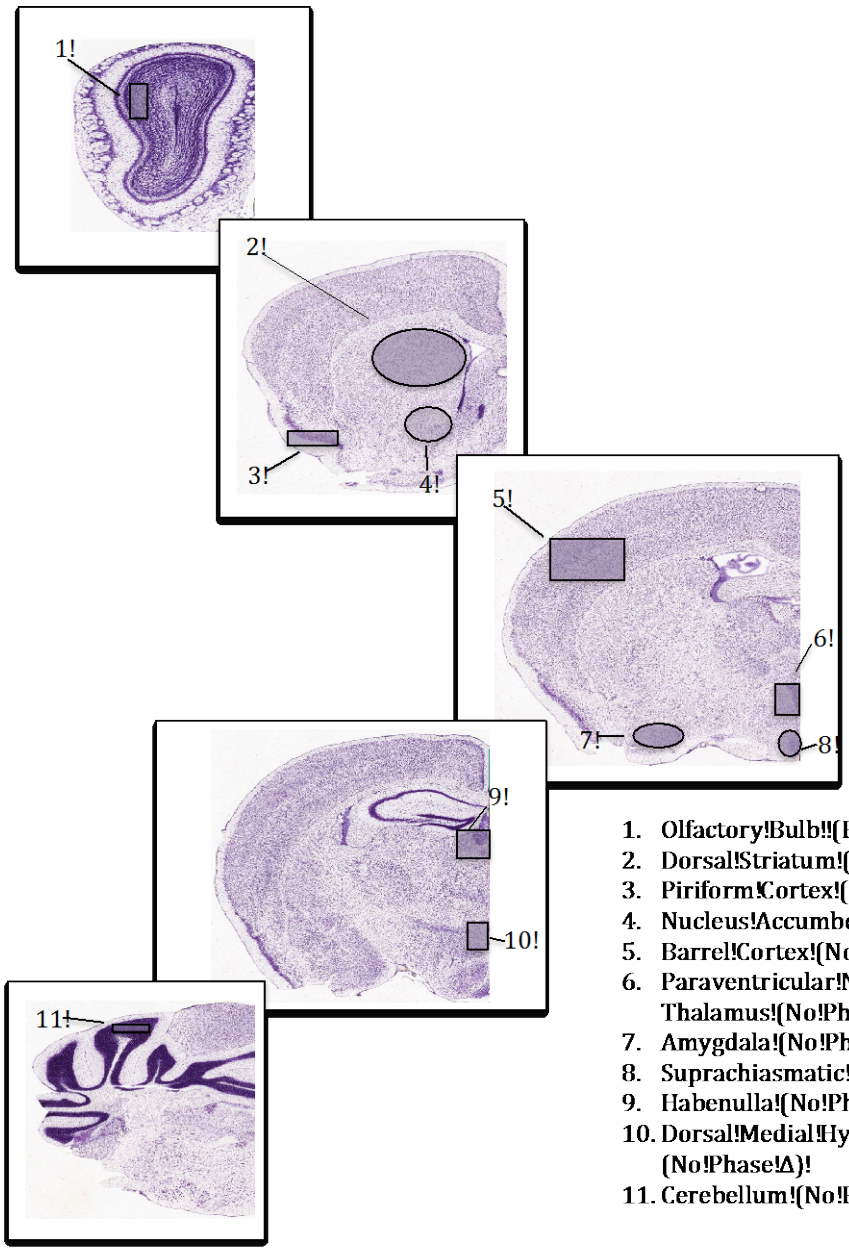
2.3. *In situ* hybridization

Prehybridization: Slides were briefly fixed (5min) in 4% paraformaldehyde in 0.1M Phosphate buffer (PB). Rinsed twice in 2XSSC and then acetylated with 0.25% Acetic Anhydride triethanolamine HCl for 10 min. Slides were rinsed again in 2XSSC and dehydrated as follows (all ethanols were prepared in DEPC-treated water): 1 min in 70% ethanol, 1 min in 80% ethanol, 2 min in 95% ethanol, 1 min in 100% ethanol, 5 min in chloroform, 1 min in 100% ethanol and then 1 min in 95% ethanol. Slides were then air dried in preparation for application of the pre-hybridization buffer (50% deionized formamide and 50% 4XSSC). Slides were cover slipped with 50ul of pre-hybridization buffer for 1 hr at 37°C. The coverslips were then removed and re-covered slipped using 35ul of hybridization buffer containing a 35S-labelled riboprobe was transcribed from a rat *bmal-1* template and incubated overnight at 52°C.

Post Hybridization: Slides were washed in series of 1XSSC baths (3 quick rinses and then two 10 min baths). Slides were then bathed twice in 50% formamide/50% 4XSSC at 52°C for 5 and 20 min each. The slides were then quickly rinsed in 2XSSC at room temperature and incubated in RNase buffer for 35 min at 37°C. Slides were again washed in 50% formamide/ 50% 4XSSC at 52°C for 5 min and subsequently dehydrated as follows: 70% ethanol in 0.1XSSC for 3 min, 80% ethanol in 0.1XSSC for 3 min, 95% ethanol in 0.1XSSC for 3 min, quickly washed in ddH_20, 70% ethanol in water for 3 min

and then air-dried. Slides where then exposed to autoradiographic films. Films were then scanned in a double-bedded, transparency scanner at 3600dpi. Scans (for examples see Appendix B) were then converted to jpegs and optical densities were calculated using image J. The average optical density was measured for the area of interest and normalized to the average background optical density for that slice. This was done by first selecting an image that best reflected the coronal level of the structure of interest by comparing to the Paxinos and Watson Rat Brain Atlas using available landmarks such as fibre tracts and ventricles. Once the images for a region were selected for all 108 animals, the same overlay was applied to the area of interest for each animal. Image j was used to calculate the optical density for that region. Then in the same section for each animal we applied the background overlay, to sample the average background optical density in a similar area across all animals using image j. The region of interest optical density was then divided by the background optical density to yield the normalized optical density for each animal in a given region. (Normalized OD= $OD_{ROI}/OD_{background}$)

Figure 3. Representative cresyl violet stained coronal sections indicating the approximate locations of brain regions analyzed for *Bmal-1* expression.



1. Olfactory!Bulb!!(Phase!Δ)!
2. Dorsal!Striatum!(No!Phase!Δ)!
3. Piriform!Cortex!(No!Phase!Δ)!
4. Nucleus!Accumbens!(Phase!Δ)!
5. Barrel!Cortex!(No!Phase!Δ)!
6. Paraventricular!Nucleus!of! Thalamus!(No!Phase!Δ)!
7. Amygdala!(No!Phase!Δ)!
8. Suprachiasmatic!Nucleus!!
9. Habenulla!(No!Phase!Δ)!
10. Dorsal!Medial!Hypothalamus! (No!Phase!Δ)!
11. Cerebellum!(No!Phase!Δ)!

2.4. Corticosterone Assays

Cardiac blood samples were rapidly cooled and then stored at 4°C overnight. Samples were then centrifuged at 4000rpm for 10 minutes after which the serum was collected and frozen at -20°C until analysis. Serum levels of corticosterone were determined using a commercially available radio-immunoassay kit (MP Biomedicals, Orangeburg, NY, USA).

2.5. Ghrelin Assays

Cardiac blood samples were added to prepared tubes containing EDTA and 100ul/ml of whole blood of apoprotenin (Phoenix Pharmaceuticals Inc, Burlingame, CA, USA) and stored on crushed ice for 45 min. Samples were then centrifuged for 15 min and plasma was collected into DNA/RNAse free tubes. 7ul/100 ul of HCL was added to the plasma to decrease the pH to 4.0. Samples were then transferred to dry ice and subsequently stored in a -80C freezer until analysis. Active ghrelin levels in plasma were determined using the commercially available Rat/Mouse Ghrelin (active) ELISA kit (Millipore, Billerica, MA, USA)

2.6. RT PCR

Stomach and Adrenal samples were analyzed using RT-PCR for *Bmal-1*, *Npas2*, *Rev-erb* α, *Per1,* and *Per2*,. Samples were homogenized using a polytron for the stomach samples or mortar and pestle for the adrenal samples, and total RNA was isolated using TRIzol reagent (Invitrogen, Burlington, ON, CA). RNA concentration and quality was measured using a nanodrop. Total RNA samples were then adjusted to 50ug/ul and cDNA was prepared from 10ul of RNA using the high capacity cDNA reverse transcription kit with RNase inhibitor (Applied Biosystems, Burnlington, ON, CA). cDNA samples (2ul) per reaction were then run in triplicate with SYBR Green (Applied Biosystems, Burnlington, ON, CA) on a real time PCR detection system (Biorad). For a list of all oligonucleotides used refer to Appendix C.

2.7. Statistical Analyses

Statistics for in situ samples were conducted as follows. Optical densities were collected using image j under blind conditions. The data were then decoded and organized based on feeding condition (ZT4 fed, ZT16 fed, or ZT4 and ZT16 fed) and time of tissue sampling (ZT0, 4, 8, 12, 16 or 20). Using GraphPad Prism 6 (La Jolla, CA) a regular (not repeated measures) 2 way analysis of variance (2 way ANOVA) with Bonferroni correction was run for each of the 11 brain regions analyzed. The p value of 0.05 was used throughout the experiment.

Statistics for the RT PCR samples were as follows. First, triplicates for each animal were assessed with respect to variability. Each triplicate value was required to be within 0.3CT's of one another to help correct for possible pipetting error adding to the variability of the data. If a single triplicate varied by more than 0.3 it was discarded. Following this the data were then normalized to the lowest CT on the plate by subtracting it from each CT value ($\Delta Ct=Ct_{observed}-CT_{min}$). Triplicates for each animal were averaged. This normalization procedure was used due to significant variability of housekeeping gene expression with time of day. For comparative purposes, in a separate analysis, normalization with respect to the housekeeping gene *Gapdh* was also done ($\Delta\Delta Ct=\Delta Ct_{gene\ of\ interest}-\Delta Ct_{housekeeping}$). The data are then placed into the following equation: 2^-$\Delta\Delta$Ct.

These values were then decoded and sorted based on feeding schedule (ZT4 fed, ZT16 fed, or ZT4 and 16 fed) and time of tissue collection (ZT0, 4, 8, 12, 16, and 20). Using GraphPad Prism 6 (La Jolla, CA) a regular (not repeated measures) 2 way analysis of variance (2 way ANOVA) with Bonferroni correction was run for each clock gene (*Per1, Per2, Bmal-1, Npas2, Rev-erb α, and Gapdh*). The p value of 0.05 was used throughout the experiment.

Statistics for the Corticosterone and Ghrelin samples were similar. The data were decoded with respect to feeding condition and tissue sampling time. Using GraphPad Prism 6 (La Jolla, CA) a regular (not repeated measures) 2 way analysis of variance (2 way ANOVA) with Bonferroni correction was run for each hormone. The p value of 0.05 was used throughout the experiment.

Chapter 3.

Peripheral Correlates of Food Anticipation

3.1. Adrenal gland: Introduction

In 1974 it was demonstrated that daytime restricted feeding schedules alter the secretion profile of corticosterone, such that a preprandial increase of corticosterone is observed (Kreiger et al., 1974). Clock gene rhythms in the adrenal gland of ad lib fed animals have been reported. The rhythm of *Bmal-1* as measured by either *in situ hybridization,* qPCR or microarray, peaks at the end of the mid subjective night (~ ZT22) (Oster et al., 2006; Girotti et al., 2009). *Rev-erb*α as expected peaks in anti-phase to *Bmal-1* during the mid subjective day around ZT6. Both *Per1* and *Per2* peaked close to the light dark transition with *Per1* peaking slightly earlier than *Per2* at about ZT8 and 13 respectively. This rhythm is markedly shifted by restricting food to the middle of the day (Girotti et al., 2009). To date, the phase of clock gene oscillations within the adrenal gland have not been assessed under night-time restricted feeding.

In the case of two daily meals separated by 6 hrs or more, behavioural FAA is exhibited to both meals and persists during multiple days of food deprivation (Mistlberger et al., 2012). In animals entrained to a two daily meal schedule with meals 6h apart, and then food deprived, the corticosterone rhythm peaked in anticipation of the first scheduled meal and then remained high until the end of the second meal (Honma et al., 1984). Whether this unimodal rhythm during food deprivation is characteristic of feeding schedules with longer intermeal intervals is unknown. The phase of circadian clock genes within the adrenal gland of animals anticipating two daily meals has also not been examined.

Our aim was to examine clock gene expression in the adrenal gland in animals anticipating an AM meal at ZT4, a PM meal at ZT16, and two meals one at ZT4 and the

other at ZT16. Using these clock gene profiles it becomes possible to examine the relationship between adrenal clock gene expression on RF and corticosterone release. Our hypothesis is that alterations in clock gene expression in the adrenal gland induced by restricted feeding would be reflected in the rhythmic profiles of serum corticosterone.

Our study did not include an ad lib control group so we therefore cannot compare measurement of clock gene expression in the adrenal gland of ad lib rats. However, our ZT16 fed group should be reasonably close to the ad lib fed condition as our rats consumed their daily meal approximately 2 hours after ad lib controls would have engaged in their largest feeding bout. However, it remains possible that intermittent feeding throughout the night in ad lib fed animals may contribute to the phase of clock gene expression in the adrenal gland.

3.1.1. Adrenal gland: Results

Here we will first present data normalized to *Gapdh* (Fig 4) followed by non-normalized data (Fig 5). In rats fed at ZT 16 the *bmal-1* rhythm peaked at ZT20, and the peak of *Npas2* occurred shortly after at ZT0. *Rev-erb*α peaked in antiphase at ZT8, while *Per1* and *Per2* peaked at ZT12. These results are consistent with the hypothesis that animals on a restricted feeding schedule during the dark phase from ZT 14-16 show clock gene profiles in the adrenal gland that are similar to ad lib controls, based on previously published data. Importantly, these findings make sense in the context of the described oscillations of the feed-forward and feed-back loops, i.e., *Bmal-1* and *Npas2* peak in phase with one another and out of phase with *Rev-erb*α.

In rats fed at ZT4 the peak in *Bmal-1* and *Npas2* expression occurred at ZT12. Therefore the peak in *Bmal-1* was 8 hours in advance of the peak observed in the ZT16 fed group, while the peak in *Npas2* was approximately 10hrs earlier. Again, *Rev-erb*α peaked out of phase with *Bmal-1* and *Npas2* somewhere between ZT20 and ZT0. Both *Per1* and *Per2* peaked at meal time (ZT4). These results are consistent with a major shift in the timing of the adrenal gland clock by daytime feeding, compared to nocturnal feeding.

In animals fed two daily meals, rhythms of clock gene expression in the stomach, liver, and kidney have been reported to remain unimodal (Davidson et al., 2003; Kuroda et al., 2012). Our findings are in agreement with these earlier studies, as no bi-modal rhythms of clock gene expression were observed in the adrenal gland of animals anticipating a meal at ZT4 and ZT16.

Under this feeding condition, *Bmal-1* and *Npas2* peaked at ZT20, similar to the ZT16 fed group. Interestingly, the profile of *Rev-erb*α expression looks very similar to that of the ZT4 fed condition with a peak at ZT0 and therefore is not peaking out of phase with *Bmal-1* and *Npas2.* Expression of *Per1* occurred at ZT4, while *Per2* peaked at ZT8. This suggests that under a two meal feeding schedule the profiles of *Per1* and *Per2* become slightly dissociated. The normalized data in the two meal fed group, for *Bmal-1, Npas2,* and *Rev-erb*α profiles are not in agreement with the usual profiles for these genes. It is difficult to determine why the phase relationship of clock genes within the adrenal is not maintained in animals fed two daily meals. It may be that the duplicity of the entraining stimulus generated by this feeding condition dissociates the phase relationship of these genes with one another. Although it is possible that the strength of the entraining stimulus generated by each meal may differ this would likely be reflected in the phasing of the oscillations in the adrenal being more closely aligned with the meal exerting stronger influence and not by a dissociation of components of the molecular clock.

All ten of the housekeeping genes that we assessed, including *Gapdh, Actin* and genes encoding histones and ribosomal proteins, exhibited a daily variation (see Appendix C for list and oligonucleotide sequences, for *Gapdh* data see Appendix D). Therefore, the analysis was repeated without normalizing to a housekeeping gene and were simply normalized to the lowest CT value (the number of cycles necessary to cross the threshold set during the linear phase of the curve) observed for that particular clock gene (ΔCT). The Non-normalized data in the adrenal looks slightly different. In the ZT16 single meal condition, the peak of *Bmal-1* expression occurs at ZT8, which is earlier than expected based on ad lib findings that showed a peak at ZT22 (Oster et al., 2006; Girotti et al., 2009). Again *Npas2* peaks slightly at ZT12, and in antiphase to *Rev-erb*α which peaks at about ZT20. The rhythms of the *Per* genes are dissociated with *Per1* peaking at ZT0, and *Per2* peaking at ZT4. The rhythms we observed in *Per 1* and *Per2* are quite

different from the peaks of ZT 8 for *Per1* and ZT13 for *Per2* reported previously (Oster et al., 2006; Girotti et al., 2009).

In the ZT4 fed conditions, *bmal-1* expression peaked at ZT0, while *Npas2* peaked at ZT4. As expected *Rev-erb*α peaked out of phase with *Bmal-1* and *Npas2* at ZT 12. In this condition both *Per1* and *Per2* peaked at ZT20. In the two meal condition, the peak of *Bmal-1* and *Npas2* occurs between the peaks observed in the ZT4 fed and ZT16 fed conditions, at ZT8. However, *Rev-erb* α peaks at ZT12 according to these data which is not out of phase with the oscillations of *Bmal-1* and *Npas2.* Again, the *Per1* and *Per2* show a similar profile with *Per1* peaking at ZT20 and *Per2* peaking at ZT0.

3.1.2. Conclusions

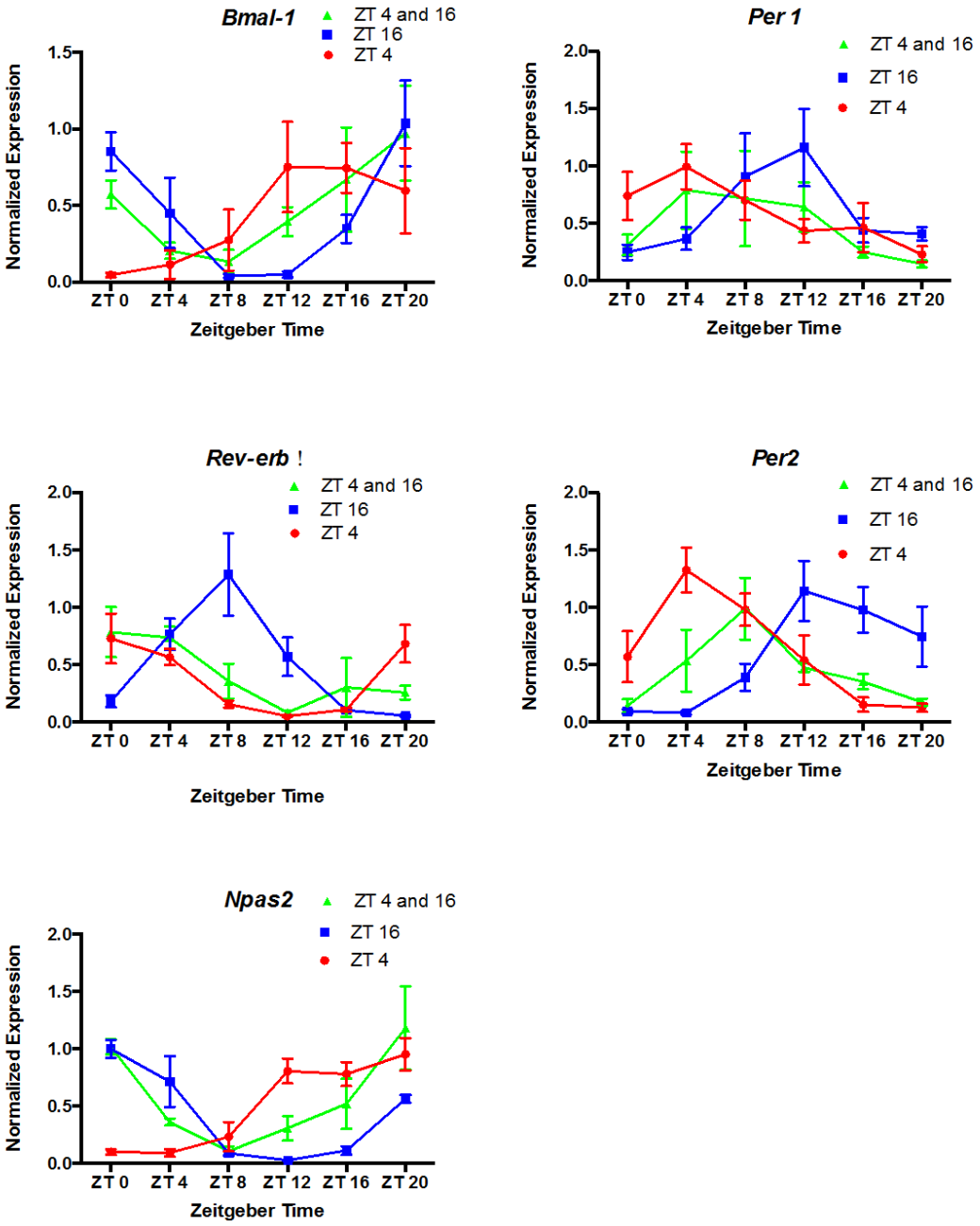
Despite the oscillations in *Gapdh* the normalized data from the ZT 16 fed animals in the adrenal more closely approximate oscillations observed under ad lib feeding conditions than the unnormalized data (ΔCT). This observation suggests that the oscillation of clock genes within the adrenals of animals under night time restricted feeding is similar to ad lib fed controls. The results from the ZT4 feeding condition indicate that the adrenal is shifted by day time RF as has been suggested previously by Girotti and colleagues. In the two daily meal condition clock gene oscillations were unimodal, which is in agreement with previous findings investigating the effect of twice daily feeding in the liver (Davidson et al., 2003). The profile of multiple clock genes from the same peripheral tissue under a twice daily feeding schedule has not been examined previously. Therefore the alteration of the phase relationship between clock genes in the same tissue as the result of twice daily feedings has not been previously reported. It is possible that RF may induce accessory loops similar to CK1 ε that alter the duration of PER accumulation in the cytoplasm for example, thereby altering the phase relationship between clock genes. It is also possible that individual populations of adrenal cells are out of phase with one another and therefore when the adrenal is examined in aggregate the phase relationship of clock genes appears to be altered when in fact they may be maintained at the single cell level.

Recall that the phase relationship between clock gene components is important for generating a ~24 hour rhythm. While the range of entrainment for FAA is within the

circadian range, the range of entrainment for peripheral tissues has not been examined. It is possible that the range of entrainment in the periphery is more variable to compensate for feeding at any time of day and as a result the phase relationship of clock genes within these tissues is not the same as central oscillators.

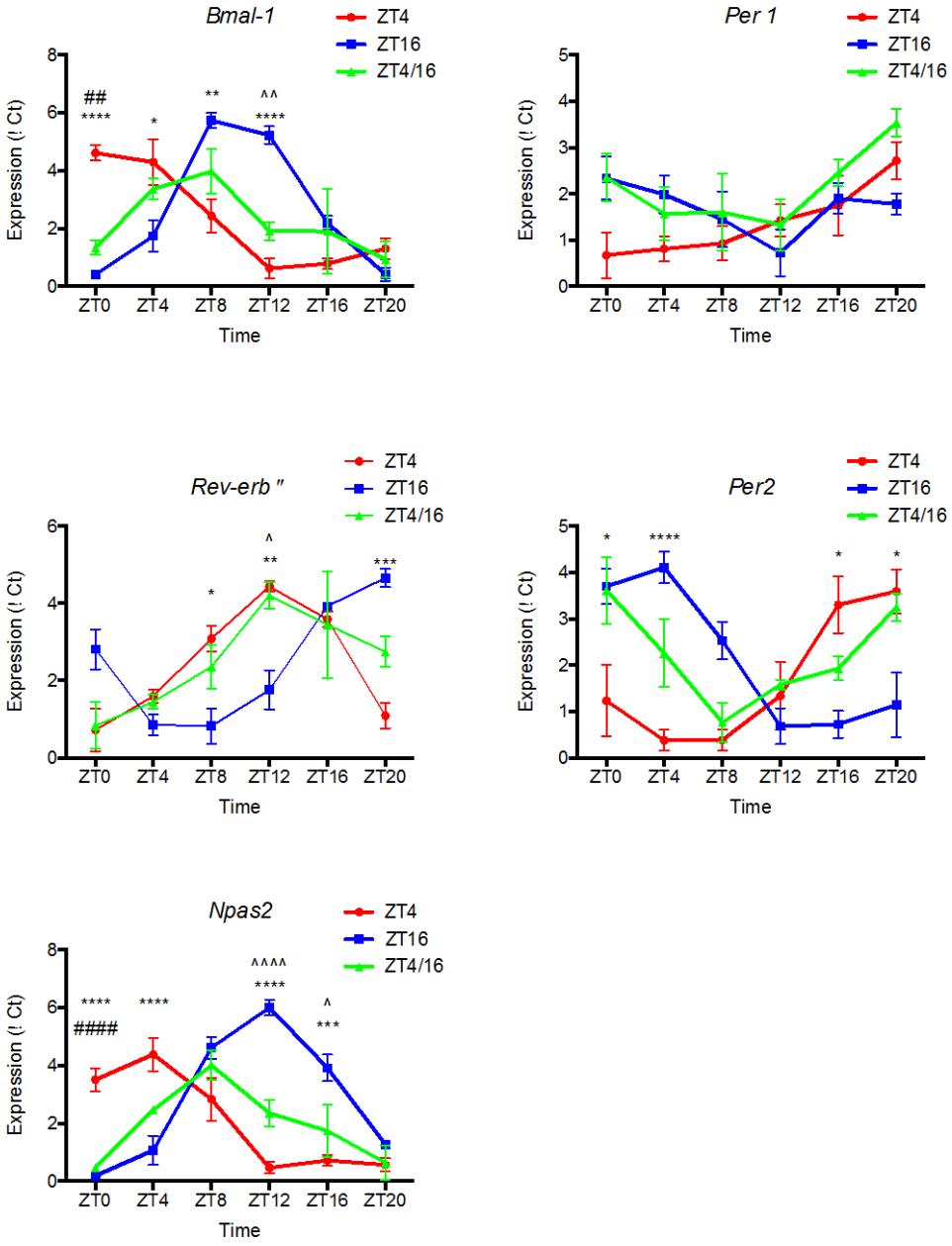
These data indicate that a single daily meal at either ZT4 or ZT16 entrains *Bmal-1, Rev-erb α, and Npas2* in the adrenal gland. When two daily meals are provided at ZT 4 and 16 the oscillation of *Bma-1l* and *Npas2* entrains to an intermediate phase between the two meals. This may be due to differential entrainment of populations of cells within the tissue to one meal or the other. Alternatively there could be inter-animal variability within this condition at each of the time points.

Figure 4. Normalized Adrenal RT PCR Data



Group mean (± sem) waveforms of clock gene expression in the adrenal gland normalized to *Gapdh*. Feeding schedule is denoted by color, ZT4 feeding condition (red), ZT16 feeding condition (blue), ZT4 and 16 feeding condition (green) n=3 animals per time point per condition. A. Bmal-1 expression for all three feeding conditions the interaction between ZT time and feeding was significant $F(10,36)= 4.190$, $p=0.0007$. B. Per1 expression for all three feeding conditions the interaction between ZT time and feeding was not significant $F(10,36)=1.495$, $p=0.18$, the effect of ZT time was significant $F(5,36)= 3.264$, $p=0.016$. C. Rev-erb expression for all three feeding conditions the interaction was significant $F(10,36)-6.050$, $p<0.0001$. D. Per2 expression for all three feeding conditions the interaction between ZT time and feeding was significant $F(10,36)=6.951$, $p<0.0001$. E. Npas2 expression for all feeding conditions the interaction between ZT time and feeding was significant $F(10,36)=7.957$, $p<0.0001$.

Figure 5. Δct Adrenal RT PCR Data.



Group mean (± sem) waveforms of clock gene expression in the adrenal gland reported as Δct. Feeding schedule is denoted by color, ZT4 feeding condition (red), ZT16 feeding condition (blue), ZT4 and 16 feeding condition (green). N=3 animals per time point per condition A. Bmal-1 expression for all three feeding conditions the interaction between ZT the and feeding was significant $F(10,36)=10.3$, $p<0.0001$. B. Per1 expression for all three feeding conditions the interaction between ZT time and feeding was not significant $F(10,36)=1.401$, $p=0.219$, the effect of ZT time was significant $F(5,36)=4.103$, $p=0.005$. C. Rev-erb expression for all three feeding conditions the interaction between ZT time and feeding was significant $F(10,36)=6.822$, $p<0.0001$ D. Per2 expression for all three feeding conditions the interaction between ZT time and feeding condition was significant $F(10,36)=8.159$, $p<0.0001$. E. Npas2 expression for all feeding conditions the interaction between ZT time and feeding condition was significant $F(10,36)=1.691$, $p<0.0001$

3.2. Stomach: Introduction

The stomach is the source of circulating ghrelin, a hormone that stimulates food seeking activity and eating (Mason et al, 2013). The stomach exhibits a daily rhythm of clock gene expression which is markedly reset by daytime restricted feeding schedules (le Sauter et al., 2009). In a study looking at entrainment of the stomach to a single daily meal mice were fed daily from ZT6-12. Under these conditions PER1 and PER2 within the oxyntic cells of the stomach peaked 6hrs earlier than the peak observed in ad lib controls. The peak in PER1 protein under RF occurred at ZT 12 and the peak in PER2 at ZT 18. They assessed only 4 time points however so the temporal resolution of these peaks is quite rough (LeSauter et al., 2009).

Two studies have examined the effect of multiple meals on the phasing of clock gene expression in the liver. Davidson et al (2003) used explants from *Per1-luc* rats, and measured the daily rhythm of bioluminescence from stomachs harvested from rats fed at ZT4, ZT16 or both ZT4 and ZT16. Rhythms from the stomach varied based on feeding condition. In a ZT4 fed group *Per1* peaked at ZT12, in the ZT 16 fed condition *Per1* peaked at ZT 20 and in the two meal condition *Per1* peaked at ZT 24. Kuroda et al (2012) examined the phasing of PER2 in the liver of animals fed 2, 3, 4 or 6 meals at equal intervals. The animals were fed 80% of their baseline total daily intake (TD1) divided by the number of meals. They report that 2, 3, 4, or 6 equally spaced meals does not alter the phase of PER2 expression in the liver. However there is no adlib fed control or single meal condition to compare the multiple meal results with. The phase of the liver in the animals fed two meals a day may have been shifted compared to ad lib or single meal controls.

3.2.1. Stomach: Results

In our animals fed at ZT 16 both *Bmal-1 and Npas2* peaked at ZT0. The profile of *Rev-erb*α peaked at ZT8. The *per* genes were more variable in the stomach than they were in the adrenal under the same feeding conditions. The peak in *Per1* expression occurred at ZT12, while the peak in *Per2* was at ZT16. The peaks of gene expression are similar to those reported by Davidson and colleagues (2003) for a ZT16 feeding schedule.

In the ZT 4 fed animals, *bmal-1* showed two peaks, one at ZT4 and the other at ZT16. However, *Npas2* showed a unimodal rhythm with a peak at ZT20. *Rev-erbα* peaked at ZT 20 under this feeding condition. Again the rhythms in *Per* expression were variable, with *Per1* showing peaks at both ZT4 and ZT16, while *Per2* peaked at ZT4. This is in contrast to Davidson and colleagues who observed a peak in *Per1* only at ZT16 under these feeding conditions. LeSauter and colleagues (2009) observed a peak of gastric PER1 protein levels at ZT12 in mice fed in the middle of the light period, which would imply a peak of *Per1* expression at about ZT6, which is close to what we observed.

Under the two meal schedule, *Bmal-1* and *Npas2* peaked at ZT20, while *Rev-erbα* peaked at ZT0. *Per1* expression was rhythmic with a peak at ZT4, while there was no obvious rhythm in *Per2.* Davidson and colleagues (2003) found the peak of *Per1* at ZT 24 in per1:luciferase expression. Fig 6 shows the profile of *Gapdh* expression in the stomach across the three feeding conditions. Given such variable results in the normalizing data, we will also present the non-normalized data.

When the data are not normalized to *Gapdh* but instead normalized using the ΔCT approach the data look quite different. In the ZT16 fed condition, *Bmal-1* peaked at ZT12 and the profile of *Npas2* is very flat. *Rev-erbα* peaked between the ZT20 and ZT0 time points. Both *Per1* and *Per2* peaked at ZT4. In the ZT4 fed condition *Bmal-1* and *Npas2* peaked at ZT 4, while *Rev-erbα* peaked between ZT12 and ZT16. Expression of the *Per1* and *Per2* genes under this feeding condition were relatively flat.

In the two meal condition *Bmal-1* peaked at a phase between the peaks observed in the ZT4 and ZT 16 fed conditions, occurring at ZT8. The profile of *Npas2* is again very flat, while *Rev-erbα* peaked at ZT12. The expression of *Per1* and *Per2,* was flat. In both the normalized and non-normalized data, the profiles of clock gene expression are puzzling in the 2 meal condition particularly because of the lack of an anti-phasic relationship between *Bmal-1* and *Rev-erbα*. Davidson and colleagues (2003) reported that after 10 days of RF the stomach was often arrhythmic in culture. In a separate group of animals maintained on RF for 20 days, of the 4 cultures that were found to be rhythmic 2 were shifted by RF while the other 2 remained at the ad lib phase. In their two daily meal condition the *per1* rhythm in the tissues assessed

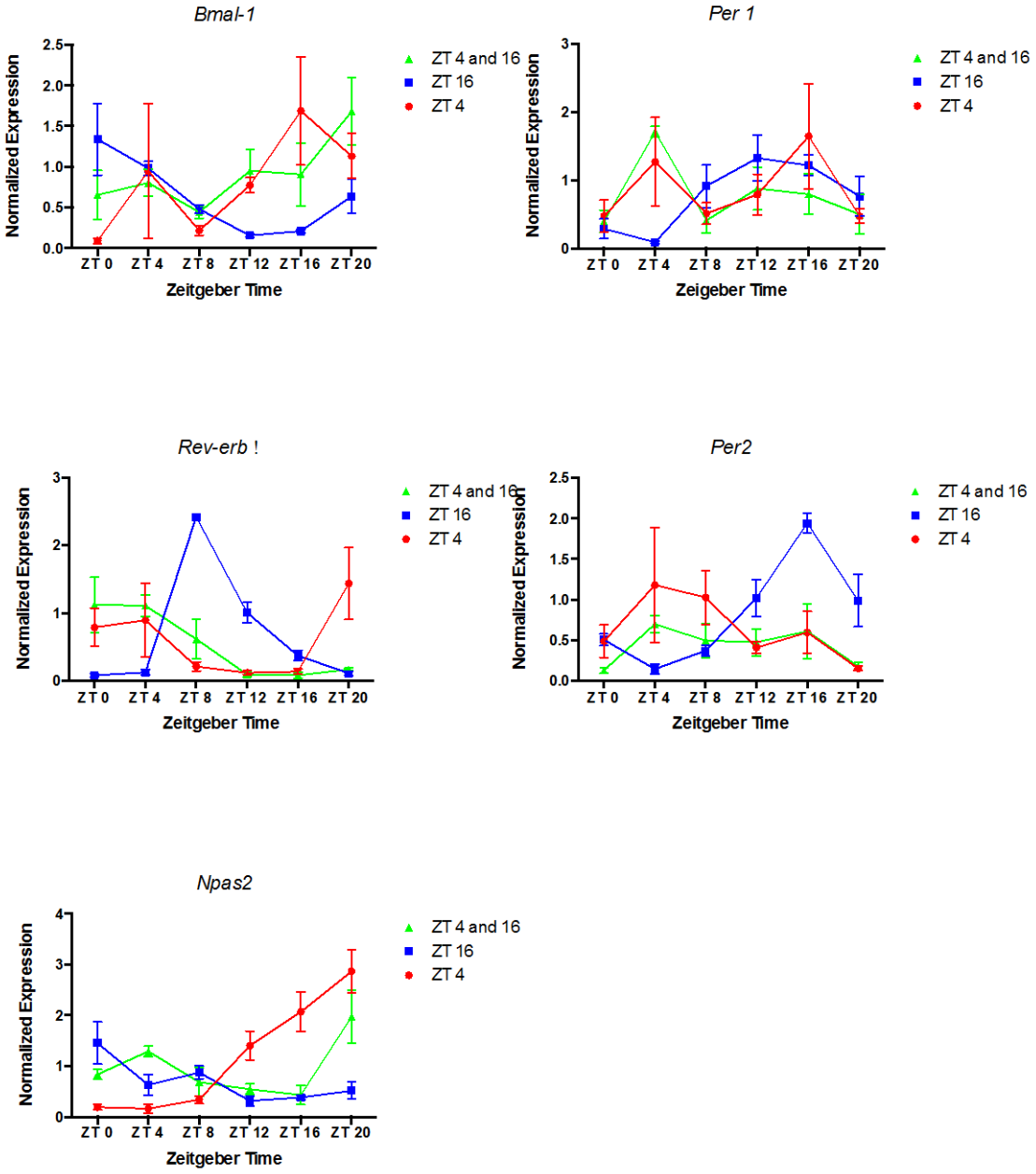
(esophagus, stomach, colon and liver) closely approximated the expression observed in rats fed one daily meal at ZT16.

There is evidence for rhythmic expression of *Per2* in the liver of mice with liver specific *Bmal-1-/-* KO (Kornmann et al., 2007; Lamina et al., 2008). In these animals in the absence of an appreciable rhythm of *Bmal-1* in the liver, *Per2* continues to show robust oscillations. This suggests that transcription of *Per2* in the liver may be less reliant on oscillations of *Bmal-1* than in the SCN for example. It is possible that the oscillation of *Per2* in the absence of *Bmal-1* oscillations may be due to oscillating systemic factors since in mice with conditional knockdown of *Bmal-1* crossed with *mPer2::luc* mice, no rhythm of *Per2* was observed in culture, suggesting that the oscillations observed using RT-PCR in the liver must be reliant on some factor that is not present in culture (Kornmann et al, 2007). Kornmann and colleagues examined transcripts that remained rhythmic in the absence of a *Bmal-1* rhythm. Rhythmic transcripts included those for the heat shock pathway and *Nocturnin.* With respect to phasing of peripheral clocks in animals in RF conditions, the heat shock pathway is of particular interest, as there are established links between heat shock proteins and the circadian clock (Buhr et al., 2010) and evidence that the transcription of these genes is altered by feeding. We attempted to assess rhythmicity of *Hsp70* with no success. However, the profile of heat shock proteins in RF fed animals is a question that should be addressed.

There are few published examples in circadian literature that show RT PCR data that is not normalized to a house-keeping gene (Hood et al, 2010). The use of one especially in experimental designs with only one time point makes for a nice control. However, we would argue that the use of housekeeping genes in experiments assessing temporal profiles of a transcript is flawed. In preparation for cDNA synthesis the concentration of input RNA was carefully measured in order to produce cDNA samples of equivalent concentration. Circadian variability in housekeeping genes has previously been reported and some authors using techniques such as western blots have opted against using loading controls in situations similar to this (Bellesi et al., 2013). Normalizing our data to housekeeping genes that show circadian variability significantly alters the waveform of clock gene expression and should be interpreted with caution. Further investigation into suitable housekeeping genes for rhythms experiments and

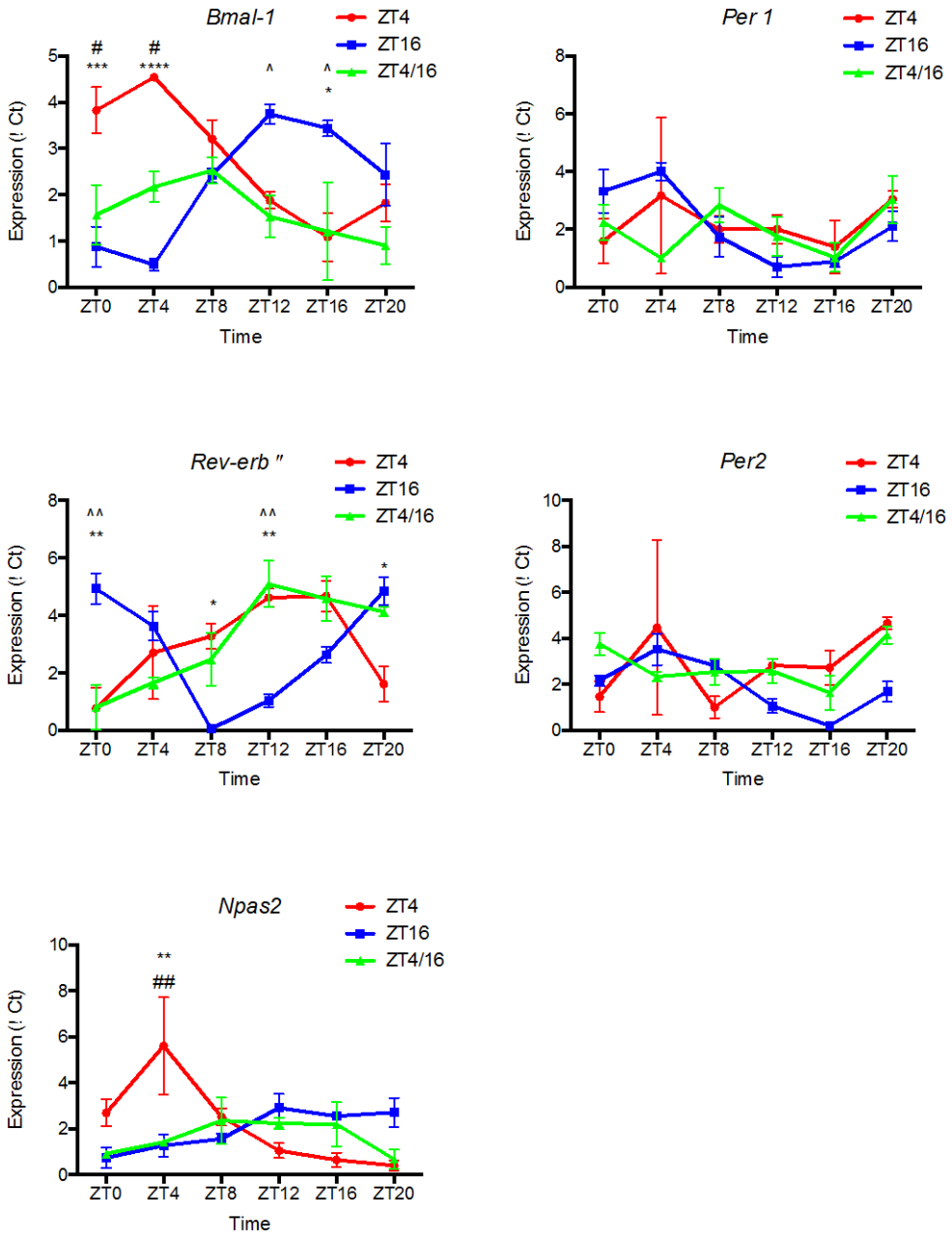
especially restricted feeding experiments is warranted.

Figure 6. Normalized stomach RT PCR data



Group mean (± sem) waveforms of clock gene expression in the stomach normalized to *Gapdh*. Feeding schedule is denoted by color, ZT4 feeding condition (red), ZT16 feeding condition (blue), ZT4 and 16 feeding condition (green) n=3 animals per time point per condition. A. *Bmal-1* expression for all three feeding conditions, the interaction between ZT time and feeding was significant F(10,36)=8424, p<0.0001. B. *Per1* expression for all three feeding conditions, the interaction between ZT time and feeding was not significant F(10,36)-1.178, p=0.337. C. *Rev-erb* expression for all three feeding conditions, the interaction between ZT time and feeding was significant F(10,36)=8.594, p<0.0001. D. *Per2* expression for all three feeding conditions was not significant F(10,36)=1.332, p=0.2515. E. *Npas2* expression for all feeding conditions the interaction between ZT time and feeding condition was significant F(10,36)= 4.372, p=0.0005.

Figure 7 Δct Somach RT PCR data



Group mean (± sem) waveforms of clock gene expression in the stomach reported as Δct. Feeding schedule is denoted by color, ZT4 feeding condition (red), ZT16 feeding condition (blue), ZT4 and 16 feeding condition (green) n=3 animals per time point per condition. A. *Bmal-1* expression for all three feeding conditions the interaction between ZT time and feeding was significant F(10,36)=2.355, p=0.02 B. *Per1* expression for all three feeding conditions the interaction between ZT time and feeding was not significant F(10,36)=2.035, p=0.06. C. *Rev-erb* expression for all three feeding conditions the interaction between ZT time and feeding was significant F(10,36)=10.17, p<0.0001. D. *Per2* expression for all three feeding conditions the interaction between ZT time and feeding was significant F(10,36)=3.689, p=0.002. E. *Npas2* expression for all feeding conditions the interaction between ZT time and feeding condition was significant F(10,36)=10.02, p<0.0001.

3.3. Corticosterone

Corticosterone is synthesized from cholesterol and released from the adrenal gland. The release of corticosterone is under the rhythmic control of adrenocorticotropin releasing hormone from the anterior pituitary (Kaneko et al., 1981). This rhythmic releasing signal acts on the autonomous oscillators within the adrenal glands resulting in the rhythmic release of corticosterone (Oster et al., 2006). Daily restricted feeding alters the circadian profile of circulating corticosterone levels (Krieger et al., 1974). In nocturnal rodents under ad lib conditions, corticosterone levels peak close to dark onset. When food is restricted to middle of the light phase, the rhythm of corticosterone secretion becomes bimodal, with a peak in anticipation of daytime feeding in addition to the nocturnal peak (Krieger et al., 1974). Interestingly, treatment with sodium pentobarbital can suppress the pre-feeding peak while sparing the nocturnal peak, suggesting that these two peaks are mediated by independent neural mechanisms (Honma et al., 1984).

The preprandial increase in corticosterone associated with RF was one of the first putative endogenous entrainment pathways investigated. However, corticosterone was ruled out as an entrainment pathway mediating FAA as adrenalectomized rats display behavioural FAA comparable to intact controls (Boulos et al., 1980; Stephan et al., 1979; Segall et al., 2008; Suijino et al., 2012). Further, under a two meal restricted feeding schedule with meals separated by at least 6 hours, animals display robust FAA to both meals. However, during food deprivation tests corticosterone rises in anticipation of the first meal but remains high until the end of the second meal (Honma et al., 1984b). Behavioural FAA persists to both meal times during total food deprivation probe days, but under these same food deprivation conditions, corticosterone only increases in anticipation of the first meal, indicating that corticosterone rhythms and FAA can be dissociated.

The phase of peripheral oscillators including the adrenal gland, liver, stomach, intestines, pancreas, kidney, heart, and lungs become entrained to feeding time in animals on RF (Damiola et al., 2000; Davidson et al., 2003; Stokkan et al., 2001). It has been proposed that glucocorticoids may be the signal that sets the phase of these peripheral oscillators in response to restricted feeding. This is suggested by the effect of

glucocorticoid administration on the phase of peripheral clocks in many of these tissues (Balsalobre et al., 2000; Reddy et al., 2007; Pezuk et al., 2012).

Adrenalectomy in *Per1-luc* mice results in a phase shift within the liver and kidneys, and also increases the rate of re-entrainment to a 6 hour phase delay in the liver, kidney, cornea, pineal, lung, and SCN (Pezuk et al., 2012). Peripheral clocks were also found to shift more rapidly in adrenalectomized mice or in mice specifically lacking glucocorticoid receptors within the liver (Balsalobre et al., 2000; Le Minh et al., 2001). The effects of corticosterone administration during the mid-subjective day (used to simulate the preprandial increase in corticosterone induced my RF) indicate that some tissues may be differentially responsive to its phase setting effects. The liver is not shifted by a daily corticosterone injection and the shift induced by RF in the liver is not prevented by a late day corticosterone injection. In contrast, shifting of the kidney and lungs in response to RF is blocked by a late day corticosterone injection (Stokkan et al., 2001). Sujino and colleagues (2012) report that adrenalectomy has no effect on the expression of *Per1, Per 2, Cry1* or *Bmal-1* in the liver, kidney or lungs, providing evidence challenging the role of glucocorticoids entraining peripheral clocks. Additionally, the liver was found to entrain to restricted feeding independent of daily corticosterone injections in adrenalectomized rats, while the kidney and lungs entrained to the daily corticosterone injections during RF. This suggests that the phase of different peripheral clocks may be set by different signals, e.g., food, glucocorticoids or temperature (Sujino et al., 2012). Together, the data suggest that the role of corticosterone in synchronizing peripheral oscillators is not clear cut and these tissues respond to multiple signals induced by RF in a tissue dependent manner.

We examined the profiles of serum corticosterone under three feeding schedules to determine whether corticosterone rises prior to two meals separated by 12h, and to relate the profiles of corticosterone secretion in animals anticipating multiple meals with the profile of clock genes within the adrenal gland. In the ZT4 one-meal condition serum corticosterone rose in anticipation of meal time, whereas in both the ZT16 one-meal and ZT4/16 two-meal conditions, corticosterone peaked near ZT12, similar to the rhythm reported for ad lib fed rats (Fig.8). The two meal results differ from those reported by Homna and colleagues (1984). In that study, during a food deprivation day, corticosterone rose in anticipation of the first meal and remained high until the end

of the second meal. One possible explanation is the duration of time between meals: in their study, the meals were separated by 6 hours during the light phase, whereas in our study the meals were separated by 12 hours with one occurring in the light phase and the other in the dark phase. It may be that feeding in the dark phase is a stronger zeitgeber for corticosterone entrainment than feeding in the light phase. Alternatively the duration between the two meals may alter the coupling of the corticosterone rhythm with food availability. It may be that when the duration between meals is longer than 6hrs corticosterone remains coupled to one of the two meals, whereas when they occur closer together (6 hrs or less) it prevents the decrease of corticosterone prior to the second meal. Whatever the explanation, the results of the Homna et al (1984) study and the present study both show a dissociation between behavioural rhythms and corticosterone release.

As previously discussed, we examined the phase of core circadian clock genes within the adrenal glands of rats receiving a meal in the either the mid subjective day, the mid subjective night, or in the mid subjective day and the mid subjective day and night. Oster et al (2006b) have proposed two putative roles for circadian clock genes in the adrenal. First, the clock within the adrenal directly regulates the synthesis of glucocorticoids. Or, second, the adrenal clock regulates the sensitivity of the adrenal to adrenocorticotropin releasing hormone. If in fact the adrenal clock is functionally relevant for the rhythmic release of corticosterone it is currently unknown what clock gene(s) regulate this rhythm. In our single meal conditions at either ZT 4 or 16, the peak in corticosterone secretion coincides with the peak of the *per* genes in the adrenal at ZT4 and 12 respectively. In the two daily meal condition the phase relationship between clock genes and also the expression of the *per* genes is more variable, this variability is also observed in corticosterone levels under this feeding condition.

Recall that the FAA is relatively robust to perturbations of the molecular clock and that *Per2-/-* and *Per2*^{bdrm} mice have different phenotypic responses to RF with the latter showing no FAA in response to RF. Rhythms of corticosterone in response to RF have been investigated in *Per2*^{bdrm}, *Per2*^{bdrm}*;Cry1-/-* and *Per2*^{bdrm}*; Cry2-/-* mice. In the absence of behavioural FAA corticosterone still increases in anticipation of the scheduled meal indicating that physiological anticipation persists in the absence of behavioural anticipation. Oster et al (2006) have investigated the sensitivity of the

adrenal to adrenocorticotropin releasing hormone in WT mice or *Per2/Cry1* deficient mice. They examined the effect of transplanting *Per2/Cry1* adrenals into WT hosts and visa versa on profiles of adrenocorticotropin releasing hormone and corticosterone release, and rhythms of clock gene expression in the adrenal. They report in WT animals the rhythm of adrenocorticotropin releasing hormone is not dependent on the genotype of the transplanted adrenal. In mutant hosts, a WT adrenal cannot restore the rhythm of adrenocorticotropin releasing hormone or corticosterone release. Based on these data the authors claim that in the absence of a light entrained SCN a WT adrenal cannot sustain rhythmic corticosterone release (Oster et al., 2006). Interestingly, in DD conditions, under RF *Per2/Cry1* mice display rhythmic release of corticosterone. This result indicates that the interpretation by Oster et al. (2006) may be due to the control of the LD cycle on feeding rhythms in their arrhythmic genotype.

3.4. Ghrelin

Acyl-ghrelin and des-acyl ghrelin are peptide hormones synthesized peripherally in the oxyntic cells of the stomach from precursor preproghrelin (Kojima et al., 1999) and centrally by neurons of the medial and lateral hypothalamic nuclei (Cowley et al., 2003). The two forms of ghrelin differ in their orexigenic effects. Both systemic and intracerebroventriclar administration of acyl-ghrelin promotes feeding in rats and mice (Nakazato et al., 2001; Toshinani et al., 2006). Des-acyl ghrelin can also promote feeding when administered intracerebroventricular, but not when administered systemically (Toshinai et al., 2006). Ghrelin's orexigenic action is primarily mediated via activation of agouti-related peptide and neuropeptide Y neurons (AgRP/NPY) in the arcuate nucleus, resulting in the release of AgRP and NPY. Ghrelin also exerts an inhibitory action at proopriomelanocortin neurons (POMC), preventing the release of anoxerigenic peptides. The growth hormone secretagogue 1 receptor (GHSR) is the only known ghrelin receptor and is found in high levels in the arcuate nucleus. GHSR mRNA has also been localized to multiple hypothalamic areas involved in feeding and arousal and extra hypothalamic regions including the hippocampus, ventral tegmental area, substantia nigra, tuberomammilary nucleus, Edinger-Westphal nucleus, dorsal and median raphe nuclei, lateraldorsal tegmental nuclei and the facial nucleus (Guan et al., 1997).

Plasma ghrelin increases prior to meal time in rats and mice maintained on RF (Bodosi et al., 2004; Drazen et al., 2006; LeSauter et al., 2009). Gastric oxyntic cells exhibit a daily rhythm of clock gene expression that is entrained by RF, suggesting a function of gastric FEOs may be rhythmic ghrelin release (LeSauter et al., 2009). It has been suggested that gastric ghrelin may contribute to the regulation of FAA, by acting to stimulate appetite and activity or as an entraining signal to central clocks that control behavioural rhythms. Multiple studies employing gene knockout models and administration of exogenous ghrelin have examined this hypothesis.

The results of five studies indicate that ghrelin signalling is not required for FAA prior to a scheduled meal. Three of these studies reported FAA in ghrelin ligand deficient mice (Szentirmai et al., 2010; Gunapala et a., 2011) or GHSR KO mice (Gooley et al., 2006) that was equivalent with the FAA of WT mice. In two other studies, FAA was either decreased in duration (Le Sauter et al., 2009) or in amplitude (Blum et al., 2009).

Conversely two studies report that ghrelin receptor knock out mice do not anticipate RF. The first of these studies compared activity in the 2hrs prior to the daily meal during 1 baseline day and on day 14 of the RF schedule in a separate recording cage. Without normalizing total activity and longer behavioural tests (multiple days of baseline and experimental conditions) it is difficult to assess whether the mice were anticipating. Further, transferring the animal to an independent recording cage could serve as a cue of upcoming food availability and this variable has been documented to decrease FAA and therefore confounds these results (Terman et al, 1984). In the second study, a severe caloric restriction schedule was utilized to induced “activity based anorexia” (Verhagen et al., 2011). This induced a daytime hyperactivity in the WT mice that was not observed in the KO mice. However, the feeding schedule was only in place for 3 days, and the extreme caloric restriction can result in immediate non-specific hyperactivity throughout the day, which appears to be the case in this study. Failure of the KO mice to exhibit non-specific hyperactivity does not mean that FEOs are disabled or that an entrainment signal (e.g., ghrelin) is lacking . Given the extreme nature of this food restriction schedule the results are not comparable to other RF studies.

Together, ghrelin ligand and GHSR KO studies indicate that ghrelin signaling is not required for FAA. Further evidence for this can come from observing dissociations

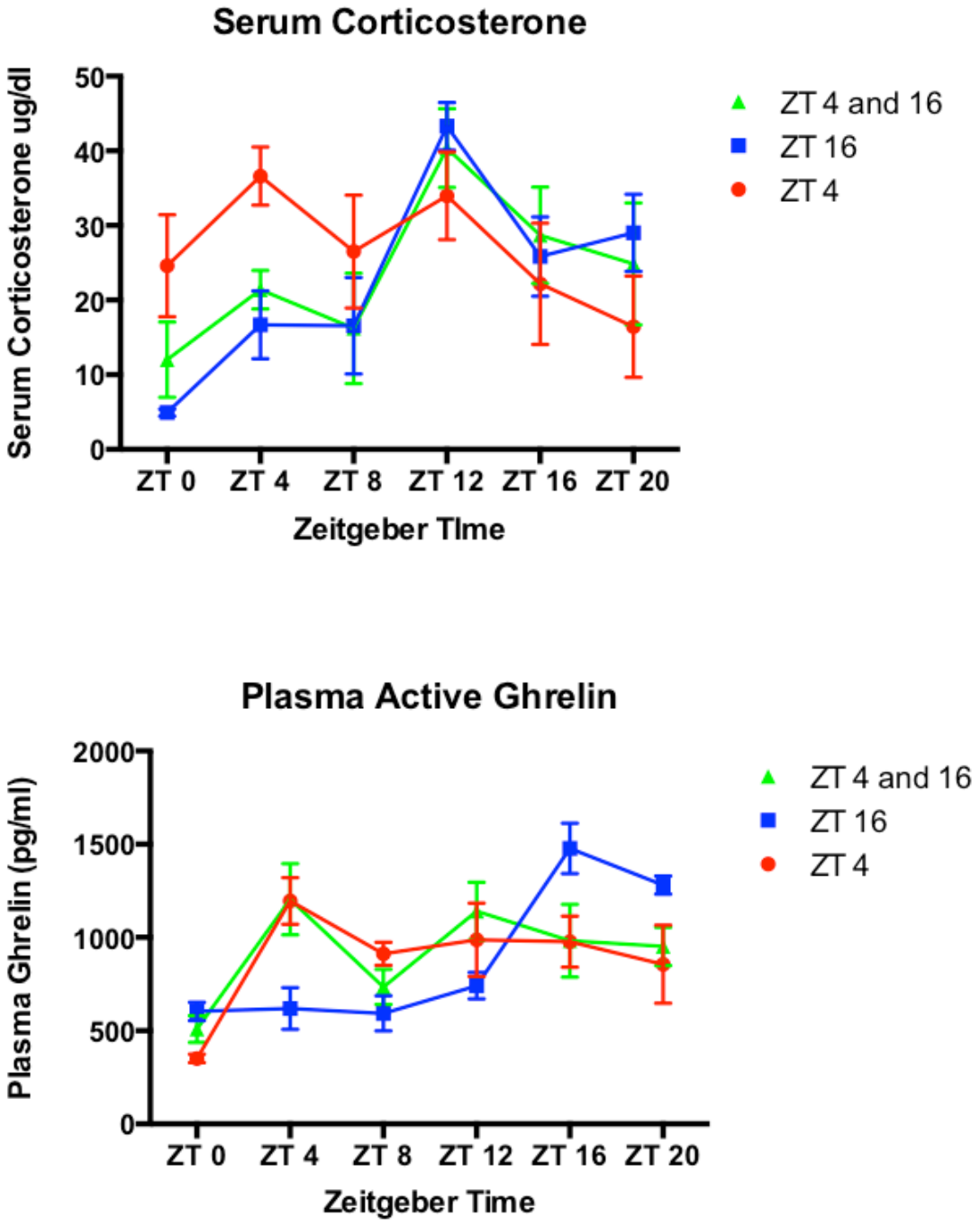
between ghrelin secretion, the clocks within the oxyntic cells of the stomach and behavioural FAA. In animals entrained to a mid day meal, returned to ad lib feeding for a week, and then subsequently food deprived for 1-4 days, behavioural FAA re-emerges at the prior mealtime, but the stomach clock as measured by *Per1-luc* reverts to a nocturnal phase consistent with nocturnal food intake during adlib food access. Gastric ghrelin release during these food deprivation days should peak at night if it is controlled by the oscillations of the oxytinic cells and not during the middle of the day. The persistence of anticipation after skipped meals is a critical test of a self-sustaining oscillator. Acyl-ghrelin rises prior to one skipped daily meal, but it then decreases and does not increase again prior to a second skipped meal (Liu et al., 2008, Kirchner et al., 2009). This may be due to the activity of the enzyme GOAT which requires ingested nutrients and is responsible for the acylation of ghrelin.

Our two meal anticipation schedule provides an additional test of this interpretation. Circadian FAA persists to both meals in a rat entrained to a 2 meal a day schedule under food deprivation. If ghrelin is mediating this FAA then a bimodal pattern of ghrelin secretion in advance of both meals would be expected during food deprivation. Ghrelin levels peaked prior to the meal time in both the ZT4 and ZT16 one-meal conditions. In the 2 meal condition, ghrelin peaked before the ZT4 meal and remained high through the rest of the day (Fig. 8). This dissociation adds further evidence against a role for ghrelin in mediating FAA to RF. When comparing the profiles of clock gene expression in the stomach with that of ghrelin levels in the single meal conditions the peak of *per2* coincides with the peak of plasma ghrelin. RF has been reported to shift the peak of ghrelin, PER1 and PER2 protein in the oxytinic cells of the stomach (LeSauter et al., 2009) however plasma ghrelin levels were not assessed in that study so it is difficult to compare the temporal patterns of plasma ghrelin with PER histology in this case. Under the two daily meal paradigm the expression of *Per2* is very low amplitude and variable throughout the day, it peaks at ZT4 as does plasma ghrelin however ghrelin levels remain generally high after this peak.

Collectively, the results of ghrelin knockout studies indicate that ghrelin secretion is not required for anticipation of a daily meal. If ghrelin does participate in circadian expression of food anticipatory activity under some experimental conditions, it is likely acting downstream from or in parallel with FEOs hypothesized to drive FAA, or it is

redundant with other FEOs or with other signals that can entrain FEOs. The site of action of ghrelin may be both hypothalamic and mesocorticolimbic, given recent observations that ghrelin receptor KO mice exhibit lower levels of food-anticipatory C-FOS activation (a correlate of neuronal activity) in lateral hypothalamic orexin neurons, the ventral tegmental area and the nucleus accumbens shell (Lamont et al, 2012). Whether ghrelin is responsible for controlling the phase of circadian oscillators in these areas, or in any peripheral tissues, has not yet been demonstrated.

Figure 8 Serum Corticosterone and Plasma Ghrelin Data



Group mean (± sem) waveforms of (A). Serum Corticosterone levels in (ug/dl). The interaction between ZT time and feeding condition was not significant $F(10,90)=1.701$, $p=0.09$, the effect of ZT time was significant $F(5,90)=6.224$, $p<0.0001$. and (B). Plasma Active Ghrelin the interaction between ZT time and feeding was significant $F(10,85)=22.38$, $p<0.0001$. Feeding schedule is denoted by color, ZT4 feeding condition (red), ZT16 feeding condition (blue), ZT4 and 16 feeding condition (green). N=6 animals per time point per condition.

Chapter 4.

Central Correlates of Food Anticipation

4.1. The SCN: Introduction

The master light-entrained circadian oscillator of the hypothalamus was ruled out as the site of the FEO decades ago (Stephen et al., 1979). However, since clock gene expression and function has been examined in the SCN extensively, including clock gene expression in the SCN when examining other brain regions of interest as a useful positive control. Additionally, information about the phase of the SCN may be useful for elucidating what signals are contributing to the phasing of the oscillation in question. For example, the oscillations of clock gene expression seen within extra-SCN brain regions is often in antiphase to the SCN under adlib conditions (Amir and Robinson 2006). However, some regions like the central nucleus of the amygdala and bed nucleus of the stria terminalus are in phase with the SCN under ad lib conditions (Amir et al. 2004). This appears to be due to neural projections from the SCN, since a unilateral SCN lesion results in ipsilateral arhythmicity in these structures. Under RF, however, the central nucleus of the amygdala and bed nucleus of the stria terminalus uncouple from the SCN, indicating that the FEO exerts some effect on these structures, (whether this is neural or diffusible is not known). This example illustrates the utility of examining the SCN when looking for oscillators altered by RF, as it provides a reference point for comparison.

The majority of studies examining the affect of RF on clock gene expression in the SCN have observed no changes due to RF (Damiola et al, 2000, Hara et al., 2001, Wakamatsu et al., 2001, Verwey et al., 2007). However, Minana-Solis et al 2009, reported a phase advance of the *Per1* oscillation in the SCN of RF fed rats. Additionally, PER1 protein has been found to be increased in anticipation of meal time in the SCN (Angeles-Castellanos et al., 2008), and alterations in C-FOS have been reported (Escobar et al., 2007). Behaviourally, the SCN is not necessary for the emergence of

FAA. However, RF does change the daily organization of the nocturnal activity controlled by the SCN. It is possible that changes to nocturnal activity may feed back on the SCN in a way that affects clock gene expression, but in the majority of cases oscillation of clock gene expression within the SCN was unaltered by RF.

Methodological differences between studies that could account for the shift or change in *Per1* expression observed in a few cases could include the duration of the food availability window, the number of days of RF, or the diet itself. In the Minana-Solis (2009) study, the daily meal was 2 hrs long. This is at the low end of the usual meal duration for an RF protocol. Some of the studies that show no influence of RF on the SCN used much longer feeding windows, up to 12 h in the light phase. Also, depending on the species and the starting weight of the animal, behavioural anticipation emerges after a variable number of days on RF. Oscillations of clock gene expression within peripheral tissues varies by structure, but in no case is it immediately re-set by RF. Therefore, it is possible that changes to clock gene expression within central oscillators does not emerge for several days under RF. It may be possible that changes were not observed in the SCN in some studies because the duration of the RF schedule was not long enough. For example, only 6-8 days were used in the most widely cited studies (Damiola et al 2000, Hara et al 2001 and Wakamatsu et al 2001). Multiple unit recordings suggest that restricted feeding or the activity associated with restricted feeding may have an inhibitory effect on the SCN. Whether this inhibition has a functional significance for clock gene expression and clock phase in the SCN is not known.

Clock gene expression within the SCN in animals anticipating multiple meals has not been examined previously. Behaviourally, animals readily anticipate two meals a day, and as previously discussed it is not known whether this requires one or two clocks. Given that the SCN is sensitive to locomotor activity feedback and changes in metabolic state, it is possible that the SCN is differentially affected by anticipation of two daily meals compared to one meal a day.

4.1.1. SCN: Results

The SCN showed variable levels of *Bmal-1* expression across feeding conditions (Fig 9). In the ZT4 fed rats no clear circadian rhythm of *Bmal-1* expression was observed. Visual inspection of the data showed a peak at ZT 20, but this was not significant. Additionally during the light phase *Bmal-1* expression was higher than the ZT 16 fed rats.

The ZT16 fed rats did not show a significant rhythm in *Bmal-1* expression however the highest level occurred at ZT 16 (feeding time). *Bmal-1* levels were low during the light phase in this group. As anticipated this waveform is similar to the oscillation of *Bmal-1* observed in the SCN of ad-lib fed animals.

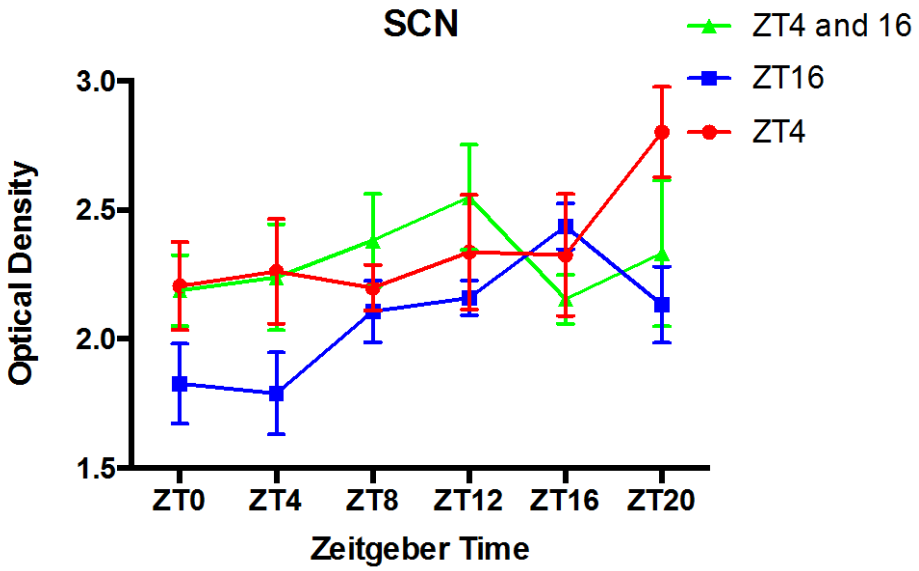
In the ZT 4 and 16 fed rats, *Bmal-1* expression was not rhythmic. Similar to the ZT4 feeding condition *Bmal-1* expression was elevated during the light phase, suggesting that daytime feeding increases *Bmal1* expression when it is usually low in ad-lib fed animals.

The SCN has generally been reported as impervious to the phase resetting effects of RF. RF in both the mid subjective day and night has little impact on the phase of *Per1* or *Per2* expression in the SCN (Damiola et al 2000, Wakamatsu 2001, Hara et al., 2001). However, Minana-Solis and colleagues (2009) reported a 3h phase delay in *Per1* with no change observed for *Per2* in rats fed from ZT6-8 for 3 weeks. In the Hara and Damiola reports the animals were on a restricted feeding schedule for 6 and 9 days, respectfully. In the Minana-Solis study the animals were on restricted feeding for 21 days. Therefore it might be that the lack of impact on the SCN in some studies is due to the short RF duration.

Four studies have examined *Bmal1* expression in the SCN of animals on restricted feeding, two using rats and two using mice. Minana-Solis (2009) reported that *Bmal-1* expression in the SCN of rats on RF is unchanged compared to ad lib controls. Inspection of the wave form suggests a delayed peak of about 8h but this does not reach significance. Again Girotti (2009) report no change in *Bmal-1* expression in the SCN of rats in RF, but they examined only 2 time points which limits the ability to detect shifts. Mendoza and colleagues (2005) report that in mice *Bmal-1* expression in the SCN as

measured by ISH is not different between ad lib, normocalorie or hypocalorie feeding schedules. Similarly, Moriya and colleagues (2009) found no alterations in the phase of *Bmal-1* expression in the SCN between ad lib and RF fed mice. There are no data available to compare our two meal waveforms with. Further investigation into the effects of multiple meal anticipation on the SCN is needed. From the ZT4 and two meal fed data it appears that RF during the mid subjective day results in an increase of *Bmal-1* expression at the time of day when it is usually low.

Figure 9 Suprachiasmatic Nucleus *Bmal-1* In Situ Data



. Group mean (± sem) waveforms of *Bmal-1* optical density as measured by in situ hybridization in the SCN. Feeding schedule is denoted by color, ZT4 feeding condition (red), ZT16 feeding condition (blue), ZT4 and 16 feeding condition (green) n=5-6 animals per time point per condition. In the SCN the interaction between ZT time and feeding schedule was not significant $F(10,82)=1.193$, $p=0.31$. The effect of ZT time was also not significant $F(5,82)=1.859$, $p=0.11$.

4.2. The Olfactory Bulb: Introduction

Robust endogenous oscillations of *Per1* have been reported in the olfactory bulb (Abe et al 2002). Subsequently, it was reported that the *Per1* rhythms in cultured olfactory bulbs persist in SCN-ablated animals. This rhythm can be entrained by 1.5˚C temperature cycles, but the period of the olfactory bulb is nonetheless temperature-

compensated, as warming or cooling the culture media does not speed or slow the rate of the *Per1* oscillation in the cultured olfactory bulb. Further, the olfactory bulb remains rhythmic in rats that are behaviourally arrhythmic due to constant light exposure (LL) or SCN lesion (Granados-Fuentes et al., 2004). Previous evidence has suggested a role for the olfactory bulb in the circadian timing system. First, olfactory bulbectomy has been reported to lengthen tau in hamsters and mice (Possidente et al 1990, Pieper and Lobocki, 1999) and shorten tau in rats (Granados-Fuentes et al, 2006). Second, olfactory bulbectomy has been shown to alter the rate of re-entrainment to shifts in the LD cycle. The rate of re-entrainment to phase advances was increased, whereas the rate of re-entrainment to delays was decreased (Granados-Fuentes et al 2006). Re-entrainment to phase shifts in the LD cycle is also slower in olfactory bulb-ablated *Octagon degus* (Goel and Lee, 1995, 1997; Lee and Lebyak, 1997; Goel et al., 1998). Third, light induced phase shifts in locomotor activity and SCN C-FOS expression can be potentiated using olfactory stimulation (Amir et al, 1999a). Fourth, odor-evoked activity within the SCN displays a circadian pattern (Amir et al., 1999b). In mice presented with an olfactory cue at 4 different times of day, neural response as measured by C-FOS counts were nearly 4 times higher in the subjective night compared to the subjective day in both the olfactory bulb and the piriform cortex (Granados-Fuentes et al., 2006). In SCN-ablated mice, this rhythmic modulation in the response to olfactory cues was maintained, but peaked 8hrs earlier, suggesting that the SCN is not required for a functional rhythm driven by the oscillation localized to the olfactory bulb (Granados-Fuentes et al., 2006). Due to their similar function, the existence of neural projections between the olfactory bulb and the piriform cortex, and the similar phasing of a spontaneous peak of C-FOS expression in the two regions at CT0, Granados-Fuentes and colleagues speculated that the olfactory bulb clock may drive rhythms in the piriform cortex (although Abe et al, 2002, documented a lack of rhythmicity in the piriform cortex in culture, it is possible this was due to the severing of connections between it and the olfactory bulb). It was found that bulbectomy significantly attenuated the rhythm of C-FOS in the piriform cortex and that unilateral bulbectomy attenuated the C-FOS rhythm in the ipsilateral piriform cortex suggesting that the olfactory bulb exerts control of rhythms in the piriform cortex via a neural connection, thereby indicating a rationale for the lack of rhythmicity in the piriform cortex observed in culture.

FAA as measured by either wheel running activity (Coleman and Hay, 1990) or food bin-directed activity (Davidson et al., 2003) develops normally in olfactory bulbectomized animals. Further, the amplitude of FAA is similar between controls and olfactory bulb ablated rats, and FAA persists in olfactory bulb-ablated animals through 3 days of total food deprivation (Davidson et al., 2003). However, there is evidence that the olfactory bulb can be entrained by restricted feeding, suggesting that the olfactory system may play a role as one of many FEOs within the brain. In rabbit pups provided access to their mother in either the subjective day or night, PER1 and C-FOS levels in the olfactory bulb were found to increase prior to mealtime. However, the PER1 increase was much more pronounced in the pups fed during the subjective night. When the pups were fasted the subjective day rhythm was slightly advanced, while in the subjective night group, the rhythm became bimodal and decreased in amplitude (Nolasco et al., 2012). In Per2:luciferase rats however, the olfactory bulbs from ad lib and RF fed rats were found to oscillate in culture at the same phase, suggesting that RF did not have an impact on the *Per2* rhythm in the olfactory bulb (Natsubori et al., 2013).

4.2.1. The Olfactory Bulb: Results

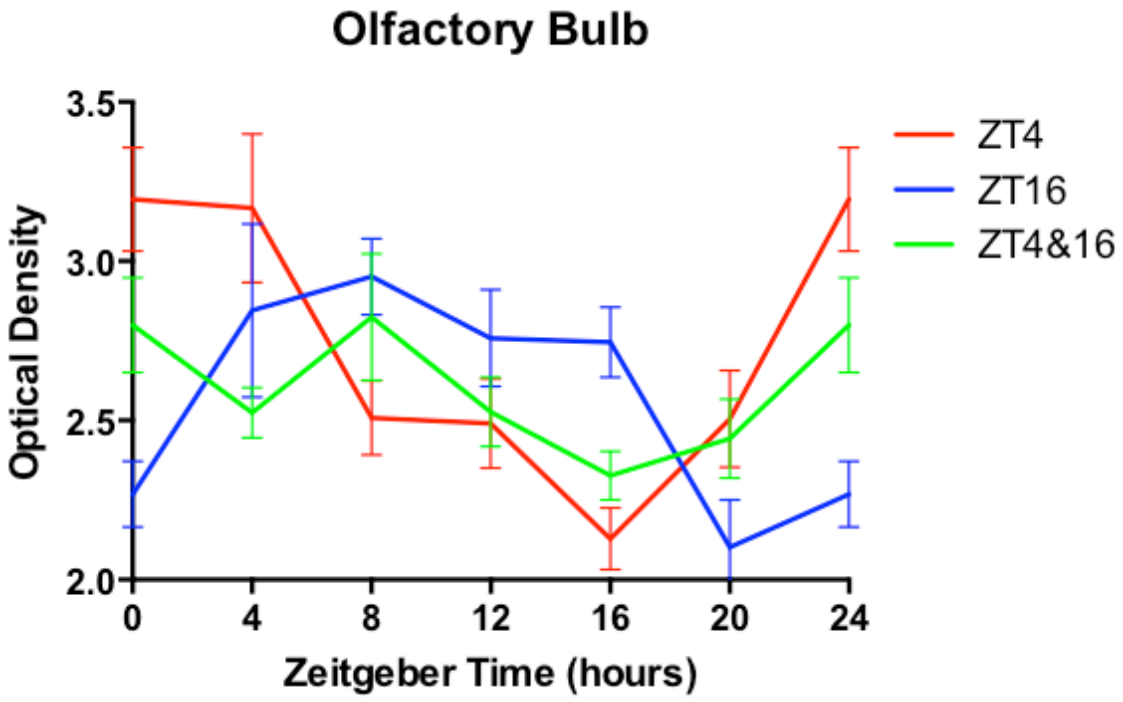
The olfactory bulb showed a circadian oscillation that was altered by restricted feeding (Fig 10). In the ZT4 condition, *bmal-1* peaked at ZT0, and remained high until meal-time at ZT4, and troughed at ZT16. The amplitude of *Bmal-1* expression in the ZT4 fed animals was slightly higher than the ZT16 or two meal feeding conditions. In the ZT16 feeding condition, *Bmal-1* peaked at ZT8 and remained elevated until feeding time at ZT16. Similar to the ZT4 feeding condition, there was a steep decrease in *Bmal-1* after meal time in the ZT16 condition with the trough level occurring at ZT20. In the two daily meal condition, there were two peaks in *Bmal-1* expression although neither of these precede either mealtime. The two peaks occurred at ZT8 and ZT24 and the troughs occur at the ZT4 and 16 meal times .

These data suggest that the circadian oscillator located in the olfactory bulb is entrained by RF. This is indicated by the antiphasic peaks in *Bmal-1* expression observed in the ZT4 and ZT16 single meal feeding conditions. The two daily meal condition is more difficult to interpret. The waveform is more flat and variable than the single meal conditions. This could be explained in multiple ways. Firstly, individual clock

cells in the olfactory bulb may become dissociated from one another, where some cells are set by the AM meal and others are set by the PM meal, thereby flattening the rhythm. To clarify this issue, clock gene expression in the olfactory bulb would need to be analyzed using a technique that provided better spatial resolution, for example ICC or single cell recordings. Secondly, inter-animal variation may contribute to the observed results: Some animals may have entrained to the AM meal while others entrained to the PM meal and therefore in aggregate, the rhythm is dampened. Since behavioural data were not collected for every animal, correlations between FAA amplitude prior to the two meals and clock gene expression cannot be run.

Nolasco et al 2012, reported that PER1 expression was higher in rabbit pups that were nursed in the subjective night. Furthermore, in mice exposed to olfactory stimuli at 4 different times of day (2 in the subjective day and 2 in the subjective night), the C-FOS response was significantly greater during the subjective night. This suggests that the olfactory system is more sensitive when animals are behaviourally active (Granados-Fuentes et al., 2006). Since in this experiment the olfactory stimuli were given acutely, it may be that repeated stimulation at the same time of day (as in RF) may result in a greater response at the time of presentation, independent of the rest activity cycle. Recall that FAA is still observed in olfactory bulbectomized rats (Colman and Hay 1990; Davidson et al 2003). Therefore the olfactory bulb is not necessary for the expression of FAA, however it is likely that the olfactory bulb is a component of a distributed system of FEOs. Due to its role in olfaction, the olfactory bulb may provide an input signal to other FEOs. There is evidence that the olfactory bulb can entrain rhythms of C-FOS in other brain regions including the piriform cortex and the SCN. Odour presentation can entrain C-FOS expression in the piriform cortex and these rhythms are lost in olfactory bulbectomized animals (Granados-Fuentes et al., 2006). Further, olfactory bulbectomy increases the rate of re-entrainment to phase advances of the LD cycle, and presentation of olfactory stimuli increases the amplitude of C-FOS rhythms in the SCN (Granados-Fuentes et al., 2006). These data provide rationale for further investigation into the role of the olfactory bulb and olfaction in FAA.

Figure 10 Olactory Bulb *Bmal-1* In Situ Data



Group mean (± sem) waveforms of *Bmal-1* optical density as measured by in situ hybridization in the mitral cell layer of the olfactory bulb. Feeding schedule is denoted by color, ZT4 feeding condition (red), ZT16 feeding condition (blue), ZT4 and 16 feeding condition (green) n=5-6 animals per time point per condition. In the olfactory bulb the interaction between ZT time and feeding condition was significant $F(10,82)=4.692$, $p<0.0001$.

4.3. The Piriform Cortex: Introduction

The piriform cortex (or olfactory cortex) is implicated in olfaction. Given that olfaction is implicated in mammalian feeding behaviour by helping animals to locate a food source (Giachetti et al., 1970), it is possible that all or various parts of the olfactory system may become entrained by RF. As discussed, the olfactory bulb contains a self-sustained circadian oscillator, and the piriform cortex receives a large amount of olfactory bulb efferent fibres (Brunjes et al., 2005). These inputs appear to be partially responsible for the synchronization of C-FOS rhythms in the piriform cortex. Indirect evidence also suggests that these inputs may be important for the rhythmic expression of clock genes in the piriform cortex, since the piriform cortex is found to be arrhythmic in culture (Abe et al., 2002), but rhythmic when examined using in situ for *Bmal-1, Per1*

and, *Per2* (Abe et al., 1998; Homna et al 1998; Wakamatsu et al, 2001; Reick et al, 2001) or immunocytochemistry for PER1, but not PER2 (Feillet et al., 2008).

The effect of RF on the piriform cortex has not been widely investigated. Feillet and colleagues (2008) reported that the rhythm of PER1 protein in the piriform cortex was advanced by 3 hr in RF animals vs. ad lib controls. Wakamatsu and colleagues 2001, assessed a mid subjective day and mid subjective night time point and showed that in RF animals *Per1 and Per2* were higher than controls in the mid subjective day and lower than controls in the mid subjective night. Together, these studies suggest that clock gene rhythms in the piriform cortex are sensitive to RF.

As previously mentioned, there is a daily rhythm in the number of C-FOS cells in the olfactory bulb and the piriform cortex. The response to an olfactory stimulus (cedar oil) is maximal during the subjective night and lowest during the subjective day in both the olfactory bulb and the piriform cortex. Removal of the olfactory bulb bilaterally demonstrates that rhythmic C-FOS expression in the piriform cortex is dependent on a signal from the olfactory bulb, as olfactory bulbectomized animals no longer show a rhythm in C-FOS in the piriform cortex. Additionally, unilateral olfactory bulbectomy selectively reduces the rhythm of C-FOS in the ipsilateral piriform cortex while leaving the contralateral piriform cortex unaffected, again highlighting the importance of the olfactory bulb efferents to the piriform cortex (Granagos-Fuentes et al., 2006). As indicated previously, the olfactory bulb is not required for FAA as olfactory bulbectomized animals display normal FAA (Coleman and Hay 1990, Davidson et al., 2003). However evidence suggests that the olfactory bulb and in turn in the piriform cortex are entrained by RF. To investigate this further and explore the effects of our 2 meal paradigm on clock gene rhythms in the piriform cortex we assessed the optical density of *Bmal-1* in this region.

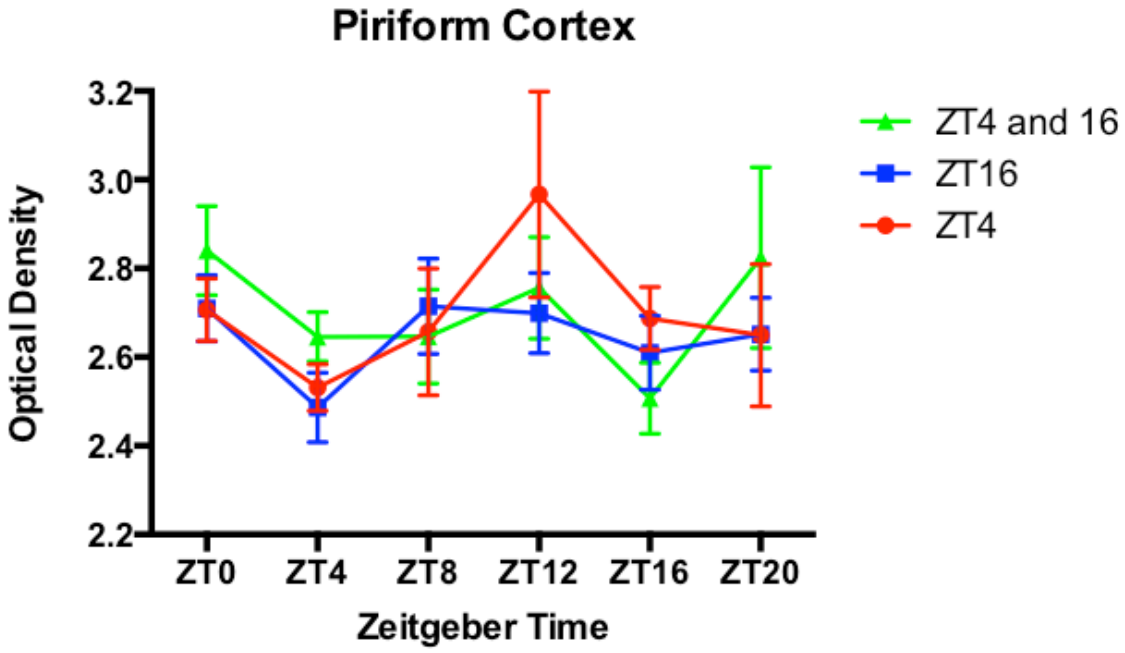
4.3.1. The Piriform Cortex: Results

The piriform cortex was not rhythmic under any of the three feeding conditions. This result is surprising given previous reports of rhythmic clock gene expression in this region. Furthermore Feillet and colleagues (2008) provided evidence that RF can alter PER protein levels within this cortical region. It has been suggested that the piriform

cortex receives entrainment signals from both the SCN and the olfactory bulb (Granagos-Fuentes et al., 2006). It is possible that under RF conditions the piriform cortex receives conflicting signals from these two oscillators. Evidence suggests that the outputs from the SCN and the olfactory bulb in animals under RF would likely be antiphasic to one another, therefore this provides a possible rationale for the lack of *Bmal-1* rhythms in the piriform cortex. If this is the case it would make sense that rhythms in this region are observed in ad lib fed animals (Abe et al., 1998; Homna et al 1998; Wakamatsu et al, 2001; Reick et al, 2001) but not in animals on RF. It may be possible to determine whether the piriform cortex is entrained by RF in SCN lesioned animals. Using this preparation the phase of clock gene expression within this region could be examined in the absence of competing inputs from the SCN.

Had we observed entrainment of the piriform cortex by RF we would have expected to see unimodal rhythms of *Bmal-1* expression in both the single meal conditions. These unimodal rhythms if entrained by RF would likely be antiphasic to one another given that the meals in each of these conditions were exactly 12 hours apart.. However, entrainment by RF was not observed in either of the single meal conditions or the two meal condition. This suggests that the piriform cortex is not a FEO.

Figure 11 Piriform Cortex *Bmal-1* In Situ Data



Group mean (± sem) waveforms of *Bmal-1* optical density as measured by in situ hybridization in the piriform cortex. Feeding schedule is denoted by color, ZT4 feeding condition (red), ZT16 feeding condition (blue), ZT4 and 16 feeding condition (green) n=5-6 animals per time point per condition. The interaction between ZT time and feeding condition was not significant $F(10, 86)=0.7$, $p=0.72$. The effect of time was also not significant $F(5,86)=2.004$, $p=0.086$.

4.4. Habenula: Introduction

Information emanating from the limbic forebrain and striatal structures converge at the habenula (Nauta, 1974; Hisosaka et al., 2008). This has led to the general hypothesis that the habenula has a functional role in regulation of movement and motivational processes (Sutherland, 1982; Bromberg-Martin et al., 2010) both of which are relevant to behavioural FAA. The habenula is composed of the medial and the lateral habenular nuclei, the latter of which is further divided into medial and lateral components. The inputs to the medial habenula and lateral differ, with the medial habenula receiving input from the septal area and to a smaller extent the limbic forebrain while the lateral habenula receives input from the limbic forebrain, septum, lateral preoptic area, lateral hypothalamus and striatum (Sutherland, 1982). Similarly, efferents from the two sub-divisions also differ, with the majority of medial habenula fibres terminating in the interpeduncular nucleus, while the lateral habenula sends fibres to the

serotonergic medial and dorsal raphe nuclei, the dopaminergic substantia nigra, medial forebrain bundle, lateral hypothalamic area, several thalamic nuclei, and the ventrolateral septum (Sutherland, 1982).

Due to the convergent nature of its inputs and wide variety of its outputs, the habenula and more specifically the lateral habenula has been implicated in a wide variety of behaviours that are modulated by emotion, motivation and behavioural state, including learning and memory and reward-based locomotor activity (Klemm, 2004; Lecourtier and Kelly 2007). Early research investigated the importance of the habenula in olfaction, however these reports were often contradictory (Rausch and Long, 1971; Rosene, 1976). Long latency-evoked potentials within the habenula have been recorded in response to electrical stimulation of the olfactory bulb, indicating that it may receive olfactory input via a multisynatpic pathway (Mok and Morgenson, 1974), as the once hypothesized projection from the olfactory bulb and piriform cortex was found to actually terminate in the dorsomedial nucleus of the thalamus (Heimer, 1972). More recent reports of involvement of the habenula in memory retrieval of an olfactory task (Tronel and Sara, 2002, Roullet et al., 2005) have renewed interest in a role for the habenula in olfactory related processes. The habenula has also been investigated with respect to feeding. The conclusion of a small number of studies was that the habenula may play a role in the limbic modulation of hypothalamic feeding centers, but is not likely to be involved in the regulation of food intake (Sutherland, 1982).

A role for the habenula in reward-based motor control has been described. Lateral habenula neurons are excited by a sensory event signaling a reward that will be less rewarding than predicted or punishment (Matsumoto and Hikosaka, 2007). This activation of lateral habenular neurons inhibits dopaminergic neurons in the dorsal and lateral striatum (Ji and Shepard, 2007). It is hypothesized that this suppression may prevent engaging in behaviours that lead to negative outcomes (Hikosaka et al., 2008). Conversely, lateral habenular neurons are inhibited by a sensory event signaling a reward that will be more rewarding than predicted. This inhibition of lateral habenular neurons results in disinhibition of dopaminergic neurons (Lecourtier et al., 2006). It is hypothesized that this disinhibition may have a functional behavioural outcome, to repeat a behaviour that results in a desirable action (Hikosaka et al., 2008). Taken together, the

habenula has been implicated in many behavioural process related to food anticipation including learning and memory, olfaction and reward prediction.

Rhythms of PER2 from PER2:luc mice have been localized to the medial part of the lateral habenula and the ependymal cell layer at the dorsal aspect of the third ventricle (Guilding et al., 2010). The periods of the PER2 rhythm in these two regions are slightly different from one another indicating the presence of two separate oscillators in this structure. The rhythms in the medial part of the lateral habenula and the ependymal cell layer dampen after 2-3 cycles in culture and can be reset using forskolin (stimulator of adenylylcyclase). The dampening of these regions suggest that the oscillators within the habenula require phase setting stimuli from the SCN or other oscillators within the brain for the generation of coherent circadian rhythms. In the rat, a circadian rhythm of spontaneous cell firing rate from the medial aspect of the lateral habenula has been reported to peak in the subjective day both in vivo and in vitro (Zhao and Rusak, 2005). In mice, the peak of cell firing rate was found in the subjective night in vitro (Guilding et al., 2010). It appears that circadian rhythms in the habenula are generated by cell autonomous clocks as treatment with tetrodotoxin, which blocks action potentials, did not alter the PER2::LUC rhythm (Guilding et al., 2010).

Circadian rhythms of neuronal activation assessed by C-FOS immunocytochemistry have also been observed in the habenula (Chastrette et al., 1991; Tavakoli-Nezhad and Schwartz, 2006). However, these two studies were conducted in different species and found different results. The C-FOS rhythm peaked during the light phase in rats and in the dark phase in hamsters. mRNA for *Per1* and *Per2* have been observed in the habenula of the rat (Shieh, 2003) but examination of PER1 and PER2 protein using ICC showed no staining in hamster tissue (Tavakoli-Nezhad and Schwartz, 2006). Whether these differences are due to technical issues or real differences in the habenula across species remains to be clarified.

4.4.1. Habenula: Results

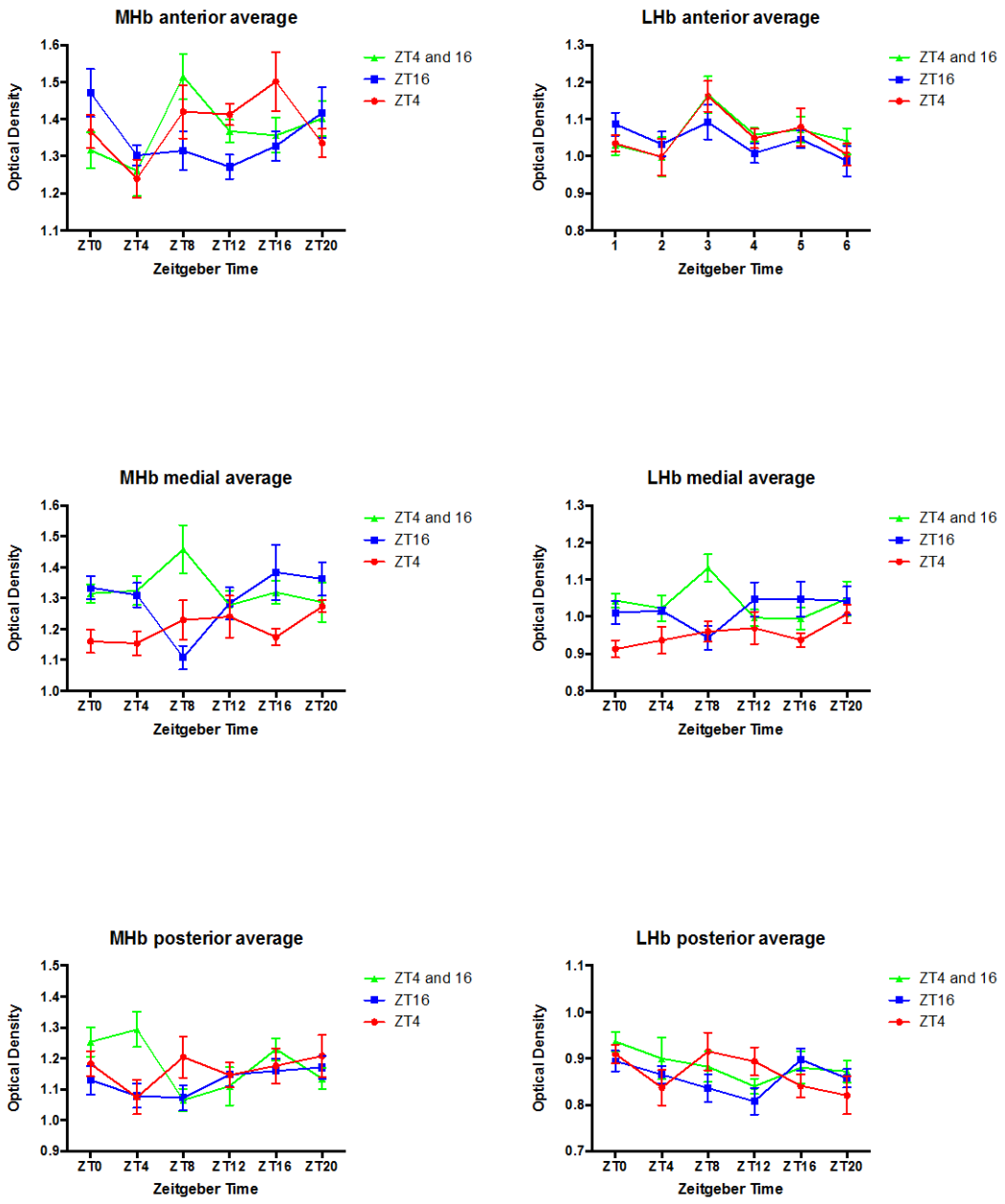
The spatial resolution of the in situ data prevented the differentiation of the lateral habenula into medial and lateral parts preventing a direct comparison with the Guilding et al 2010 data. In the anterior medial habenula circadian rhythmicity of *Bmal-1*

expression was observed under all three feeding conditions. In the ZT4 fed condition *Bmal-1* peaked at T16. In the ZT 16 condition *Bmal-1* peaked between ZT20 and ZT0. Finally in the two meal feeding condition *Bmal-1* peaked at ZT8. This pattern was not maintained in the medial or posterior sections of the medial habenula however. In rats fed at ZT 4 *Bmal-1* expression was not rhythmic in the medial or posterior aspects of the medial habenula. In the ZT 16 fed rats, *Bmal-1* was highest in the late subjective night in the medial aspect and not rhythmic in the posterior aspect of the medial habenula. Finally in the two meal fed rats *Bmal-1* expression was highest at ZT 8 in the medial aspect and not rhythmic in the posterior aspect of the medial habenula.

Contrary to Guilding et al 2010, no rhythms of *Bmal-1* expression were observed in the lateral aspect of the habenula under any of the three feeding conditions. This may be due to the heterogeneity of the lateral aspect of the habenula reported by Guilding et al 2010. Attempting to analyze clock gene expression in this region using a technique that allows the parsing of this region would help to resolve this. Entrainment of clock gene expression in the habenula by RF has not been previously examined. Therefore we cannot compare our *Bmal-1* results with another published data set.

Had we observed entrainment of the lateral or medial habenula by RF we would have expected to see unimodal rhythms of *Bmal-1* expression in both the single meal conditions. These unimodal rhythms if entrained by RF would likely be antiphasic to one another given that the meals in each of these conditions were exactly 12 hours apart. In the case of the two meal condition we sought evidence for either the multiple FEO or continuously consulted clock models. However, entrainment by RF was not observed in either of the single meal conditions or the two meal condition. This suggests that the habenula is not a FEO.

Figure 12 Habenula *Bmal-1* In Situ Data



Group mean (± sem) waveforms of *Bmal-1* optical density as measured by in situ hybridization in the medial and lateral habenula. Feeding schedule is denoted by color, ZT4 feeding condition (red), ZT16 feeding condition (blue), ZT4 and 16 feeding condition (green) n=5-6 animals per time point per condition. A. anterior aspect of the medial habenula the interaction between ZT time and feeding condition was significant $F(10,174)=2.354$, $p=0.01$. B. medial aspect of the medial habenula the interaction between ZT time and feeding was significant $F(10,182)=2.954$, $p=0.002$. C. posterior aspect of the medial habenula the interaction between ZT time and feeding condition was significant $F(10,176)=1.948$, $p=0.042$. D. anterior aspect of the lateral habenula the interaction between ZT time and feeding was not significant $F(10,174)=0.635$, $p=0.782$. E. medial aspect of the lateral habenula the interaction between ZT time and feeding was not significant $F(10,174)=0.6354$, $p=0.782$. F. posterior aspect of the lateral habenula the interaction between ZT time and feeding was not significant $F(10,175)=1.265$, $p=0.254$.

4.5. Dorsal Medial Hypothalamus: Introduction

The dorsal medial hypothalamus is thought to be important for the incorporation of rhythmicity into feeding, sleep, endocrine output and thermoregulation. This is due to its interconnectivity with structures that mediate these behaviours, including the arcuate nucleus, lateral hypothalamus, paraventricular nucleus of the hypothalamus and medial preoptic area (Elmquist et al., 2005). With respect to feeding, the dorsal medial hypothalamus responds to several feeding related hormones, including leptin, cholecystokinin and ghrelin (Elmquist et al, 2005) and is responsive to glucose (Berthoud, 2002).

The role of the dorsal medial hypothalamus in the generation of FAA has been the subject of great debate (Landry et al., 2006, Landry et al, 2007, Gooley et al., 206, Fuller et al., 2008, Mistlberger et al., 2008, Mistlberger et al., 2009, Fuller et al., 2008). The details of this debate will be summarized here only in brief. The experiment conducted by Gooley et al, 2006 examining behavioural FAA after dorsal medial hypothalamus lesion using ibotinic acid overstate the relevance of their data and cannot provide confirmation of their claims that the dorsal medial hypothalamus is essential for FAA. The experiments conducted by Fuller et al., 2008 claiming that *Bmal-1* in the dorsal medial hypothalamus was necessary for FAA were severely flawed in terms of data management, and interpretation of results (Mistlberger et al., 2008; Mistlberger et al., 2009). Three independent laboratories have demonstrated that rats and mice with dorsal medial hypothalamus ablations can show robust FAA (Moriya et al., 2009, Landry et al., 2006, 2007, 2011; Acosta-Gavin et al, 2011).

Therefore, our intent in studying the dorsal medial hypothalamus is not to further address whether the dorsal medial hypothalamus is the master FEO for FAA, but rather to examine what role it might play based on how it responds to 1 and 2-meal feeding schedules. The literature on expression of clock genes and CFOS within the dorsal medial hypothalamus is not consistent and additional data are needed.

The dorsal medial hypothalamus from PER2::LUC mice oscillates in culture. This oscillation is not dependent on intercellular signaling involving action potentials, as it persists when tetrodotoxin is applied to the culture. The oscillation does dampen out

after 2-3 cycles but can be re-instated by applying forskolin. The period of the dorsal medial hypothalamus oscillation is not altered in mice maintained on a high fat diet. It is also not phase shifted by acute short term food deprivation (Guilding et al., 2009).

Under ad lib conditions most studies have not observed a circadian rhythm in the expression of *Per1* in the dorsal medial hypothalamus (Mieda et al., 2006; Mihana-Solis et al., 2009). *Per2* has been reported to be rhythmic under ad lib conditions by Mihana-Solis and colleges (2009) but not by Mieda and colleagues (2006). Mihana-Solis and colleagues (2009) also report that *Bmal-1* expression is not rhythmic in the dorsal medial hypothalamus under ad lib conditions.

Under RF conditions *Per1* has been reported to be rhythmic with a peak at meal time (Mihana-Solis et al., 2009;) or during the meal (Mieda et al., 2006). *Per2* has been reported to be dampened by RF (Mihana-Solis et al., 2009) or rhythmic with a peak during the meal (Mieda et al., 2006). *Bmal-1* was reported to remain arrhythmic during RF (Mihana-Solis et al., 2009). Immunocytochemistry for PER1 protein has been described as rhythmic under ad lib (Feillet et al., 2008), while PER2 has been reported as arrhythmic under these same conditions (Amir et al., 2004, Lamont et al., 2005, Verwey et al., 2007, Verwey et al., 2008). Under RF conditions PER1 has been reported as rhythmic with a peak similar to ad lib fed animals at ZT11 (Feillet et al., 2008). This group did not find PER2 staining in the dorsal medial hypothalamus of their rats. RF has been reported to induce a rhythm of PER2 in the dorsal medial hypothalamus with a peak at food presentation and high levels throughout the meal (Verwey et al., 2007, 2008). Interestingly, in animals fed a palatable meal on an ad lib background PER2 rhythmicity is not induced in the dorsal medial hypothalamus although FAA is expressed (Verwey et al., 2007; Waddington-Lamont et al., 2007) and C-FOS is increased. These findings are important for two reasons. First FAA is not dependent on PER2 rhythms in the dorsal medial hypothalamus as suggested by Mieda and colleagues (2006). Second, the mechanisms driving rhythms of neural activation as measured by C-FOS and the circadian clock as measured by PER2 are not coupled.

In the majority of reports examining C-FOS protein in the dorsal medial hypothalamus from ad lib fed animals no temporal pattern was observed, with relatively low levels of C-FOS being expressed at all time points (Angeles-Catellanos et al., 2003,

Mendoza et al., 2005, Verwey et al., 2007, Renner et al., 2010). One study reported a rhythm of C-FOS expression in ad lib animals that peaked during the subjective night (Gooley et al., 2006). Under RF during the mid subjective day the general trend of C-FOS expression was an increase prior to feeding with a peak occurring mid meal or at the end of the meal. However this slight increase during consumption was not significantly different from the point at food presentation and therefore it was often cited that C-FOS peaked in anticipation of the scheduled meal (Angeles-Catellanos et al., 2003, Mendoza et al., 2005, Gooley et al., 2006; Verwey et al., 2007). When C-FOS is assessed in animals that are not provided with a meal at the expected time, the amplitude of this rhythm is decreased (Angeles-Castellanos et al., 2003) indicating that C-FOS expression within the dorsal medial hypothalamus may be related to consumption. A similar conclusion was drawn from a study of mice, which showed much more C-FOS after the mice had eaten than immediately prior to mealtime (Steele et al, 2012).

Rhythms of *Bmal-1* in the dorsal medial hypothalamus under RF have only been assessed in one study, therefore examining *Bmal-1* expression in our three feeding conditions will add significantly to the literature.

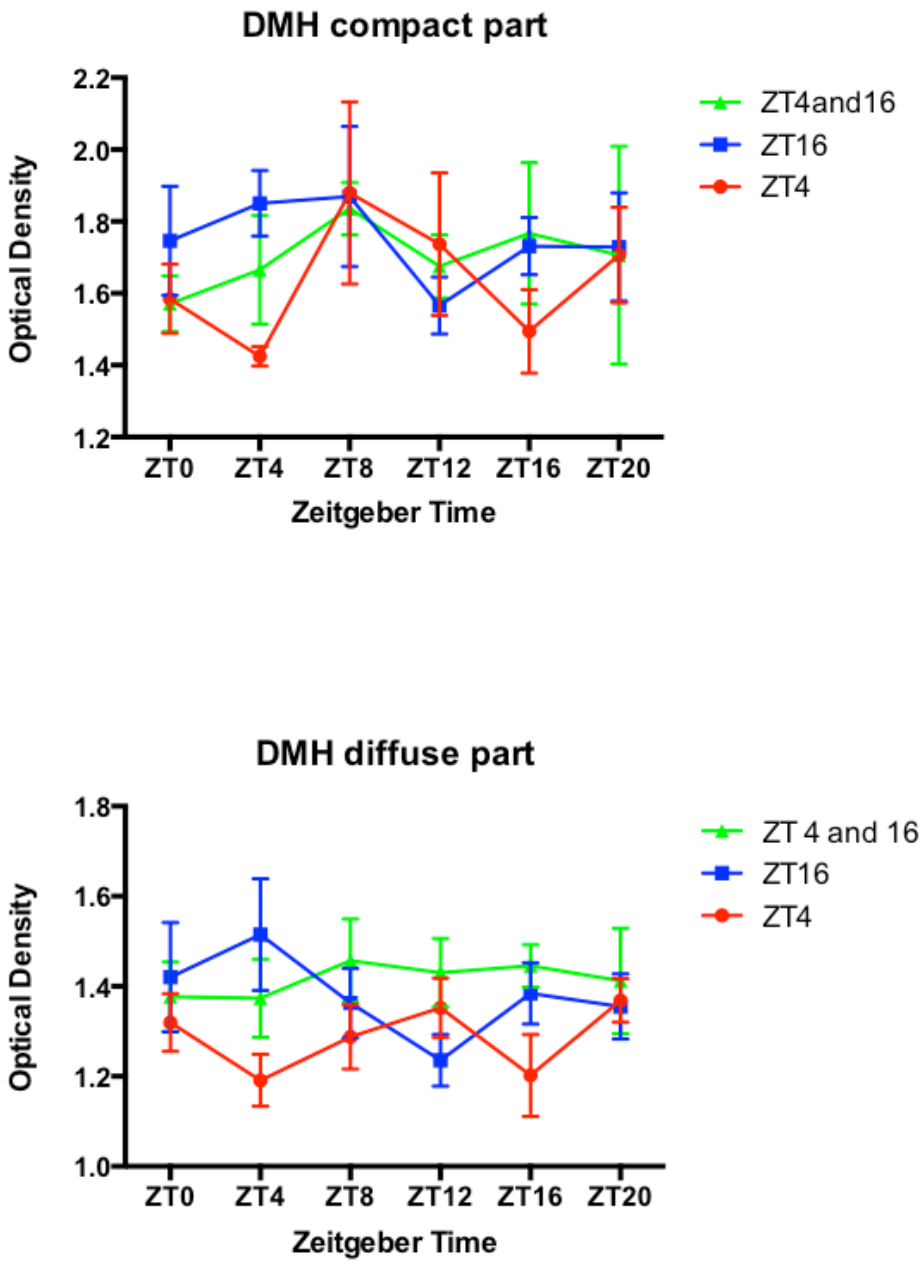
4.5.1. Dorsal medial hypothalamus: Results

Rhythms of *Bmal-1* expression within the dorsal medial hypothalamus were not observed under any restricted feeding schedule. Robust expression of *Bmal-1* was observed specifically in the compact region of the dorsal medial hypothalamus but this did not reliably vary as a function of time of day or feeding condition. Whether this is attributable to the chosen technique could be examined further using RT PCR. The result is consistent with the study of Mihana-Solis et al 2009, and does not support a hypothesis that the dorsal medial hypothalamus is a circadian oscillator that drives food anticipatory activity.

Had we observed entrainment of *Bmal-1* expression by RF in the dorsal medial hypothalamus we would have expected to see unimodal rhythms in both the single meal conditions. These unimodal rhythms if entrained by RF would likely be antiphasic to one another given that the meals in each of these conditions were exactly 12 hours apart. In

the case of the two meal condition we sought evidence for either the multiple FEO or continuously consulted clock models. However, entrainment by RF was not observed in either of the single meal conditions or the two meal condition. This suggests that the dorsal medial hypothalamus is not a FEO.

Figure 13 Dorsal Medial Hypothalamus In Situ Data



Group mean (± sem) waveforms of *Bmal-1* optical density in the dorsal medial hypothalamus as measured by in situ hybridization. Feeding schedule is denoted by color, ZT4 feeding condition (red), ZT16 feeding condition (blue), ZT4 and 16 feeding condition (green) n=5-6 animals per time point per condition. A. compact region of the dorsal medial hypothalamus here the interaction between ZT time and feeding was not significant $F(10,77)=0.844$, $p=0.589$. B. diffuse region of the dorsal medial hypothalamus here the interaction between ZT time and feeding condition was not significant $F(10,75)= 1.071$, $p=0.395$..

4.6. Nucleus Accumbens: Introduction

Located in the basal forebrain, the nucleus accumbens along with the olfactory tubercle form the ventral striatum. The Nucleus Accumbens can be divided into two components, the nucleus accumbens core, and nucleus accumbens shell. Neuronal activation as measured by C-FOS expression in rats on RF show increased C-FOS levels relative to ad lib controls in both areas. Increases of C-FOS in the core of the nucleus accumbens occur slightly before the increase in the shell of the nucleus accumbens although this difference is not very large upon inspection of the data (Mendoza et al., 2005). Extracellular dopamine levels increase in the core of the nucleus accumbens prior to food delivery, while dopamine increases in the shell of the nucleus accumbens during consumption (Bassaero and Chiara, 1999). By combining the C-FOS and extracellular dopamine data the authors hypothesize that the core of the nucleus accumbens is primarily responsive during anticipation or the “wanting” phase, while the shell of the nucleus accumbens is responsive during consumption and is more related to “liking” (Mendoza et al, 2005; Angeles-Castellanos et al., 2007). The changes in CFOS expression in response to RF were maintained during food deprivation in the core of the nucleus accumbens and are consistent with a role for this area in the circadian anticipation of food delivery (Mendoza et al., 2007).

As previously mentioned, FAA can also be observed to restricted access to palatable food on an ad lib background. The levels of C-FOS expression within the core and the shell of the nucleus accumbens are higher in rats anticipating a palatable meal compared to those observed under RF. This suggests that C-FOS within these regions may be more related to the rewarding aspects of restricted feeding than with the homeostatic deficit that accompanies RF, which may be better reflected in the activation of hypothalamic and brainstem structures (Mendoza et al., 2005). PER1 levels have also been assessed in the core and shell of the nucleus accumbens of in rats on RF. Both the core and the shell of the nucleus accumbens show rhythmic levels of PER1 peaking around ZT 18. Under RF the peak of PER1 is advanced to ZT12, which is after the meal in this experiment. This suggests that neural activation as measured by C-FOS and the molecular clock as measured by PER1 are not in phase under RF. Therefore it may be

that the nucleus accumbens may be more involved in rewarding correlates of FAA and less so with the anticipatory circadian mechanism.

Radiofrequency ablation of the entire nucleus accumbens was found to have no effect on food anticipatory wheel running or food bin activity in rats (Mistlberger and Mumby, 1992). However, lesions targeting the core of the nucleus accumbens and the shell of the nucleus accumbens independently have differential impacts on FAA. In both cases the animals exhibit FAA, however with specific lesions of the core of the nucleus accumbens development of FAA is significantly attenuated and the amplitude is reduced. With lesions of the shell of the nucleus accumbens decrease duration of FAA but the amplitude is not significantly reduced. With any lesion experiment it is important to consider not only the impact of removing the nucleus in question but also the loss of the structure's efferents. In the case of the core and shell of the nucleus accumbens of these efferents are divergent. The core of the nucleus accumbens efferents primarily reach motor systems, whereas the shell of the nucleus accumbens efferents primarily reach limbic structures (Heimer et al., 1991). This suggests that the impact on FAA maybe related more to a movement specific deficit rather than to a circadian timing deficit. Importantly, the authors report that nocturnal activity in both lesion conditions was not impacted as monitored by general cage activity. This suggests that the changes in FAA associated with lesions of the core or shell are not due to a non specific reduction in locomotor activity. Why lesions of both structures yield no effect on FAA, while lesions restricted to one or the other subregions do affect the duration or intensity of FAA, has not been addressed

The Nucleus Accumbens receives dopaminergic input from the ventral tegmental area and is heavily implicated in the reward system. Stimulation of the ventral tegmental area by feeding associated signals, including ghrelin and orexin, result in increased levels of dopamine within the nucleus accumbens. The increase of C-FOS within limbic forebrain regions including the nucleus accumbens may reflect a preferential role in reward. It may be that activation of the ventral tegmental area via hormonal rhythms induced both by RF and palatable RF and subsequent increased extracellular dopamine within the ventral striatum may reflect a reward entrained mechanism that can augment parameters of FAA (Webb et al., 2009). To date no studies have examined the neural correlates of multiple meals within the nucleus accumbens. It is conceivable that in the 2

meal paradigm 1 meal is associated with a greater metabolic deficit than the other. Hypothetically if the nucleus accumbens is associated more so with reward than metabolism it may be that differential activation is seen to the “reward associated” meal than in other structures.

4.6.1. Nucleus Accumbens: Results

Both the core and the shell of the nucleus accumbens displayed circadian variation in *bmal-1* expression that was entrained by RF. Generally, the peaks of *Bmal-1* expression in the ZT4 fed rats occurred in antiphase to the peak observed in the ZT16 fed rats. In the two meal condition, the expression of *bmal-1* throughout the day was essentially flat.

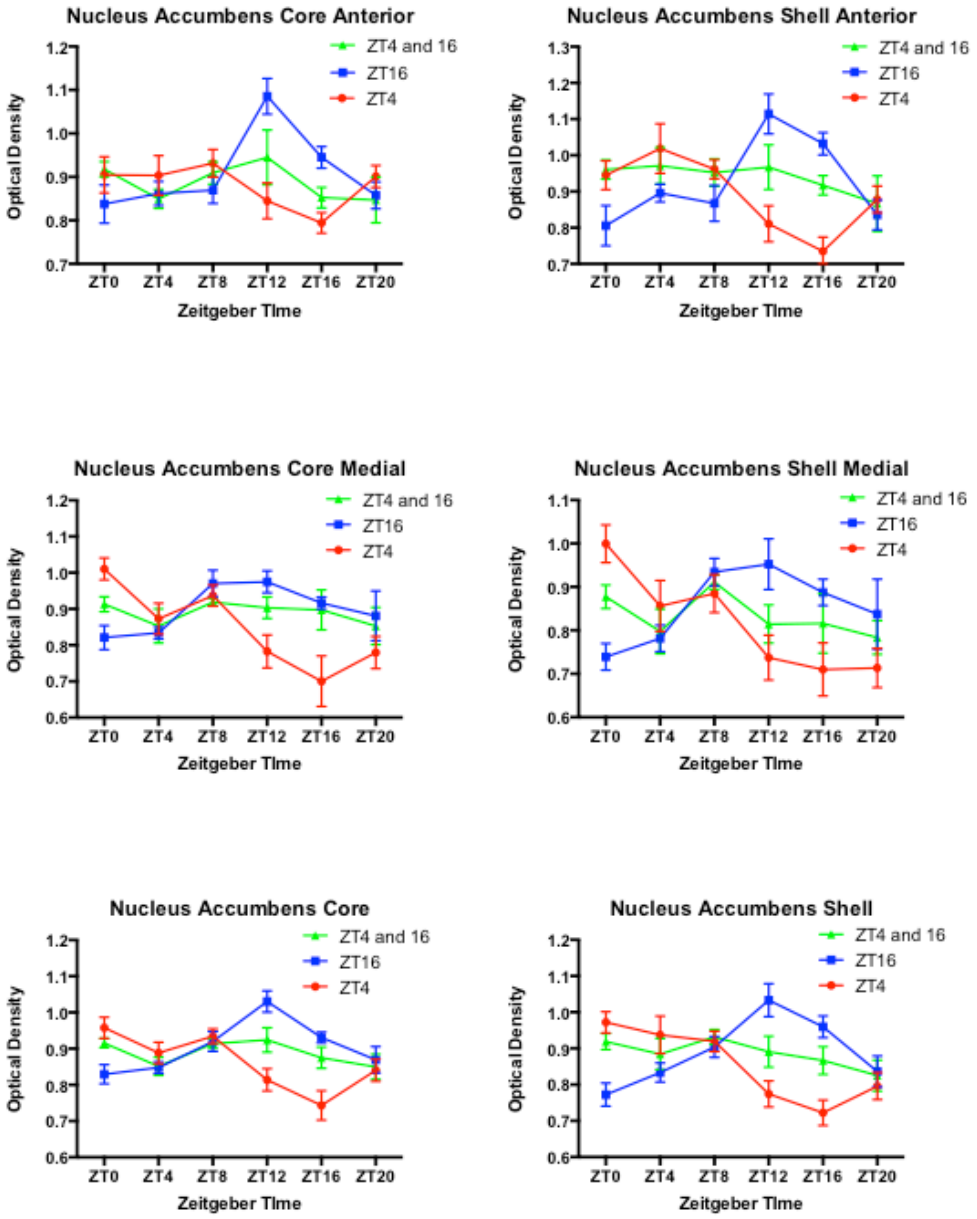
In ZT4 fed rats, the peak of *Bmal-1* expression varied slightly between anterior and medial sections. In the anterior aspect, *Bmal-1* was generally elevated between ZT0 and 8 in the core and the shell. In the medial aspect, *Bmal-1* was elevated at ZT0 and decreased prior to mealtime at ZT4 in both the core and the shell. When the structure is averaged the peak of *Bmal-1* expression occurs at ZT0 and troughs at ZT16 in both the core and the shell. In ZT16 fed rats, the peak of *Bmal-1* expression occurs at ZT16 in both the core and shell in anterior sections. In the medial aspect of the nucleus the peak occurs slightly earlier around ZT12. In aggregate, *Bmal-1* expression peaks at ZT12 in both the core and the shell of the nucleus accumbens and decreases as mealtime approaches at ZT16, finally reaching the lowest point at ZT0.

In the two meal fed rats, *Bmal-1* expression did not show a significant circadian variation and any slight peaks in the waveform were accompanied by increased variability at that data point. Instead, the *Bmal-1* waveform appeared to be the superposition of the ZT4 and ZT16 waveforms (the two component waveforms sum destructively to cancel each other out). Similar to the olfactory bulb, these data suggest that either individual clock cells or animals become dissociated, potentially via a preferential response to either the AM or PM meal. This pattern could also suggest that two distinct sets of neurons are entrained by each of the two meals within the nucleus

accumbens. Because we looked at the structure in aggregate expression from these two populations would be averaged thereby eliminating any evidence of biphasic rhythms. This would need to be examined using a technique that can provide data about clock gene expression at the level of individual neurons such as real time recording of single cells from luciferase animals.

Contratary Mendoza et al 2007, these data do not provide evidence for temporal dissociation between the core and shell of the nucleus accumbens in response to RF. Furthermore, the peak of *Bmal-1* occurred prior to feeding time in both the core and shell but was not sustained until meal time. The timing of this peak suggests that the nucleus accumbens might send signals that serve as a cue to other FEOs responsible for behavioural anticipation

Figure 14 Nucleus Accubmens *Bmal-1* In Situ Data



Group mean (± sem) waveforms of *Bmal-1* optical density in the core and shell of the nucleus accumbens as measured by in situ hybridization. Feeding schedule is denoted by color, ZT4 feeding condition (red), ZT16 feeding condition (blue), ZT4 and 16 feeding condition (green) n=5-6 animals per time point per condition. A. anterior aspect of the core of the nucleus accumbens here the interaction between ZT time and feeding was significant $F(10, 85)=3.730$, $p=0.0004$. B. medial aspect of the core of the nucleus accumbens, here the interaction between ZT time and feeding was significant $F(10,87)=3.825$, $p=0.0003$. C. Average of the core of the nucleus accumbens again the interaction between ZT time and feeding was significant $F(10,190)=6.169$, $p<0.0001$. D. anterior aspect of the shell of the nucleus accumbens here the interaction between ZT time and feeding was significant $F(10,84)=5,438$, $p<0.0001$. E. medial aspect of the shell of the nucleus accumbens here the interaction between ZT time and feeding was significant $F(10,87)=3.59$, $p=0.0005$. F. Average of the shell of the nucleus accumbens again the interaction between ZT time and feeding condition was significant $F(10, 189)= 6.8$, $p<0.0001$.

4.7. Dorsal Striatum: Introduction

Dorsal striatum explants from per1::luc mice did not show oscillations in culture in one study (Abe et al), but a more recent report did report sustained oscillations in caudate putamen explants from PER2:luc rats (Natsubori et al, 2013). Therefore there is mixed evidence for the presence of a semi-autonomous oscillator in the striatum. Using immunocytochemistry and in situ hybridization, oscillations of PER1 and PER2 (Hood et al., 2010; Feillet et al., 2008) and *Per2* (Wakamatsu et al., 2001), respectively, have been documented under ad lib conditions. There is also a circadian rhythm of dopamine release in the striatum which peaks in the mid subjective night and troughs in the mid subjective day (Paulson and Robinson, 1994; Castaneda et al, 2004). Pharmacological modulation of dopamine has been demonstrated to alter clock gene expression in the striatum, suggesting that endogenous dopamine may alter clock gene expression (Nikaido et al, 2001; Iijima et al, 2002; Imbesi et al, 2009). The endogenous rhythm of dopamine release regulates the rhythm of PER2 expression, and attenuates *Per2* transcription in the striatum (Hood et al., 2010). Altering dopamine transmission pharmacologically, using a synthesis inhibitor (tyrosine hydroxylase antagonist (AMPT) or a dopamine receptor 2 antagonist (raclopride), or by lesions (deafferentation of dopamine afferents using 6-OHDA lesions), severely attenuated rhythmicity of *Per2* transcription and PER2 protein levels. The *Per2* rhythm was not affected by a dopamine receptor 1 antagonist (SCH 23390).

Feillet and colleagues (2008) found evidence of PER1 oscillations in the dorsal striatum, but contrary to Hood et al, 2010, not of PER2. Interestingly Feillet and colleagues (2008) found that hypocaloric feeding increased the amplitude of the PER1 oscillation in the striatum. Restricted feeding has also been correlated with increased tyrosine hydroxylase in the ventral tegmental area and substantia nigra, and dopamine transporter in the ventral tegmental area at the time of feeding. This suggests that dopamine synthesis and transport is increased at meal time (Lindblom et al., 2005). Using HPLC with electrochemical detection, increased levels of dopamine and its metabolite 3,4-hydroxphenylacetic acid (DOPAC) was observed in the striatum of RF animals during FAA (Liu et al., 2012). Additionally they showed that dopamine receptor 1 (SCH 23390) and dopamine receptor 2 (raclopride) antagonists decreased the

amplitude of FAA. This attenuation is increased when the two antagonists were co-applied. Recent observations from our laboratory also suggest a role for dopamine in FAA. Treatment with the dopamine receptor 2 antagonist quinpirole can alter the phase of FAA, inducing phase delays in the onset of anticipation (Mistlberger lab, unpublished observations). Additionally, dopamine 1 receptor knock out mice (dirko) show markedly attenuated development of anticipation. Interestingly, this genotype appears to be metabolically challenged and displays impaired feeding behaviour. These animals must be maintained on a high fat diet upon weaning, and fed on the floor as they will not reach up to extract food from an overhead food hopper, despite exhibiting normal locomotor activity levels. This suggests a deficit in motivation to eat.

The striatum receives dopaminergic efferents from the substantia nigra via the mesostriatal pathway. Typically the striatum has been associated more with the dopaminergic control of movement than reward but more recent studies suggest a role in reward as well. With respect to feeding, both of these functions are important, locomotor activity is a measurable output of the FEO, while reward may modulate the amplitude of this behavioural output. Further, with respect to feeding and the striatum it appears that dopamine may be more important for reward prediction, learning, and incentive salience (Di Chiara et al 1998; Robinson and Berridge 2001) than the hedonic properties of reward which may be more closely linked with opiate and enkephalin signaling (Pecina and Berridge 2005).

Evidence for the predictive nature of dopamine signaling comes from observations that dopamine is released to both rewarding and non-rewarding stimuli, and is amplified when these stimuli are presented at unexpected times (Ikemoto and Panksepp 1996; Horvitz 2000). Barbano and Cador's 2005 study supports the notion that feeding can be dissociated into consummatory (defined as latency to eat), motivational (defined as the work required to gain access to food),and anticipatory (defined as the development of conditioned locomotor activity in expectation of food delivery) components. Although this anticipatory activity is cued and therefore fundamentally different from the endogenous FAA interesting observations can be taken from it. Food restricted animals in this experiment developed anticipatory activity that was highest in the 15-30 min preceding the trial and was equivalent in animals fed a palatable or "less palatable" food. However, food sated animals did not develop this

anticipatory activity even for the palatable food. The authors conclude that anticipatory activity is not controlled by the hedonic properties of food but rather by a homeostatic process. This is confirmed by studies by Mistlberger and Rusak (1987) and later Escobar and colleagues, (2005) that document significantly reduced FAA to a palatable food on an adlib background in rats. This anticipation is also not associated with entrainment of peripheral tissues and with differential C-FOS staining patterns. The hypothalamus is impervious to signals in this condition but the nucleus accumbens does show anticipatory increase in C-FOS. Mice will show anticipation to a palatable food on an ad lib background, however only to a high fat diet (Hsu et al., 2010).

Currently it is not known whether RF alters the rhythm in dopamine levels in the striatum or induces an additional peak in dopamine levels in RF rats, similar to what is observed in corticosterone levels for example. Interestingly, decreasing corticosterone levels decreases dopamine receptor 1 mRNA and decreases dopamine receptor 1 binding (Czyrak et al., 1997). Aside from the up regulation of PER1 it is not understood how RF affects the phase of clock gene expression within the striatum to single or multiple meals.

4.7.1. Dorsal Striatum: Results

In the anterior dorsal striatum *Bmal-1* expression was not rhythmic. Similarly, rhythmic expression of *Bmal-1* was not observed in the medial dorsal striatum. Additionally feeding schedule had no overall effect on the observed *Bmal-1* expression in the dorsal striatum.

Reports of clock gene rhythms within the dorsal striatum have been limited to *Per1 and Per2* and the PER1 protein product. The amplitude of the oscillation reported by Hood et al (2010) using RT PCR was extremely low. Interestingly Challet and colleagues (2008) report not only a rhythm of PER1 but an increase it is amplitude in mice on hypocaloric feeding. The striatum is not a homogeneous structure. DiFeliceantonio and colleagues (2012) report that specific areas within the dorsal striatum, and especially the anterior dorsal medial aspect of the dorsal striatum is responsive to palatable foods. To address the possibility of specific regions of the dorsal striatum displaying rhythms it was first analyzed in quadrants (dorsal and ventral medial

and dorsal and ventral lateral). However, as there was no evidence of rhythmicity in any of these compartments the quadrants were averaged. It remains a possibility that rhythmicity could be observed in specific types or populations of dorsal striatal neurons with a technique offering higher spatial resolution

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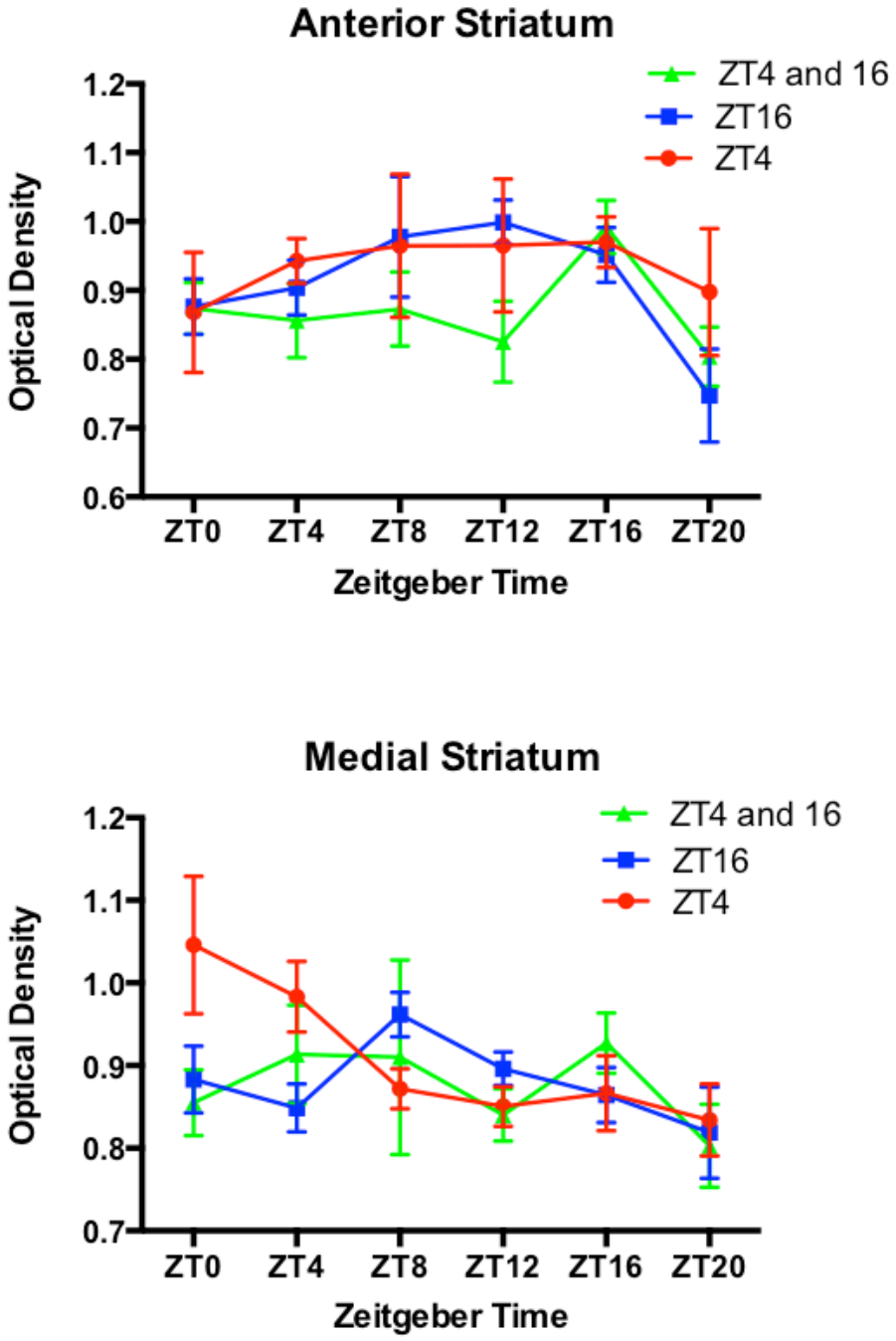
Figure 15 Anterior Striatum *Bmal-1* In Situ Data



Group mean (± sem) waveforms of *Bmal-1* optical density in the dorsal striatum as measured by in situ hybridization. Feeding schedule is denoted by color, ZT4 feeding condition (red), ZT16 feeding condition (blue), ZT4 and 16 feeding condition (green) n=5-6 animals per time point per condition. A. anterior aspect of the dorsal striatum here the interaction between ZT time and feeding was not significant $F(10,86)=0.705$, $p=0.718$. B. medial aspect of the dorsal striatum where the interaction between ZT time and feeding was not significant $F(10,85)=1,474$, $p=0.163$.

4.8. Amygdala: Introduction

Located in the medial temporal lobe the amygdala integrates emotion, cognition and autonomic signals. It is central in the responses to and perception of fear. Additional roles include the stress response and applications in drug abuse. The amygdala is comprised of many different subregions, some of which express circadian clock genes in a rhythmic fashion. Cultures of amygdala from *Per1* luciferase mice did not show rhythmicity in the central nucleus of the amygdala or bed nucleus of the stria terminalus. Further evidence against regions of the amygdala exhibiting self sustained oscillations comes from a study that examined the effect of unilateral and bilateral lesion of the SCN and subsequent rhythmicity within the central nucleus of the amygdala, bed nucleus of the stria terminalus and the basal nucleus of the amygdala. In rats under LD12:12 conditions the central nucleus of the amygdala and bed nucleus of the stria terminalus show PER2 rhythmicity that is in phase with PER2 oscillations in the SCN, peaking around lights out at ZT12 (Amir et al., 2004). This is uncharacteristic for most oscillations outside of the SCN, which tend to peak in antiphase to the SCN. Bilateral lesion of the SCN or housing the rats in LL abolished the PER2 rhythm in the bed nucleus of the stria terminalus and central nucleus of the amygdala, but left the rhythm in the basal nucleus of the amygdala intact (Amir et al., 2004; Waddington Lamont et al., 2005). Importantly, unilateral lesions of the SCN blunt the PER2 rhythms in the central nucleus of the amygdala and bed nucleus of the stria terminalus but only on the ipsilateral side. This suggests that neural connections from the SCN are involved in rhythmicity of PER2 expression within these regions (Amir et al, 2004). The PER2 rhythm in these unilaterally lesioned animals was not completely abolished, however, suggesting that another rhythm, most likely humoural in nature, also contributes to the rhythms of PER2 in these areas.

Adrenalectomy which results in the abolishment of the corticosterone rhythm does not affect rhythmicity of the SCN, but did blunt the PER2 rhythm in the central nucleus of the amygdala and bed nucleus of the stria terminalus while sparing PER2 rhythms in the basal nucleus of the amygdala (Amir et al, 2004; Waddington Lamont 2005). This indicates that the corticosterone rhythm which is under the control of the SCN in ad lib fed rats also contributes to the PER2 rhythms in these two nuclei.

Replacing corticosterone in adrenalectomized rats by administration in drinking water indirectly results in a rhythmic replacement of corticosterone as a circadian rhythm in water intake is tightly coupled to the LD cycle in ad lib fed rats. Replacing corticosterone using subcutaneous steady release implants results in a relatively constant level of corticosterone throughout the day. Interestingly, in adrenalectomized rats corticosterone replacement via administration in drinking water re-instated the PER2 rhythms in the central nucleus of the amygdala and bed nucleus of the stria terminalus, while steady release tablets did not (Segall et al., 2006). This finding indicates that the corticosterone signal to the central nucleus of the amygdala and bed nucleus of the stria terminalus may directly drive PER2 rhythms in these structures.

It was demonstrated in (1974) that RF alters the phase of the corticosterone rhythm inducing a peak prior to meal time in addition to the nocturnal peak corresponding to activity onset at ZT12. This daily anticipatory increase in corticosterone is also observed in arrhythmic SCN lesioned animals. Therefore the rhythmic changes in corticosterone in animals on RF provides a mechanism by which RF could control the phase of PER2 in the central nucleus of the amygdala and bed nucleus of the stria terminalus in SCN lesioned animals. RF does synchronize PER2 rhythms in the central nucleus of the amygdala and bed nucleus of the stria terminalus but interestingly the phase of these oscillations is synchronized with the basal nucleus of the amygdala and in antiphase to the SCN (Verwey et al., 2007; Waddington Lamont et al., 2007). This suggests that under RF input related to feeding, whether neural or humoural, overrides the signal from the SCN and any differential effects of corticosterone on the central nucleus of the amygdala and bed nucleus of the stria terminalus are no longer observed under RF conditions.

Using restricted access to a palatable treat (Verwey et al., 2007; Waddington Lamont et al., 2007) Amir and colleagues attempted to differentiate between motivation to eat and metabolic state. The effects of these feeding correlates on PER2 rhythms in the central nucleus of the amygdala, bed nucleus of the stria terminalus and basal nucleus of the amygdala were not the same. PER2 rhythms were found to be unaltered by palatable treat access when chow was available ad lib. The conclusion they drew from this was that PER2 oscillations in these regions are sensitive to the alleviation of a negative metabolic state. However there is an alternative explanation for their findings.

Restricted access to a palatable treat does not alter the rhythm of corticosterone release and therefore in these two experiments the lack of a preprandial increase in corticosterone could explain why they saw no change in PER2 oscillations.

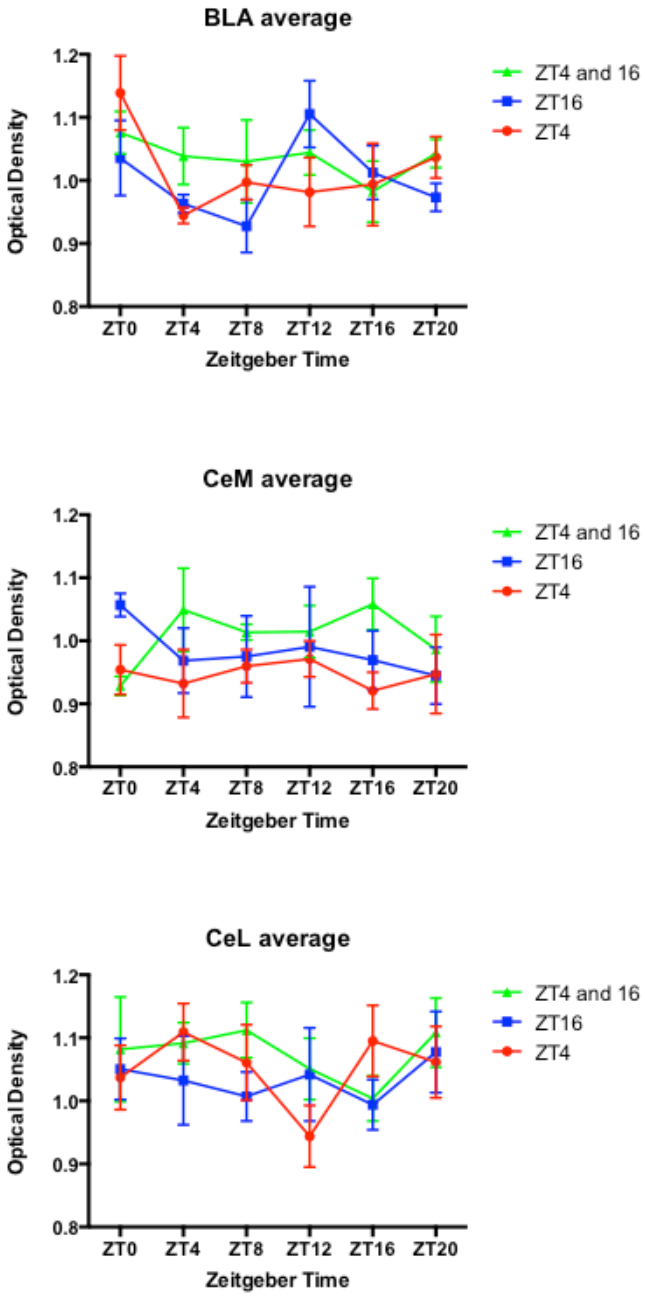
The story presented by Amir and colleagues appears relatively straightforward. However, Angeles-Castellanos and colleagues (2007) do not report oscillations of PER1 protein in the central nucleus of the amygdala or basal nucleus of the amygdala of rats fed ad lib. Also C-FOS was altered in the central nucleus of the amygdala of animals given restricted access to a palatable meal (Mendoza et al., 2005). Multiunit activity recording from the bed nucleus of the stria terminalus does show a circadian pattern in neural firing that is in phase with the SCN (Yamazaki et al., 1998). No study has evaluated the response of clock genes in the amygdala to a 2-meal feeding schedule.

4.8.1. Amygdala: Results

Bmal-1 expression in the basal lateral amygdala (basal nucleus of the amygdala) did not appear rhythmic under any of the feeding conditions. However the lack of significant rhythms appears to be attributable to high levels of variability and with an increased number of animals significant rhythms may be observed. The waveforms suggest that entrainment by RF in the basal nucleus of the amygdala may occur and that similar to other regions examined two daily meals may result in the dampening of clock gene expression within this region.

Under all feeding conditions both the medial and lateral divisions of the central nucleus of the amygdala failed to exhibit rhythmic *Bmal-1* expression. It is not clear why previous experiments examining PER histology report rhythms within these regions (Waddington-Lamont et al., 2007; Verwey et al., 2007; Segall et al., 2008, Verwey et al., 2011a, 2011b) while others do not (Angeles-Castellanos et al., 2007, 2008) as PER protein rhythms should be dependent on *Bmal-1* expression according to the TTFL. To date *Bmal-1* expression within the amygdala in response to RF has not been examined in a published report. Therefore we cannot compare our data with another data set.

Figure 16 Amygdala *Bmal-1* In Situ *Data*



Group mean (± sem) waveforms of *Bmal-1* optical density in the Amygdala as measured by in situ hybridization. Feeding schedule is denoted by color, ZT4 feeding condition (red), ZT16 feeding condition (blue), ZT4 and 16 feeding condition (green) n=5-6 animals per time point per condition. A. Basal lateral nucleus of the amygdala here the interaction between ZT time and feeding was not significant $F(10,84)=1.126$, $p=0.353$. B. Medial aspect of the central nucleus of the amygdala here the interaction between ZT time and feeding condition was not significant $F(10,69)=1.008$, $p=0.446$. C. Lateral aspect of the central nucleus of the amygdala here the interaction between ZT time and feeding condition was again not significant $F(10,83)=0.632$, p=0783

4.9. Paraventricular nucleus of the thalamus: Introduction

The paraventricular nucleus of the thalamus is medially located inferior to the third ventricle. The paraventricular nucleus of the thalamus receives afferents from many regions including the SCN, amygdala, prefrontal cortex, parabrachial nucleus, and raphe nuclei (Freedman and Castle, 1994). Efferents from the paraventricular nucleus of the thalamus project to the nucleus accumbens ,the SCN and the central, basolateral, and basomedial amygdala (Moga et al., 1995). It therefore receives signals from brainstem and hypothalamic nuclei including the master circadian pacemaker and sends information to limbic centers. Rhythmic expression of *Per1* has been detected in 40 % of cultured paraventricular nucleus of the thalamus sections (Abe et al., 2002). Additionally rhythms of glucose utilization (Pereira et al., 2006), C-FOS and PER1 protein have been reported (Peng et al., 1995; Novak and Nunez, 1998; Angeles-Castellanos et al., 2007; Mendoza et al,., 2005). Studies examining the basal rhythm of C-FOS in the paraventricular nucleus of the thalamus have been relatively consistent, with one study reporting that C-FOS levels are higher during the dark phase than the light phase, a second reporting a peak between ZT17 and ZT1 under LD 12:12 conditions, and the third at peak at CT16 in DD. Rhythms of glucose utilization, C-FOS and PER1 are altered by food restriction in the paraventricular nucleus of the thalamus, becoming synchronized with feeding (Periera et al., 2006, Nakahara et al., 2004, Mendoza et al., 2005). However, two reports from the same group of authors report different effects on the pattern of C-FOS expression in rats on RF. Mendoza and colleagues (2005) report a peak in C-FOS at meal time, whereas Angeles-Castellanos and colleagues (2007) show a preprandial peak that decreases at meal time and then peaks again when the food is removed.

Mendoza and colleagues 2004 reported that PER1 protein in the paraventricular nucleus of the thalamus normally peaks at CT20 in DD. When animals are provided with access to a palatable meal in constant light where meal time is therefore treated as ZT0 (the onset time of the only zeitgeber in their environment) PER1 then peaks at CT0. In aggregate it appears that the paraventricular nucleus of the thalamus receives circadian signals from the SCN that can be integrated with visceral information from the brainstem and applied to limbic centers. Further the paraventricular nucleus of the thalamus is

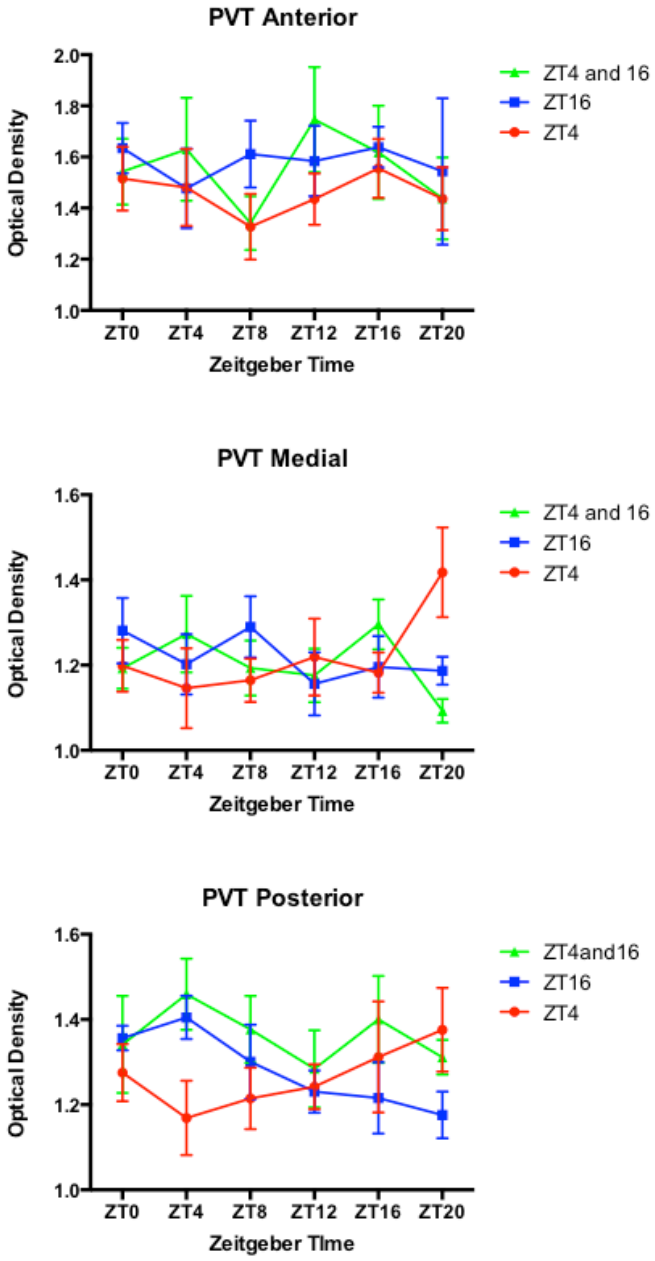
directly affected by feeding. Although it appeared a great candidate for the FEO, radiofrequency lesion of the paraventricular nucleus of the thalamus did not alter FAA compared to unlesioned controls (Landry et al., 2007). However, it remains a possibility that the RF sensitive paraventricular nucleus of the thalamus may be part of a distributed network of FEO's that together orchestrate FAA.

4.9.1. Paraventricular nucleus of the thalamus: Results

The PVN did not show significant rhythms of *Bmal-1* expression in any of the levels examined. The posterior aspect of the PVN appears to be nearly rhythmic however any inferences based on this waveform would be purely speculative.

Evidence for entrainment of PER1 or PER2 within the paraventricular nucleus of the thalamus has been mixed. Angeles-Castellanos and colleagues (2007) report that the paraventricular nucleus of the thalamus is arrhythmic under ad lib feeding but when placed on RF from ZT 5-7 PER1 levels become rhythmic within this region. In 2008 the same group published a study reporting rhythmic levels of PER1 peaking at ZT0 in ad lib fed animals and at ZT6 in animals on RF from ZT6-8. Feillet and colleagues (2008) reported that daytime RF at ZT6 does not change the rhythm of PER1 levels within the paraventricular nucleus of the thalamus but does induce a 4h phase advance in PER2 levels within this region. No study to date has examined *Bmal-1* expression in response to RF within the paraventricular nucleus and therefore we cannot compare our results with another published data set.

Figure 17 PVT *Bmal-1* In Situ Data



Group mean (± sem) waveforms of *Bmal-1* optical density in the paraventricular nucleus of the thalamus as measured by in situ hybridization. Feeding schedule is denoted by color, ZT4 feeding condition (red), ZT16 feeding condition (blue), ZT4 and 16 feeding condition (green) n=5-6 animals per time point per condition. A. anterior aspect of the paraventricular nucleus of the thalamus, here the interaction between ZT time and feeding condition was not significant $F(10,73)=0.443$, $p=0.920$. B. medial aspect of the paraventricular nucleus of the thalamus here the interaction between ZT time and feeding condition was not significant $F(10,79)=1.536$, $p=0.143$.. C. lateral aspect of the paraventricular nucleus of the thalamus where again the interaction between ZT time and feeding condition was not significant $F(10, 76)=0.972$, $p=0.474$.

4.10. Cerebellum: Introduction

The cerebellum is known for its role in coordination of motor behaviours (Ebner, 1998). It is also implicated in motivational processes (D'Agata et al., 1993) and in spatial learning (Lalonde and Botex, 1990), and appears to participate in the timing of short intervals in the seconds to minutes range (Ivry et al, 2002). Until 2010 its involvement in circadian rhythms had not been studied (Mendoza et al., 2010). It is conceivable that the cerebellum may play a role in the coordination of FAA expression. The cerebellum is very responsive to changes in levels of physical activity (Gross et al, 1980). It also has receptors for feeding associated hormones such as leptin (Guan et al 1997) and Glu4 which is sensitive to insulin (Choeiri et al, 2002)..

Rhythmic clock gene expression has been observed in the cerebellum of both mice and rats using in situ hybridization for *Per1, Per2, Bmal-1*, *Clock Rev-erbα, and Dbp* (Sun et al., 1997; Namihira et al., 1999; Mendoza et al., 2010). Using immunocytochemistry Mendoza and colleagues (2010) showed that a peak of PER2 expression in the cerebellum at ZT 20, approximately 5hrs after the peak they observed in *per2* expression using in situ hybridization. In culture, the cerebellum from PER1::luc mice was reported to be arrhythmic (Abe et al., 2002). A subsequent report showed that 5/9 cerebellar cultures were rhythmic, showing a peak in the early subjective night (ZT14). In mice placed on RF from ZT6-12, rhythms of *Per1, Per2, Rev-erb α, and Dbp* were altered. The per genes were both phase advanced by 9h, while *Rev-erb α, and Dbp* were phase advanced by 3 and 6 hrs respectively. The peak in PER2 protein was also shown to shift ZT20 and the presence of the PER2 protein was limited to the purkinje cells of the cerebellum. Cerebellums cultured from PER1::luc mice also showed phase advances in response to RF, peaking approximately 7h earlier in RF animals vs. ad lib controls. Using OX7-saporin, purkinje cell specific lesions of the cerebellum were made. Upon recovery these cerebellar lesioned mice showed delayed FAA that was attenuated in amplitude. Mice that were either heterozygous or homozygous for the nancy hotfoot mutation (*Grid2*$^{ho/+}$*, Grid2*$^{ho/ho}$), which results in the deletion of the coding sequence of the purkinje cell specific ionotropic glutamate receptor gene (*Grid2*), were challenged with RF. FAA was attenuated in a genotype specific manner; it was normal in WT, attenuated in *Grid2*$^{ho/+}$, and even further reduced in *Grid2*$^{ho/ho}$. This was not

attributable to a non-specific affect on activity as *Grid2*$^{ho/+}$, *Grid2*$^{ho/ho}$ showed more activity at night than their WT controls. When RF was scheduled at night to rule out increased masking by light in the *Grid2*$^{ho/+}$, *Grid2*$^{ho/ho}$ mice showed similar FAA to that observed under LD indicating that the affects on FAA were due to the genotype dependent depletion of *Grid2* in the cerebellum.

A recent study examined whether rhythmic gene expression in the cerebellum was transduced into a functional circadian electrical output signal to other brain regions (Mordel et al., 2013). The SCN shows a circadian pattern in the mean firing rate of neurons (Green and Gillette 1982). It was hypothesized that when the cerebellum is entrained by RF it may send signals to food sensitive regions like the hypothalamus. Although a rhythm in firing rate could be induced by changing the culture media, no evidence was obtained for a circadian pattern in the firing of cerebellar purkinje cells. Therefore whether the cerebellum harbours a self-sustaining FEO remains unclear .

Aside from Mendoza and colleagues 2010 study, the effects of RF on the cerebellum have not been explored further. Therefore we chose to examine the cerebellum to explore their findings and in addition add to the RF literature with respect to the effects on the cerebellum of two daily meals.

4.10.1. Cerebellum: Results

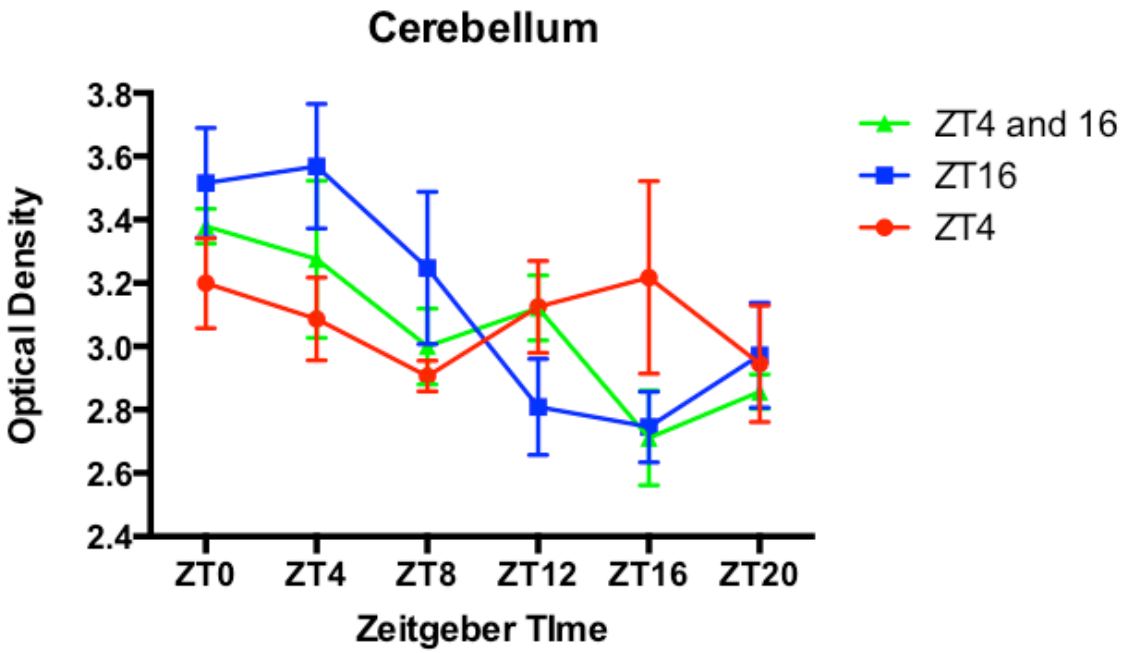
The cerebellum exhibited circadian expression of *Bmal-1* expression in the 16 fed animals peaking at ZT 4. In the ZT 4 fed animals the peak of *Bmal-1* expression occurred at ZT 16 however this is not a significant circadian oscillation. Similarly in the two meal condition *Bmal-1* expression was not expressed with a circadian variation.

Therefore rhythmicity of *Bmal-1* within the cerebellum was not altered by restricted feeding schedule. But, visual inspection of the waveforms show that the ZT4 and ZT 16 feeding conditions that the highest levels of *Bmal-1* occur at opposite phases to one another. Similar to other brain regions the two meal animals did not show a coherent circadian rhythm and the amplitude of *Bmal-1* expression was dampened. This leaves open the possibility of differential entrainment of populations of cell within the

cerebellum by different meals, or inter animal variability within the animals at each time point within this two meal condition.

Evidence of entrainment of *Per1, Per2, Rev-erbα, and Dbp* by RF in the cerebellum has previously been reported (Mendoza et al, 2010). Day time RF at ZT6 induced a 7 hour phase advance in *Per1* and *Per2* expression, but only a 2 hour phase advance in *Rev-erbα.* This raises the possibility that the *Per* genes are readily rephrased in the cerebellum in response to RF while other genes like *Rev-erbα* are not. Therefore it is possible that *Per* gene expression may be directly affected by stimuli associated with scheduled meals, without rhythmicity or resetting of *Bmal-1* by food.

Figure 18 Cerebellum *Bmal-1* In Situ Data



Group mean (± sem) waveforms of *Bmal-1* optical density in the granual cell layer of the cerebellum as measured by in situ hybridization. Feeding schedule is denoted by color, ZT4 feeding condition (red), ZT16 feeding condition (blue), ZT4 and 16 feeding condition (green) n=5-6 animals per time point per condition. The interaction between ZT time and feeding was not significant in the cerebellum F(10, 71)=1.566, p=0.135.

4.11. Barrel Cortex: Introduction

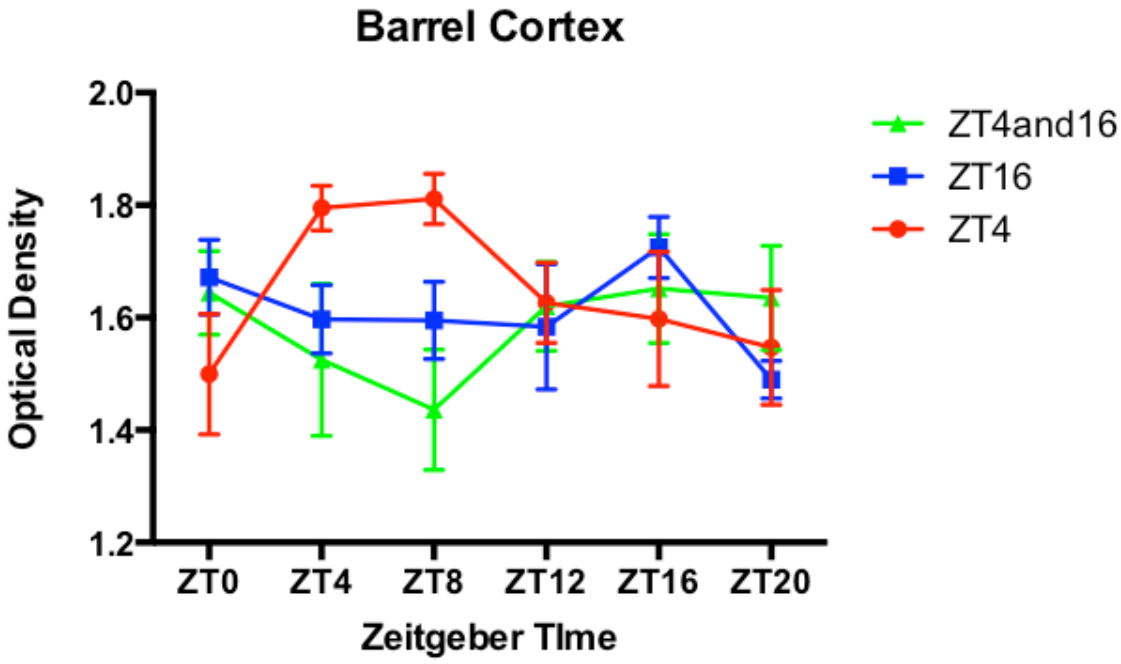
The barrel cortex is the area of sensory cortex dedicated to the barrel fields of the whiskers. It processes sensory information generated by movement of the whiskers in rodents (Petersen, 2007). As a qualifying statement, decorticate rats readily display FAA under RF (Mistlberger, 1994, Fig. 7b). Therefore, the barrel cortex is not necessary for FAA, but may be part of a distributed system of FEO's. Rhythmic clock gene expression in the cortex has been reported previously (Wakamatsu et al., 2001). There are no reports on the rhythmicity of the barrel cortex specifically, but there is one report showing that *c-Fos* mRNA was significantly increased in the barrel cortex before mealtime in WT and 5-H2CR-KO mice fed from Z4-8, relative to ad lib fed controls. Additionally there was a positive correlation between *c-Fos* mRNA and the amount of FAA observed (Hsu et al., 2010). It seems likely that the increase in *c-Fos* mRNA in the

RF fed groups is higher during FAA because they are engaged in increased locomotor activity and presumably searching through their cages for food, thereby stimulating their whiskers more than their ab lib counterparts. A significant correlation of *c-Fos* mRNA and locomotor activity was not observed during the activity associated with dark onset, but its possible that sniffing behaviour is greater prior to an expected mealtime than it is at times of day not associated with food access. These observations necessitate further inquiry and our multiple meal paradigm is interesting for two reasons. First, the effect of multiple meals has not been explored in this region. Second, the observation that *c-Fos* mRNA in the barrel cortex is higher during the daytime FAA in RF rats may be time of day dependent, that is activity at a time of day when rats are usually not active may induce *c-Fos* mRNA in the barrel cortex. To assess this question, activation of the barrel cortex during FAA during the subjective day must be compared with FAA during the subjective night, which our AM and PM fed conditions allow for.

4.11.1. Barrel Cortex: Results

Bmal-1 within the barrel cortex did not show circadian rhythmicity. No significant effect of time of day or feeding schedule was observed in *Bmal-1* expression within the barrel cortex. The barrel cortex contains individual receptive fields for individual whiskers. it is possible that by analyzing the barrel cortex in its entirety variability within the receptive fields of individual whiskers has decreased the ability to observe a significant rhythm. A technique with increased spatial resolution may allow for a more detailed analysis of individual barrel fields or clusters of barrel fields to examine whether rhythmicity is observed within smaller functional parts of the barrel cortex. No other study to date has examined clock gene expression within the barrel cortex of animals on RF. Therefore we cannot compare our *Bmal-1* data with other published data sets. Evidence for entrainment of *c-Fos* by RF that correlated with FAA has been published (Hsu et al., 2010). However it is not currently known if *c-Fos* expression within the barrel cortex correlates at all with expression of clock genes within this region.

Figure 19 Barrel Cortex *Bmal-1* In Situ Data



Group mean (± sem) waveforms of *Bmal-1* optical density in the barrel cortex as measured by in situ hybridization. Feeding schedule is denoted by color, ZT4 feeding condition (red), ZT16 feeding condition (blue), ZT4 and 16 feeding condition (green) n=5-6 animals per time point per condition. In the barrel cortex the interaction between ZT time and feeding condition was not significant $F(10,81)=1.568$, $p=0.131$.

Chapter 5. Conclusions

The primary objective of this research was to gain insight into the circadian clock mechanism by which rats anticipate two regularly scheduled daily mealtimes. Four questions were posed, as follows:

1. Where in the brain are daily rhythms of clock genes expressed and in which of these areas is the rhythm rephased by mealtime?

2. In animals fed two daily meals, are clock gene rhythms in the brain unimodal or bimodal, or does this vary by region?

3. Does the daily rhythm of hormone secretion (ghrelin and corticosterone) match the daily rhythm of food anticipatory activity in rats anticipating one or two daily meals?

4. Does the daily rhythm of hormone secretion (ghrelin and corticosterone) match the daily rhythm of clock gene expression in the source gland (stomach and adrenal gland, respectively)?

Where in the brain are daily rhythms of clock genes expressed and in which of these areas is the rhythm rephased by mealtime? Based on a survey of the literature (Appendix A) 11 brain regions were examined in an attempt to identify correlates of one and two-meal anticipation in rats. Each of these brain regions have been reported in at least one study to show circadian oscillations that can be reset by restricting food to the middle of the light period. We observed significant rhythms of *Bmal-1* expression in two of these areas, the olfactory bulb and nucleus accumbens. In both regions, *Bmal-1* expression exhibited a unimodal rhythm in rats fed one meal per day, and the phase of the rhythm was shifted in the AM group relative to the PM group. Lesion studies have shown that neither of these areas are necessary for rats to express food anticipatory activity rhythms. Resetting of the olfactory bulb circadian clock by meal timing presumably ensures that olfactory sensitivity is maximum at times of day when food is most likely to be available. The functional significance of the nucleus accumbens circadian clock is uncertain. It is most likely to be involved in generating daily rhythms of

responsiveness to reward stimuli (Webb et al, 2009), but the lack of effect of complete nucleus accumbens lesions on food anticipatory activity rhythms suggests that this plays little or no role in control of behavioural rhythms by meal timing.

Lack of significant rhythmicity in the other 9 regions sampled was unexpected. However, it is important to note that most studies that have mapped clock gene expression in the brain in rodents entrained to one daily meal used ISH or ICC to measure *Per1* and *Per2*. Only 2 studies measured *Bmal-1* expression, and of those only 1 study used rats (Minana-Solis et al, 2009). Notably, in that study, shifting of clock gene rhythms by daytime feeding was observed in the dorsal medial hypothalamus, paraventricular nucleus of the hypothalamus, and ventral medial hypothalamus, but only in *Per1* or *Per2*, and not in *Bmal-1*, which was arrhythmic in each of these areas. *Bmal-1* is an essential component of the circadian clock in the SCN and other tissues; it is the only single clock gene knockout that eliminates self-sustaining circadian rhythms at the behavioural, tissue and single cell level. However, its role in the expression of circadian food-anticipatory rhythms has been controversial, with multiple studies reporting that *Bmal-1* knockout mice robustly anticipate a daily meal (Storch and Weitz, 2009; Pendergast et al, 2009). The most recent analysis of food anticipation in *Bmal-1* null mice indicates that *Bmal-1* does play a role in the regulation of food anticipatory rhythms (Takasu et al, 2012). Wildtype mice can anticipate daily meals only if these recur at circadian intervals (which defines this as a circadian clock controlled process). By contrast, *Bmal-1* null mice can anticipate meals at any interval. This implies that food anticipatory rhythms are generated independently of the *Bmal-1*-dependent circadian clock, but that the *Bmal-1*-dependent clock normally imposes circadian constraints on the food-entrained clock. A similar conclusion was reached by another recent study showing that mice with triple knockouts of *Per1, Per2* and *Per2* anticipate meals best when these recur at 21 h intervals, rather than 24h intervals (Pendergast et al, 2012). Whether or not the known circadian clock genes mediate food anticipatory rhythms, *Per1* and *Per2* expression in the brain has been shown repeatedly to exhibit circadian rhythmicity entrained by daily mealtimes. Our choice of *Bmal-1* to assess food-entrained rhythmicity in the brain may in hindsight have been unfortunate, if this clock gene is not only unnecessary for food anticipatory rhythms, but also less likely to exhibit rhythmicity

in response to restricted feeding schedules. To evaluate this further, the brain tissue used in this study can be processed further to measure *Per1* or *Per2* expression.

It is also possible that our failure to see significant rhythmicity of *Bmal-1* expression in most brain regions reflects a limitation of the in situ hybridization technique. The method works well for determining the presence or absence of gene expression in the brain, and has been used to quantify daily rhythms of clock gene expression in various brain areas, including the SCN. Neurons in the SCN are small and very densely packed. Other areas of the brain in which daily rhythms of clock gene expression have been reliably observed using in situ hybridization include the compact part of the dorsal medial hypothalamus, the mitral cell layer of the olfactory bulb and the cerebellum. Neurons within these regions are also more tightly clustered than in some of the other target areas that we examined, such as the dorsal striatum and paraventricular nucleus. In our analysis of *Bmal-1* expression, many regions showed "trends" towards rhythmicity but the variability of the measurements was high and the trends did not reach significance. Its possible that within more diffusely populated brain regions the signal to background ratio may not be as robust as when cell bodies are tightly packed thereby resulting in increased variability and an overall lack of significant rhythms. This could be examined further by using other techniques, such as RT-PCR, or ICC for measuring clock gene products.

The second major question addressed in this study is whether clock gene rhythms in rats anticipating two daily meals is unimodal or bimodal. In both the stomach and adrenal gland, clock genes exhibited an intermediate phase in the 2-meal group. In the olfactory bulb and nucleus accumbens, *bmal-1* expression did not exhibit a daily rhythm in the 2-meal group, and fell at levels intermediate between the AM and PM single meal groups at each time point sampled. This outcome could result from averaging of unimodal rhythms that were shifted by different amounts in different rats, or averaging across two populations of FEOs within each area, one population aligned with the AM meal, and the other aligned to the PM meal. A third possibility is that the lack of rhythmicity in group data is real at the individual rat level, due to a disruptive effect of ingesting two large daily meals at 12h intervals. To resolve this issue, future experiments will need to use measures of clock gene rhythmicity that have greater spatial resolution, ideally at the single cell level. Employing single cell luciferase analysis or

immunocytochemistry may help to reveal any spatially distinct populations of cells that become entrained to RF.

A third question addressed in this study is whether the daily rhythm of two major metabolic hormones, gastric ghrelin and adrenal corticosterone, match the daily rhythm of food anticipatory activity in rats anticipating one or two daily meals. The answer, for ghrelin, is no. Ghrelin has been proposed to be a food-entrained gastric signal that regulates food anticipatory activity (Le Sauter et al, 2009). However, we found that ghrelin levels were increased at the AM mealtime in rats fed at this time in 1-meal and 2-meal conditions, but did not increase at the PM time in the 2-meal condition. This finding argues against a critical role for ghrelin in temporal control of food anticipatory activity. This conclusion is consistent with other studies showing that mice lacking ghrelin receptors or preproghrelin are quite capable of anticipating a daily meal (Blum et al., 2009; Szentirmai et al., 2010; Gunapala et al., 2011). A similar conclusion pertains to adrenal corticosterone. corticosterone levels increased at the AM mealtime in rats fed only in the AM, but did not increase at that time in rats fed in both the AM and the PM. This dissociation of corticosterone release from food anticipatory activity demonstrates that corticosterone does not regulate entrainment of circadian clocks responsible for the food anticipatory rhythm.

A final question addressed by this study concerns the relationship between ghrelin and corticosterone and the circadian clocks in the stomach and adrenal gland, respectively. Are the circadian rhythms of circulating hormone driven by the circadian clock in the organ of origin, or is secretion at one or both meals stimulated by autonomic inputs or circulating factors? The 2-meal condition provides an interesting model for answering this question. In both the stomach and the adrenal gland, circadian clock genes exhibited unimodal rhythms in all conditions, with an intermediate phase in the 2-meal condition. The hormone rhythms essentially matched this pattern, suggesting that food-entrained rhythms of hormone release reflect the phase of the circadian clock cycling in these organs. This argues against the idea that there is a master food-

entrainable pacemaker that drives food anticipatory behavioural and physiological rhythms. Rather, it seems more likely that behavioural food anticipatory rhythms are controlled by a circadian mechanism specific to the control of behaviour. This suggests that regularly scheduled meals entrain circadian oscillators in the brain and periphery by multiple routes in parallel, rather than via a single route involving a master food-entrainable pacemaker. In this respect, food-entrainment appears to be mediated in a very different way than is light-entrainment, which involves a master light-entrainable circadian pacemaker in one brain region, the SCN. It is becoming increasingly likely that there is no analogous master food-entrainable clock, but rather a broadly distributed system of oscillators in the brain and periphery that are independently entrained by correlates of daily meals.

References

Abe, M., Herzog, E. D., Yamazaki, S., Straume, M., Tei, H., Sakaki, Y., Menaker, M., & Block, G. D. (2002c). Circadian rhythms in isolated brain regions. *Journal of Neuroscience*, *22*, 350–356.

Adelmant, G. G., Bègue, A. A., Stéhelin, D. D., & Laudet, V. V. (1996). A functional Rev-erb alpha responsive element located in the human Rev-erb alpha promoter mediates a repressing activity. *Proceedings of the National Academy of Sciences of the United States of America*, *93*, 3553–3558.

Akhtar, R. A., Reddy, A. B., Maywood, E. S., & Clayton, J. D. (2002). Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus. *Current Biology*, 12, 540-550.

Albrecht, U. (2012). Timing to Perfection: The Biology of Central and Peripheral Circadian Clocks. *Neuron*, *74*, 246–260.

Alvarez JD & Sehgal A. (2005). The thymus is similar to the testis in its pattern of circadian clock gene expression. *Journal of Biological Rhythms*, 20, 111-121.

Amir, S., Cain, S., Sullivan, J., Robinson, B., & Stewart, J. (1999a). Olfactory stimulation enhances light-induced phase shifts in free-running activity rhythms and Fos expression in the suprachiasmatic nucleus. *Neuroscience*, *92*, 1165–1170.

Amir, S., Cain, S., Sullivan, J., Robinson, B., & Stewart, J. (1999b). In rats, odor-induced Fos in the olfactory pathways depends on the phase of the circadian clock. *Neuroscience Letters*, *272*, 175–178.

Amir, S., Lamont, E. W., & Robinson, B. (2004). A circadian rhythm in the expression of PERIOD2 protein reveals a novel SCN-controlled oscillator in the oval nucleus of the bed nucleus of the stria terminalis. *The Journal of Neuroscience*, 24, 781-790.

Angeles-Castellanos, M., Aguilar-Roblero, R., & Escobar, C. (2004). c-Fos expression in hypothalamic nuclei of food-entrained rats. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, *286*, R158–65.

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

Angeles-Castellanos, M., Salgado-Delgado, R., Rodriguez, K., Buijs, R. M., & Escobar, C. (2008). Expectancy for food or expectancy for chocolate reveals timing systems for metabolism and reward. *Neuroscience*, *155*, 297–307.

Asher, G., Gatfield, D., Stratmann, M., Reinke, H., Dibner, C., Kreppel, F., et al. (2008). SIRT1 Regulates Circadian Clock Gene Expression through PER2 Deacetylation. *Cell*, *134*, 317–328.

Bae, K., Jin, X., Maywood, E. S., Hastings, M. H., Reppert, S. M., & Weaver, D. R. (2001). Differential Functions of mPer1, mPer2, and mPer3 in the SCN Circadian Clock. *Neuron*, *30*, 525–536.

Baggs, J. E., & Green, C. B. (2003). Nocturnin, a deadenylase in Xenopus laevis retina: a mechanism for posttranscriptional control of circadian-related mRNA. *Current Biology*, *13*, 189–198.

Balsalobre, A., Damiola, F., & Schibler, U. (1998). A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell*, 93, 929-937.

Balsalobre, A., Brown, S. A., & Marcacci, L. (2000). Resetting of circadian time in peripheral tissues by glucocorticoid signaling. *Science*, 289, 2344-2347.

Bassareo, V., & Di Chiara, G. (1999). Differential responsiveness of dopamine transmission to food-stimuli in nucleus accumbens shell/core compartments. *Neuroscience*, *89*, 637–641.

Bellesi, M., Pfister-Genskow, M., Maret, S., Keles, S., Tononi, G., & Cirelli, C. (2013). Effects of sleep and wake on oligodendrocytes and their precursors. *The Journal of Neuroscience*, 33, 14288-14300.

Berridge, K. C., & Robinson, T. E. (2003). Parsing reward. *Trends in Neurosciences*, *26*, 507–513.

Berthoud, H.R. (2002). Multiple neural systems controlling food intake and body weight. *Neuroscience and Biobehavioural Reviews*, *26*, 393–428.

Bolles, R. C., & Moot, S. A. (1973). The rat's anticipation of two meals a day. *Journal of Comparative and Physiological Psychology*, 83, 510-514.

Boulos, Z., & Terman, M. (1980). Food availability and daily biological rhythms. *Neuroscience and Biobehavioural Reviews*, *4*, 119–131.

Boulos, Z., Rosenwasser, A. M., & Terman, M. (1980). Feeding schedules and the circadian organization of behaviour in the rat. *Behavioural Brain Research*, *1*, 39–65.

Böttger, K. (1972). Vergleichend biologisch-ökologische Studien zum Entwicklungszyklus der Süßwassermilben (Hydrachnellae, Acari). I. Der Entwicklungszyklus von Hydrachna …. *Internationale Revue der gesamten Hydrobiologie …*.

Brown, S. A., Zumbrunn, G., Fleury-Olela, F., Preitner, N., & Schibler, U. (2002). Rhythms of mammalian body temperature can sustain peripheral circadian clocks. *Current Biology*, *12*, 1574–1583.

Brunjes, P. C., Illig, K. R., & Meyer, E. A. (2005). A field guide to the anterior olfactory nucleus (cortex). *Brain Research. Brain Research Reviews*, *50*, 305–335.

Buhr, E. D., Yoo, S.-H., & Takahashi, J. S. (2010). Temperature as a universal resetting cue for mammalian circadian oscillators. *Science*, *330*, 379–385.

Bunger, M. K., Wilsbacher, L. D., Moran, S. M., & Clendenin, C. (2000). Mop3 is an essential component of the master circadian pacemaker in mammals. *Cell*, 103, 1009-1017.

Castañeda, T. R., de Prado, B. M., Prieto, D., & Mora, F. (2004). Circadian rhythms of dopamine, glutamate and GABA in the striatum and nucleus accumbens of the awake rat: modulation by light. *Journal of pineal research*, *36*, 177–185.

Cermakian, N. N., Monaco, L. L., Pando, M. P. M., Dierich, A. A., & Sassone-Corsi, P. P. (2001). Altered behavioural rhythms and clock gene expression in mice with a targeted mutation in the Period1 gene. *The European Molecular Biology Organization Journal*, *20*, 3967–3974.

Challet, E., Mendoza, J., Dardente, H., & P vet, P. (2009). Neurogenetics of food anticipation. *European Journal of Neuroscience*, *30*, 1676–1687.

Chastrette, N., Pfaff, D. W., & Gibbs, R. B. (1991). Effects of daytime and nighttime stress on Fos-like immunoreactivity in the paraventricular nucleus of the hypothalamus, the habenula, and the posterior paraventricular nucleus of the thalamus. *Brain Research*, *563*, 339–344.

Clarke, J. D., & Coleman, G. J. (1986). Persistent meal-associated rhythms in SCN-lesioned rats. *Physiology & Behaviour*, 36, 105-113.

Coleman, G. J., Harper, S., Clarke, J. D., & Armstrong, S. (1982). Evidence for a separate meal-associated oscillator in the rat. *Physiology & Behaviour*, 29, 107-115.

Coleman, G. J., & Hay, M. (1990). Anticipatory wheel-running in behaviourally anosmic rats. *Physiology & Behaviour*, *47*, 1145–1151.

Cowley MA, Smith RG, Diano S, Tschop M, Pronchuk N, Grove KL, Strasburger CJ, Bidlingmaier M, Esterman M, Heiman ML, Garcia-Segura LM, Nillni EA, Mendez P, Low MJ, Sotonyi P, Friedman JM, Liu H, Pinto S, Colmers WF, Cone RD, Horvath TL. (2003). The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. *Neuron*, 37, 649-661.

Crystal, J. D. (2009). Theoretical and conceptual issues in time-place discrimination. *The European journal of neuroscience*, *30*, 1756–1766.

Czyrak, A., Wedzony, K., Michalska, B., Fijał, K., Dziedzicka-Wasylewska, M., & Maćkowiak, M. (1997). The corticosterone synthesis inhibitor metyrapone decreases dopamine D1 receptors in the rat brain. *Neuroscience*, *79*, 489–495.

Daan, S and Pittendrigh CS. (1976). A Functional Analysis of circadian pacemakers in nocturnal rodents III. Heavy water and constant light: Homeostasis of frequency? *Journal of Comparative Physiology* 106, 267-290.

D'Agata, V., Drago, F., Serapide, F., & Cicirata, F. (1993). Effects of cerebellectomy on motivation-related behaviour: a time-course study. *Physiology & Behaviour*, *53*, 173–176.

Damiola, F. (2000). Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes & Development*, *14*, 2950–2961.

Davidson, A. J., Cappendijk, S. L., & Stephan, F. K. (2000). Feeding-entrained circadian rhythms are attenuated by lesions of the parabrachial region in rats. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, *278*, R1296–304.

Davidson, A. J., Aragona, B. J., Houpt, T. A., & Stephan, F. K. (2001). Persistence of meal-entrained circadian rhythms following area postrema lesions in the rat. *Physiology & Behaviour*, *74*, 349–354.

Davidson, A. J., Aragona, B. J., Werner, R. M., Schroeder, E., Smith, J. C., & Stephan, F. K. (2001). Food-anticipatory activity persists after olfactory bulb ablation in the rat. *Physiology & Behaviour*, *72*, 231–235.

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

de Vasconcelos, A. P., Bartol-Munier, I., Feillet, C. A., Gourmelen, S., Pevet, P., & Challet, E. (2006). Modifications of local cerebral glucose utilization during circadian food-anticipatory activity. *Neuroscience*, *139*, 741–748.

DeCoursey, P. J. (1960). Phase control of activity in a rodent. *Cold Spring Harbor symposia on quantitative biology*, 25, 49-55.

de la Iglesia, H. O., Meyer, J., Carpino, A., & Schwartz, W. J. (2000). Antiphase oscillation of the left and right suprachiasmatic nuclei. *Science*, *290*, 799–801.

Dibner, C., Sage, D., Unser, M., Bauer, C., d'Eysmond, T., Naef, F., & Schibler, U. (2008). Circadian gene expression is resilient to large fluctuations in overall transcription rates. *The European Molecular Biology Organization Journal*, *28*, 123–134.

Vitaterna M.H., King, D.P., Chang, A.M., Kornhauser, JM, Lowrey PL, McDonald, JD, Dove WF, Pinto LH, Turek FW, & Takahashi JS. (1994). Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behaviour. *Science*, 264, 719-725.

Dudley, C. A., Erbel-Sieler, C., Estill, S. J., Reick, M., Franken, P., Pitts, S., & McKnight, S. L. (2003). Altered patterns of sleep and behavioural adaptability in NPAS2-deficient mice. *Science*, *301*, 379–383.

Ebner, T. J. (1998). A role for the cerebellum in the control of limb movement velocity. *Current opinion in neurobiology*, *8*, 762–769.

Edgar, R. S., Green, E. W., Zhao, Y., Van Ooijen, G., & Olmedo, M, Qin X, Xu Y, Pan M, Valekunja UK, Feeney KA, Maywood ES, Hastings MH, Baliga NS, Merrow M, Millar AJ, Johnson CH, Kyriacou CP, O'Neil JS & Reddy, AB. (2012). Peroxiredoxins are conserved markers of circadian rhythms. *Nature*, 485, 459-464.

Edmonds, S. C., & Adler, N. T. (1977). The multiplicity of biological oscillators in the control of circadian running activity in the rat. *Physiology & Behaviour*, *18*, 921–930.

Ehrenfried, J. A., Evers, B. M., Chu, K. U., Townsend, C. M., & Thompson, J. C. (1996). Caloric restriction increases the expression of heat shock protein in the gut. *Annals of surgery*, *223*, 592–7– discussion 597–9.

Elmquist, J. K., Coppari, R., Balthasar, N., Ichinose, M., & Lowell, B. B. (2005). Identifying hypothalamic pathways controlling food intake, body weight, and glucose homeostasis. *The Journal of comparative neurology*, *493*, 63–71.

Feillet, C. A., Mendoza, J., Albrecht, U., Pévet, P., & Challet, E. (2008). Forebrain oscillators ticking with different clock hands. *Molecular and Cellular Neuroscience*, *37*, 209–221.

Feillet, C. A., Ripperger, J. A., Magnone, M. C., Dulloo, A., Albrecht, U., & Challet, E. (2006). Lack of Food Anticipation in Per2 Mutant Mice. *Current Biology*, *16*, 2016–2022.

Freedman, L. J., & Cassell, M. D. (1994). Relationship of thalamic basal forebrain projection neurons to the peptidergic innervation of the midline thalamus. *The Journal of comparative neurology*, *348*, 321–342.

Frisch, von, K. (1974). Decoding the language of the bee. *Science*, *185*, 663–668.

Fuller, P. M., Lu, J., & Saper, C. B. (2008). Differential rescue of light- and food-entrainable circadian rhythms. *Science*, *320*, 1074–1077.

Fuller, P. M., Lu, J., & Saper, C. B. (2009). Standards of evidence in chronobiology: A response. *Journal of Circadian Rhythms*, *7*, 9.

Gachon, F., Olela, F. F., Schaad, O., Descombes, P., & Schibler, U. (2006). The circadian PAR-domain basic leucine zipper transcription factors DBP, TEF, and HLF modulate basal and inducible xenobiotic detoxification. *Cell Metabolism*, *4*, 25–36.

Giachetti, I., Mac Leod, P., & Le Magnen, J. (1970). [Influence of hunger and satiety states on responses of the olfactory bulb in rats]. *Journal de physiologie*, *62 Suppl 2*, 280–281.

Girotti, M., Weinberg, M. S., & Spencer, R. L. (2009). Diurnal expression of functional and clock-related genes throughout the rat HPA axis: system-wide shifts in response to a restricted feeding schedule. *AJP: Endocrinology and Metabolism*, *296*, E888–E897.

Goel, N., & Lee, T. M. (1996). Relationship of circadian activity and social behaviours to reentrainment rates in diurnal Octodon degus (Rodentia). *Physiology & Behaviour*, *59*, 817–826.

Goel, N., & Lee, T. M. (1997). Olfactory bulbectomy impedes social but not photic reentrainment of circadian rhythms in female Octodon degus. *Journal of Biological Rhythms*, *12*, 362–370.

Goel, N., Lee, T. M., & Pieper, D. R. (1998). Removal of the olfactory bulbs delays photic reentrainment of circadian activity rhythms and modifies the reproductive axis in male Octodon degus. *Brain Research*, *792*, 229–236.

Gooley, J. J., Schomer, A., & Saper, C. B. (2006). The dorsomedial hypothalamic nucleus is critical for the expression of food-entrainable circadian rhythms. *Nature Neuroscience*, *9*, 398–407.

Granados-Fuentes, D. D., Saxena, M. T. M., Prolo, L. M. L., Aton, S. J. S., & Herzog, E. D. E. (2004). Olfactory bulb neurons express functional, entrainable circadian rhythms. *The European journal of neuroscience*, *19*, 898–906.

Granados-Fuentes, D., & Prolo, L. M. (2004). The suprachiasmatic nucleus entrains, but does not sustain, circadian rhythmicity in the olfactory bulb. *The Journal of Neuroscience*, 24, 615-619.

Granados-Fuentes, D., Tseng, A., & Herzog, E. D. (2006). A Circadian Clock in the Olfactory Bulb Controls Olfactory Responsivity. *Journal of Neuroscience*, *26*, 12219–12225.

Green, D. J., & Gillette, R. (1982). Circadian rhythm of firing rate recorded from single cells in the rat suprachiasmatic brain slice. *Brain Research*.

Guilding, C., Hughes, A., Brown, T. M., & Namvar, S. Piggins, HD. (2009). A riot of rhythms: neuronal and glial circadian oscillators in the mediobasal hypothalamus. *Molecular Brain*, 2: 28, doi 10.1186/1756-6606-2-28.

Guilding, C., Hughes, A. T. L., & Piggins, H. D. (2010). Circadian oscillators in the epithalamus. *Neuroscience*, *169*, 1630–1639.

Guo, H. (2006). Suprachiasmatic Regulation of Circadian Rhythms of Gene Expression in Hamster Peripheral Organs: Effects of Transplanting the Pacemaker. *Journal of Neuroscience*, *26*, 6406–6412.

Hara, R., Wan, K., Wakamatsu, H., Aida, R., & Moriya, T. (2001). Restricted feeding entrains liver clock without participation of the suprachiasmatic nucleus. *Genes to Cells,* 6, 269-278.

Hardin, P. E., Hall, J. C., & Rosbash, M. (1990). Feedback of the Drosophila period gene product on circadian cycling of its messenger RNA levels. *Nature*, *343*, 536–540.

Heimer, L. (1972). The olfactory connections of the diencephalon in the rat. An experimental light- and electron-microscopic study with special emphasis on the problem of terminal degeneration. *Brain, behaviour and evolution*, *6*, 484–523.

Heimer, L., Zahm, D. S., Churchill, L., Kalivas, P. W., & Wohltmann, C. (1991). Specificity in the projection patterns of accumbal core and shell in the rat. *Neuroscience*, *41*, 89–125.

Hoffmann K. (1969). Circadiane Periodik bei Tupaias (tupaia glis) in konstanten Bedingungen. *Zool. Anz* 33(suppl.): 171-177.

Honma, K. I., Honma, S., & Hiroshige, T. (1984). Feeding-associated corticosterone peak in rats under various feeding cycles. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, *246*, R721–R726.

Honma, S., Ikeda, M., Abe, H., & Tanahashi, Y. (1998). Circadian Oscillation of BMAL1, a Partner of a Mammalian Clock Gene Clock, in Rat Suprachiasmatic Nucleus. *Biochemical and Biophysical Research Communications*, 250, 83-87.

Hood, S., Cassidy, P., Cossette, M. P., Weigl, Y., Verwey, M., Robinson, B., et al. (2010). Endogenous Dopamine Regulates the Rhythm of Expression of the Clock Protein PER2 in the Rat Dorsal Striatum via Daily Activation of D2 Dopamine Receptors. *Journal of Neuroscience*, *30*, 14046–14058.

Horvitz, J. C. (2000). Mesolimbocortical and nigrostriatal dopamine responses to salient non-reward events. *Neuroscience*, *96*, 651–656.

Hsu, C. T., Patton, D. F., Mistlberger, R. E., & Steele, A. D. (2010). Palatable Meal Anticipation in Mice. *PloS one*, *5*, e12903.

Hughes, M. E., DiTacchio, L., Hayes, K. R., Vollmers, C., Pulivarthy, S., Baggs, J. E., et al. (2009). Harmonics of circadian gene transcription in mammals. *PLoS genetics*, *5*, e1000442.

Iijima, M., Nikaido, T., & Akiyama, M. (2002). Methamphetamine-induced, suprachiasmatic nucleus-independent circadian rhythms of activity and mPer gene expression in the striatum of the mouse. *European Journal of Neuroscience*, 16, 921-929.

Iijima, M., Yamaguchi, S., van der Horst, G, Bonnefont, X., Okamura, H., Shibata S. (2005). Altered food-anticipatory activity rhythm in Cryptochrome-deficient mice. *Neuroscience* Research*,* 52, 166-173*.*

Ikemoto, S., & Panksepp, J. (1996). Dissociations between appetitive and consummatory responses by pharmacological manipulations of reward-relevant brain regions. *Behavioural neuroscience*, *110*, 331–345.

Imbesi, M., Yildiz, S., Dirim Arslan, A., Sharma, R., Manev, H., & Uz, T. (2009). Dopamine receptor-mediated regulation of neuronal "clock" gene expression. *Neuroscience*, *158*, 537–544.

Ivry, R. B., Spencer, R. M., Zelaznik, H. N., & Diedrichsen, J. (2002). The cerebellum and event timing. *Annals of the New York Academy of Sciences*, *978*, 302–317.

Jasper, M. S., & Engeland, W. C. (1994). Splanchnic neural activity modulates ultradian and circadian rhythms in adrenocortical secretion in awake rats. *Neuroendocrinology*, *59*, 97–109.

Kriegsfeld, LJ, Korets R, & Silver R. (2003). Expression of the circadian clock gene Period 1 in neuroendocrine cells: an investigation using mice with a Per1::GFP transgene. *European Journal of Neuroscience,* 17, 212-220.

Kohsaka, A., Laposky, A. D., Ramsey, K. M., Estrada, C., Joshu, C., Kobayashi, Y., et al. (2007). High-Fat Diet Disrupts Behavioural and Molecular Circadian Rhythms in Mice. *Cell Metabolism*, *6*, 414–421.

Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, & Kanagawa K. (1999). Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature*, 402, 656-660.

Konopka, R. J., & Benzer, S. (1971). Clock mutants of Drosophila melanogaster. *Proceedings of the National Academy of Sciences of the United States of America*, *68*, 2112–2116.

Kornmann, B., Schaad, O., Bujard, H., Takahashi, J. S., & Schibler, U. (2007). System-driven and oscillator-dependent circadian transcription in mice with a conditionally active liver clock. *PLoS Biology*, *5*, e34.

Krieger, D. T. (1974). Food and water restriction shifts corticosterone, temperature, activity and brain amine periodicity. *Endocrinology*, *95*, 1195–1201.

Kuroda, H., Tahara, Y., Saito, K., Ohnishi, N., Kubo, Y., Seo, Y., Otsuka M., Ohura Y., Hirao A., Shibata S. (2012). Meal frequency patterns determine the phase of mouse peripheral circadian clocks. *Scientific reports*, *2*, 711.

Lalonde, R., & Botez, M. I. (1990). The cerebellum and learning processes in animals. *Brain research. Brain research reviews*, *15*, 325–332.

Lamia, K. A., Storch, K.-F., & Weitz, C. J. (2008). Physiological significance of a peripheral tissue circadian clock. *Proceedings of the National Academy of Sciences*, *105*, 15172–15177.

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

Landry, G. J., & Mistlberger, R. E. (2007). Food Entrainment: Methodological Issues. *Journal of Biological Rhythms*, *22*, 484–487.

Landry, G. J., Simon, M. M., Webb, I. C., & Mistlberger, R. E. (2006). Persistence of a behavioural food-anticipatory circadian rhythm following dorsomedial hypothalamic ablation in rats. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, *290*, R1527–34.

Landry, G. J., Yamakawa, G. R., Webb, I. C., Mear RJ, Mistlberger, RE. (2007a). The dorsomedial hypothalamic nucleus is not necessary for the expression of circadian food-anticipatory activity in rats. *Journal of Biological Rhythms*, 22, 467-478.

Landry, G. J., Yamakawa, G., & Mistlberger, R. E. (2007b). Robust food anticipatory circadian rhythms in rats with complete ablation of the thalamic paraventricular nucleus. *Brain Research*, 1141, 108-118.

Le Martelot, G., Claudel, T., Gatfield, D., Schaad, O., Kornmann, B., Sasso, Lo, G., et al. (2009). REV-ERBalpha participates in circadian SREBP signaling and bile acid homeostasis. *PLoS Biology*, *7*, e1000181.

Le Minh, N., Damiola, F., Tronche, F., & Schütz, G., & Schibler U. (2001). Glucocorticoid hormones inhibit food-induced phase-shifting of peripheral circadian oscillators. *The European Molecular Biology Organization Journal*, 20, 7128-7136.

Lee, C., Etchegaray, J. P., Cagampang, F. R., Loudon, A. S., & Reppert, S. M. (2001). Posttranslational mechanisms regulate the mammalian circadian clock. *Cell*, *107*, 855–867.

Lehman, M. N. M., Silver, R. R., Gladstone, W. R. W., Kahn, R. M. R., Gibson, M. M., & Bittman, E. L. E. (1987). Circadian rhythmicity restored by neural transplant. Immunocytochemical characterization of the graft and its integration with the host brain. *Journal of Neuroscience*, *7*, 1626–1638.

LeSauter, J., Hoque, N., Weintraub, M., Pfaff, D. W., & Silver, R. (2009). Stomach ghrelin-secreting cells as food-entrainable circadian clocks. *Proceedings of the National Academy of Sciences*, *106*, 13582–13587.

Lindblom, N., de Villiers, S. H. L., Semenova, S., Kalayanov, G., Gordon, S., Schilström, B., et al. (2005). Active immunisation against nicotine blocks the reward facilitating effects of nicotine and partially prevents nicotine withdrawal in the rat as measured by dopamine output in the nucleus accumbens, brain reward thresholds and somatic signs. *Naunyn-Schmiedeberg's archives of pharmacology*, *372*, 182–194.

Liu, Y.-Y., Liu, T.-Y., Qu, W.-M., Hong, Z.-Y., Urade, Y., & Huang, Z.-L. (2012). Dopamine is involved in food-anticipatory activity in mice. *Journal of Biological Rhythms*, *27*, 398–409.

Lowrey, P. L., & Takahashi, J. S. (2004). Mammalian circadian biology: elucidating genome-wide levels of temporal organization. *Annual Review of Genomics and Human Genetics*, *5*, 407–441.

Marchant, E. G., & Mistlberger, R. E. (1997). Anticipation and entrainment to feeding time in intact and SCN-ablated C57BL/6j mice. *Brain Research*, 765, 273-282.

McCarthy, J. J., Andrews, J. L, McDearmon EL, Campbell KS, Barber BK, Miller BH, Walker JR, Hogenesch JB, Takahashi JS, Esser KA. (2007). Identification of the circadian transcriptome in adult mouse skeletal muscle. *Physiological Genomics*, 31, 86-95.

Meijer JH, Daan S, Overkamp GLF, Hermann PM. (1990). The two-oscillator system of tree shrews (Tupaia belangeri) and its response to light and dark pulses. *Journal of Biological Rhtyhms*, 5, 1-6.

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

Mendoza, J., Graff C., Dardente H, Pevet P, & Challet E. (2005). Feeding Cues Alter Clock Gene Oscillations and Photic Responses in the Suprachiasmatic Nuclei of Mice Exposed to a Light/Dark Cycle. *Journal of Neuroscience*, *25*, 1514–1522.

Mendoza, J. (2007). Circadian clocks: setting time by food. *Journal of Neuroendocrinology*, 19, 127-137.

Mendoza, J., Albrecht, U., & Challet, E. (2010). Behavioural food anticipation in clock genes deficient mice: confirming old phenotypes, describing new phenotypes. *Genes, brain, and behaviour*, *9*, 467–477.

Mendoza, J., Pevet, P., Felder-Schmittbuhl, M. P., Bailly, Y., & Challet, E. (2010). The Cerebellum Harbors a Circadian Oscillator Involved in Food Anticipation. *Journal of Neuroscience*, *30*, 1894–1904.

Mieda, M., Williams, S. C., & Richardson, J. A. Tanaka K, Yanagisawa M. (2006). The dorsomedial hypothalamic nucleus as a putative food-entrainable circadian pacemaker. *Proceedings of the National Academy of Scienceof the United States of America*, 103, 12150-12155.

Miñana-Solis, M. C., Angeles-Castellanos, M., Feillet, C., Pevet, P., Challet, E., & Escobar, C. (2009). Differential Effects of a Restricted Feeding Schedule on Clock-Gene Expression in the Hypothalamus of the Rat. *Chronobiology International*, *26*, 808–820.

Mistlberger, R. E., & Rechtschaffen, A. (1984). Recovery of anticipatory activity to restricted feeding in rats with ventromedial hypothalamic lesions. *Physiology & Behaviour*, 33, 227-235.

Mistlberger, R.E., & Rusak, B. (1987). Palatable daily meals entrain activity rhythms in free-feeding rats: dependence on meal size and nutrient content. *Physiology and Behaviour*, 34, 17-22.

Mistlberger, R. E., & Rusak, B. (1988). Food-anticipatory circadian rhythms in rats with paraventricular and lateral hypothalamic ablations. *Journal of Biological Rhythms*, 3, 277-291.

Mistlberger RE, Houpt TA, Moore-Ede MC. (1990). Food-anticipatory rhythms under 24-hour schedules of limited access to single macronutrients. *Journal of Biological Rhythms,* 5, 35-46.

Mistlberger, R. E. (1992). Anticipatory Activity Rhythms under Daily Schedules of Water Access in the Rat. *Journal of Biological Rhythms*, *7*, 149–160.

Mistlberger, R. E., & Mumby, D. G. (1992). The limbic system and food-anticipatory circadian rhythms in the rat: ablation and dopamine blocking studies. *Behavioural Brain Research*, 47, 159-168.

Mistlberger, R. E., & Antle, M. C. (1999). Neonatal monosodium glutamate alters circadian organization of feeding, food anticipatory activity and photic masking in the rat. *Brain Research*, *842*, 73–83.

Mistlberger, R.E, Marchant, EG, & Kippin, TE. (2001). Food-entrained circadian rhythms in rats are insensitive to deuterium oxide. *Brain Research*, *919*, 283–291.

Mistlberger, R. E., Yamazaki, S., Pendergast, J. S., Landry, G. J., Takumi, T., & Nakamura, W. (2008). Comment on “Differential Rescue of Light- and Food-Entrainable Circadian Rhythms.” *Science*, *322*, 675a–675a.

Mistlberger, R. E. (2009). Food-anticipatory circadian rhythms: concepts and methods. *European Journal of Neuroscience*, *30*, 1718–1729.

Mistlberger, R. E., Buijs, R. M., & Challet, E., Escobar, C., Landry GJ, Kalsbeek A., Pevet P., Shibata S. (2009). Standards of evidence in chronobiology: critical review of a report that restoration of Bmal1 expression in the dorsomedial hypothalamus is sufficient to restore circadian food anticipatory rhtyhms in Bmal1-/- mice. *Journal of Circadian Rhythms*, 26

Mistlberger, R. E., Kent, B. A., Chan, S., Patton, D. F., Weinberg, A., & Parfyonov, M. (2012). Circadian Clocks for All Meal-Times: Anticipation of 2 Daily Meals in Rats. *PloS one*, *7*, e31772.

Moga, M. M., Weis, R. P., & Moore, R. Y. (1995). Efferent projections of the paraventricular thalamic nucleus in the rat. *The Journal of Comparative Neurology*, *359*, 221–238.

Mok, A. C., & Mogenson, G. J. (1974). Effects of electrical stimulation of the lateral habenular nucleus and lateral hypothalamus on unit activity in the upper brain stem. *Brain Research*, *78*, 425–435.

Moore, R. Y. R., & Eichler, V. B. V. (1972). Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. *Brain Research*, *42*, 201–206.

Moriya, T., Aida, R., Kudo, T., Akiyama, M., Doi, M., Hayasaka, N., Nakahata N, Mistlberger R, Okamura H., & Shibata S. (2009). The dorsomedial hypothalamic nucleus is not necessary for food-anticipatory circadian rhythms of behaviour, temperature or clock gene expression in mice. *The European Journal of Neuroscience*, *29*, 1447–1460.

Mrosovksy N., & Salmon P.A. (1987). A behavioural method for accelerating re-entrainment of rhythms to new light-dark cycles. *Nature*, 372-373.

Nakahara, K. K., Fukui, K. K., & Murakami, N. N. (2004). Involvement of thalamic paraventricular nucleus in the anticipatory reaction under food restriction in the rat. *Journal of Veterinary Medical Science*, *66*, 1297–1300.

Natsubori, A., Honma, K., & Honma, S. (2013). Differential responses of circadian Per2 expression rhythms in discrete brain areas to daily injection of methamphetamine and restricted feeding in rats. *The European Journal of Neuroscience*, ahead of print.

Nauta, H. J. (1974). Evidence of a pallidohabenular pathway in the cat. *The Journal of Comparative Neurology*, *156*, 19–27.

Nicholson SA, Adrian TE, Bacarese-Hamilton AJ, Gillham B, Jones MT, Bloom SR. (1983). 24-hour variation in content and release of hypothalamic neuropeptides in the rat. *Regulatory Peptides*, 7, 385-397.

Nikaido, T. T., Akiyama, M. M., Moriya, T. T., & Shibata, S. S. (2001). Sensitized increase of period gene expression in the mouse caudate/putamen caused by repeated injection of methamphetamine. *Molecular Pharmacology*, *59*, 894–900.

Nolasco, N., Juárez, C., Morgado, E., Meza, E., & Caba, M. (2012). A Circadian Clock in the Olfactory Bulb Anticipates Feeding during Food Anticipatory Activity. *PloS one*, *7*, e47779.

Novak, C. M., & Nunez, A. A. (1998). Daily rhythms in Fos activity in the rat ventrolateral preoptic area and midline thalamic nuclei. *The American Journal of Physiology*, *275*, R1620–R1626.

O'Neill, J. S., Van Ooijen, G., Dixon, L. E., & Troein, C., Corellou F., Bouget FY., Reddy AB., Millar AJ. (2011). Circadian rhythms persist without transcription in a eukaryote. *Nature*, 469, 554-558.

Oishi, K., Amagai, N., Shirai, H., Kadota, K., Ohkura, N., & Ishida, N. (2005). Genome-wide expression analysis reveals 100 adrenal gland-dependent circadian genes in the mouse liver. *DNA Research : an International Journal for Rapid Publication of Reports on Genes and Genomes*, *12*, 191–202.

Oster, H., Damerow, S., Kiessling, S., Jakubcakova, V., Abraham, D., Tian, J., Hoffmann, MW, Eichele G. (2006). The circadian rhythm of glucocorticoids is regulated by a gating mechanism residing in the adrenal cortical clock. *Cell Metabolism*, *4*, 163–173.

Panda, S., Antoch, M. P., Miller, B. H., Su, A. I., Schook, A. B., Straume, M., et al. (2002). Coordinated Transcription of Key Pathways in the Mouse by the Circadian Clock. *Cell*, *109*, 307–320.

Pando, M. P., Morse, D., Cermakian, N., & Sassone-Corsi, P. (2002). Phenotypic rescue of a peripheral clock genetic defect via SCN hierarchical dominance. *Cell*, *110*, 107–117.

Paulson, P. E., & Robinson, T. E. (1994). Relationship between circadian changes in spontaneous motor activity and dorsal versus ventral striatal dopamine neurotransmission assessed with on-line microdialysis. *Behavioural Neuroscience*, *108*, 624–635.

Pecina, S., & Berridge, R.C. (2005). Hedonic hot spot in nucleus accumbens sheel: where do μ- opiods cause increased hedonic impact of sweetness? *The Journal of Neuroscience*, 25, 11777-11786.

Pendergast, J. S. J., Oda, G. A. G., Niswender, K. D. K., & Yamazaki, S. S. (2012). Period determination in the food-entrainable and methamphetamine-sensitive circadian oscillator(s). *Proceedings of the National Academy of Sciences of the United States of America*, *109*, 14218–14223.

Pendergast, J. S., Nakamura, W., Friday, R. C., & Hatanaka, F. (2009). Robust food anticipatory activity in BMAL1-deficient mice. *PloS one* 4(3):e4860.

Peng, Z. C. Z., Grassi-Zucconi, G. G., & Bentivoglio, M. M. (1995). Fos-related protein expression in the midline paraventricular nucleus of the rat thalamus: basal oscillation and relationship with limbic efferents. *Experimental Brain Research*, *104*, 21–29.

Pezuk, P., Mohawk, J. A., Wang, L. A., & Menaker, M. (2012). Glucocorticoids as Entraining Signals for Peripheral Circadian Oscillators. *Endocrinology*, *153*, 4775–4783.

Pieper, D. R., & Lobocki, C. A. (1991). Olfactory bulbectomy lengthens circadian period of locomotor activity in golden hamsters. *The American journal of physiology*, *261*, R973–8.

Pittendrigh CS & Daan S.(1976a). A functional analysis of circadian pacemakers in nocturnal rodents.V. Pacemaker as clock. *Comparative Physiology,*106, 333-355.

Pittendrigh CS & Daan S (1976b). A functional analysis of circadian pacemakers in nocturnal rodents: V Pacemaker Structure: a clock for all seasons. *Journal of Comparative Physiology*, 106-333.355.

Pitts, S., Perone, E., & Silver, R. (2003). Food-entrained circadian rhythms are sustained in arrhythmic Clk/Clk mutant mice. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, *285*, R57–67.

Possidente, B., Lumia, A. R., McGinnis, M. Y., Teicher, M. H., deLemos, E., Sterner, L., & Deros, L. (1990). Olfactory bulb control of circadian activity rhythm in mice. *Brain Research*, *513*, 325–328.

Preitner, N., Damiola, F., Lopez-Molina, L., Zakany, J., Duboule, D., Albrecht, U., & Schibler, U. (2002). The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell*, *110*, 251–260.

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

Rausch, L.J., & Long, C.J. (1971). Habenular nuclei: a crucial link between the olfactory and motor systems. *Brain Research*, *29*, 146–150.

Recabarren, M. P., Valdés, J. L., Farías, P., Serón-Ferré, M., & Torrealba, F. (2005). Differential effects of infralimbic cortical lesions on temperature and locomotor activity responses to feeding in rats. *Neuroscience*, *134*, 1413–1422.

Rehfeld, J. F. (1998). The new biology of gastrointestinal hormones. *Physiological reviews*, *78*, 1087–1108.

Reick, M., Garcia, J. A., Dudley, C., & McKnight, S. L. (2001). NPAS2: an analog of clock operative in the mammalian forebrain. *Science*, *293*, 506–509.

Renner, E., Szabó-Meltzer, K. I., Puskás, N., Tóth, Z. E., Dobolyi, A., & Palkovits, M. (2010). Activation of neurons in the hypothalamic dorsomedial nucleus via hypothalamic projections of the nucleus of the solitary tract following refeeding of fasted rats. *The European journal of neuroscience*, *31*, 302–314.

Richter, C. P. (1922). A Behaviouristic Study of the Activity of the Rat. *Comparative Psychology Monographs*.

Richter, C. P. (1977). Heavy water as a tool for study of the forces that control length of period of the 24-hour clock of the hamster. *Proceedings of the National Academy of Science of the United States of America, 74, 1295-1299.*

Rosenfeld, P., Van Eekelen, J. A. M., Levine, S., & De Kloet, E. R. (1988). Ontogeny of the Type 2 glucocorticoid receptor in discrete rat brain regions: an immunocytochemical study. *Developmental Brain Research*, *42*, 119–127.

Rosenwasser, A. M., Pelchat, R. J., & Adler, N. T. (1984). Memory for feeding time: possible dependence on coupled circadian oscillators. *Physiology & Behaviour*, 32, 25-30.

Rusak, B. B., & Groos, G. G. (1982). Suprachiasmatic stimulation phase shifts rodent circadian rhythms. *Science*, *215*, 1407–1409.

Rutter, J. J., Reick, M. M., Wu, L. C. L., & McKnight, S. L. S. (2001). Regulation of clock and NPAS2 DNA binding by the redox state of NAD cofactors. *Science*, *293*, 510–514.

Rutter, J., Reick, M., & McKnight, S. L. (2002). Metabolism and the control of circadian rhythms. *Annual Review of Biochemistry*. 71, 307.

Schibler, U., Ripperger, J., & Brown, S. A. (2003). Peripheral circadian oscillators in mammals: time and food. *Journal of Biological Rhythms*, 18, 250-260.

Segall, L. A., Perrin, J. S., Walker, C.-D., Stewart, J., & Amir, S. (2006). Glucocorticoid rhythms control the rhythm of expression of the clock protein, Period2, in oval nucleus of the bed nucleus of the stria terminalis and central nucleus of the amygdala in rats. *Neuroscience*, *140*, 753–757.

Simpson, E. R., & Waterman, M. R. (1988). Regulation of the synthesis of steroidogenic enzymes in adrenal cortical cells by ACTH. *Annual Review of Physiology*, *50*, 427–440.

Stephan, F. K., & Zucker, I. (1972). Circadian rhythms in drinking behaviour and locomotor activity of rats are eliminated by hypothalamic lesions. *Proceedings of the National Academy of Sciences of the United States of America*, *69*, 1583–1586.

Stephan, F. K., Swann, J. M., & Sisk, C. L. (1979a). Entrainment of circadian rhythms by feeding schedules in rats with suprachiasmatic lesions. *Behavioural and Neural Biology*, 25, 545-554.

Stephan, F. K., Swann, J. M., & Sisk, C. L. (1979b). Anticipation of 24-hr feeding schedules in rats with lesions of the suprachiasmatic nucleus. *Behavioural and Neural Biology*.25, 346-363.

Stephan, F. K. (1981). Limits of entrainment to periodic feeding in rats with suprachiasmatic lesions. *Journal of Comparative Physiology*.143, 401-410.

Stephan, F. K. (1983). Circadian rhythm dissociation induced by periodic feeding in rats with suprachiasmatic lesions. *Behavioural Brain Research*, *7*, 81–98.

Stephan, F. K. (1984). Phase shifts of circadian rhythms in activity entrained to food access. *Physiology & Behaviour*.32, 663-671.

Stephan, F. K. (1989a). Entrainment of activity to multiple feeding times in rats with suprachiasmatic lesions. *Physiology & Behaviour*, *46*, 489–497.

Stephan, F. K. (1989b). Forced dissociation of activity entrained to T cycles of food access in rats with suprachiasmatic lesions. *Journal of Biological Rhythms*, *4*, 467–479.

Stephan, F. K. (1992). Resetting of a circadian clock by food pulses. *Physiology & Behaviour*, 52, 997-1008.

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

Storch, K.-F., Lipan, O., Leykin, I., Viswanathan, N., Davis, F. C., Wong, W. H., & Weitz, C. J. (2002). Extensive and divergent circadian gene expression in liver and heart. *Nature*, *417*, 78–83.

Storch, K. F., & Weitz, C. J. (2009). Daily rhythms of food-anticipatory behavioural activity do not require the known circadian clock. *Proceedings of the National Academy of Sciences*, *106*, 6808–6813.

Sujino, M., Furukawa, K., Koinuma, S., Fujioka, A., Nagano, M., Iigo, M., & Shigeyoshi, Y. (2012). Differential entrainment of peripheral clocks in the rat by glucocorticoid and feeding. *Endocrinology*, *153*, 2277–2286.

Sutherland, R. J. (1982). The dorsal diencephalic conduction system: a review of the anatomy and functions of the habenular complex. *Neuroscience and Biobehavioural Reviews*, *6*, 1–13.

Takasu NN, Kurosawa G, Tokuda IT, Mochizuki A, Todo T, Nakamura W. (2012). Circadian regulation of food-anticipatory activity in molecular clock-deficient mice. PLoS One, 7(11), e48892.

Tavakoli-Nezhad, M., & Schwartz, W. J. (2006). Hamsters running on time: is the lateral habenula a part of the clock? *Chronobiology International*, *23*, 217–224.

Tosini, G., & Menaker, M. (1996). Circadian rhythms in cultured mammalian retina. *Science*.272, 419-421.

van der Horst, G., Muijtjens, M., Kobayashi, K., & Takano, R. Kanno S, Takao M, de Wit J, Verkerk A, Eker AP, van Leenen D, Buijs R, Bootsma D, Hoeijmakers JH, Yasui A. (1999). Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. *Nature*, 15, 627-630.

Verwey, M., Khoja, Z., Stewart, J., & Amir, S. (2007). Differential regulation of the expression of Period2 protein in the limbic forebrain and dorsomedial hypothalamus by daily limited access to highly palatable food in food-deprived and free-fed rats. *Neuroscience*, *147*, 277–285.

Vielhaber, E. E., Eide, E. E., Rivers, A. A., Gao, Z. H. Z., & Virshup, D. M. D. (2000). Nuclear entry of the circadian regulator mPER1 is controlled by mammalian casein kinase I epsilon. *Molecular and cellular biology*, *20*, 4888–4899.

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

Wakamatsu, H. H., Yoshinobu, Y. Y., Aida, R. R., Moriya, T. T., Akiyama, M. M., & Shibata, S. S. (2001). Restricted-feeding-induced anticipatory activity rhythm is associated with a phase-shift of the expression of mPer1 and mPer2 mRNA in the cerebral cortex and hippocampus but not in the suprachiasmatic nucleus of mice. *The European Journal of Neuroscience*, *13*, 1190–1196.

Webb, I. C., Baltazar, R. M., Lehman, M. N., & Coolen, L. M. (2009). Bidirectional interactions between the circadian and reward systems: is restricted food access a unique zeitgeber? *European Journal of Neuroscience*, *30*, 1739–1748.

Welsh, D. K., Logothetis, D. E., Meister, M., & Reppert, S. M. (1995). Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. *Neuron*, 14, 697-706.

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

Yamazaki, S., Numano, R., Abe, M., Hida, A., & Takahashi, R. Ueda M, Block GD, Sakaki Y, Menaker M, Tei H. (2000b). Resetting central and peripheral circadian oscillators in transgenic rats. *Science*.288, 682-685.

Zhao, H., & Rusak, B. (2005). Circadian firing-rate rhythms and light responses of rat habenular nucleus neurons in vivo and in vitro. *Neuroscience*, *132*, 519–528.

Appendix A. Restricted Feeding Table

Structure Gene/PRT	Meal Onset	Meal Duration or %TDI	Peak Ad lib	PeakRF	Phase Change	Species	Number of time points	Technique	Reference
SCN									
per1	ZT4	5h	ZT7	ZT7	No Δ	Mouse	6	In situ	Wakamatsu 2001
	ZT5	4h	ZT7	ZT3-7	No Δ	Mouse	6	In situ	Hara 2001
	ZT0/12	12h	ZT6	ZT18	12h	Mouse	6	RPA	Damiola 2000*
	ZT6	2h	ZT3	ZT6	- 3h	Rat	8	In situ	Minana-Solis 2009
	ZT3	3h	ZT2	ZT2	No Δ	Rat	2	In situ	Girotti 2009
	ZT5	4h	ZT7	ZT7	No Δ	Mouse	6	In situ	Moriya 2009
per2	ZT4	5h	ZT7	ZT7	No Δ	Mouse	6	In situ	Wakamatsu 2001
	ZT5	4h	ZT11	ZT7-11	No Δ	Mouse	6	In situ	Hara 2001
	ZT0/12	12h	ZT10	ZT22	12h	Mouse	6	RPA	Damiola 2000*
	ZT4	4h	ZT7	ZT7	No Δ	mouse	4	In situ	Mieda 2006
	ZT6	2h	ZT12	ZT12	No Δ	Rat	8	In situ	Minana-Solis 2009
	ZT3	3h	ZT11	ZT11	No Δ	Rat	2	In situ	Girotti 2009
	ZT5	4h	ZT11	ZT11	No Δ	Mouse	6	In situ	Moriya 2009
cry1	ZT0/12	12h	ZT14	ZT22	-8	Mouse	6	RPA	Damiola 2000*
Rev-erbα	ZT0/12	12h	ZT6	ZT10	-4	Mouse	6	RPA	Damiola 2000*
bmal1	ZT6	2h	ZT12	ZT18	- 8h	Rat	8	In situ	Minana-Solis 2009
	ZT3	3h	ZT18	Sub night	N/A	Rat	2	In situ	Girotti 2009
	ZT5	4h	ZT15	ZT15	No Δ	Mouse	6	In situ	Moriya 2009

Structure Gene/PRT	Meal Onset	Meal Duration or %TDI	Peak Ad lib	PeakRF	Phase Change	Species	Number of time points	Technique	Reference
PER1	ZT6	66%	ZT12	ZT8	+ 4 h	Mouse	6	ICC	Mendoza 2007*
	ZT0	5g chocolate	ZT16	ZT8	+8h	Rat	6	ICC	Mendoza 2005*
	ZT6	2h	ZT12	ZT12	No Δ	Rat	4	ICC	Angeles-Castellanos 2008
	Variable (3)	2h	ZT13	ZT13	No Δ	Rat	4	ICC	Verwey 2009*
PER2	ZT4	2h	ZT13	ZT13	No Δ	Rat	6	ICC	Verwey 2007
	ZT6	66%	ZT12	ZT12	No Δ	Mouse	6	ICC	Mendoza 2007*
	ZT4	3h	ZT16	ZT17	− 1h	Rat	4	ICC	Segall 2008*
	ZT16	2h	ZT13	ZT13	No Δ	Rat	6	ICC	Verwey 2008
	Variable (3)	2h	ZT13	ZT13	No Δ	Rat	6	ICC	Verwey 2011*
	ZT4	2h	ZT13	ZT13	No Δ	Rat	6	ICC	Verwey 2011b
	ZT4	2h	Mid S. day	Mid S. day	No Δ	Rat	every	Luc	Natsubori 2013
CLOCK	ZT6	66%	No rhythm	No rhythm	No Δ	Mouse	6	ICC	Mendoza 2007
DMH									
per1	ZT4	4h	No rhythm	ZT6	Induced	Mouse	4	In situ	Mieda 2006
	ZT5	4h	ZT15	ZT3	12h	Mouse	6	In situ	Moriya 2009
	ZT6	2h	No rhtyhm	ZT6	Induced	Rat	8	In situ	Minana-Solis 2009
per2	ZT4	4h	No rhythm	ZT7	Induced	Mouse	4	In situ	Mieda 2006
	ZT5	4h	ZT15	ZT11	+ 4h	Mouse	6	In situ	Moriya 2009
	ZT6	2h	ZT18	No rhythm	Suppressed	Rat	8	In situ	Minana-Solis 2009
bmal1	ZT5	4h	ZT3	ZT19	− 7h	Mouse	6	In situ	Moriya 2009
	ZT6	2h	No rhythm	No rhythm	No Δ	Rat	8	In situ	Minana-Solis 2009

Structure Gene/PRT	Meal Onset	Meal Duration or %TDI	Peak Ad lib	PeakRF	Phase Change	Species	Number of time points	Technique	Reference
PER1	ZT6	2h	No rhythm	ZT12	Induced	Rat	4	ICC	Angeles-Castellanos 2008
	ZT6	66%	ZT12	ZT12	No Δ	Mouse	6	ICC	Feillet 2008*
	variable D	2h	No rhythm	ZT13	Induced	Rat	4	ICC	Verwey 2009*
	variable N	2h	No rhythm	ZT1	Induced	Rat	4	ICC	Verwey 2009*
	variable all day	2h	No rhythm	No rhythm	No Δ	Rat	4	ICC	Verwey 2009*
PER2	ZT4	2h	No rhythm	ZT5-9	Induced	Rat	6	ICC	Verwey 2007
	ZT6	66%	No staining	No staining	No Δ	Mouse	6	ICC	Feillet 2008
	ZT16	2h	No rhythm	ZT1	Induced	Rat	6	ICC	Verwey 2008
	ZT4	2h	ZT21	ZT9	12h	Rat	6	ICC	Verwey 2011b
	ZT4	6h	ZT21	ZT13	+8h	Rat	6	ICC	Verwey 2011b
N. Accumbens									
Core									
PER1	ZT5	2h	ZT18	ZT12	+ 6h	Rat	6	ICC	Angeles-Castellanos 2007
	ZT6	2h	ZT18	ZT12	+ 6h	Rat	6	ICC	Angeles-Castellanos 2008
Shell									
PER1	ZT5	2h	ZT18	ZT12	+ 6h	Rat	6	ICC	Angeles-Castellanos 2007
	ZT16	2h	ZT18	ZT12	+ 6h	Rat	6	ICC	Angeles-Castellanos 2008
BNST									
PER1	ZT5	2h	ZT12	ZT12	No Δ	Rat	6	ICC	Angeles-Castellanos 2007
PER2	ZT4	2h	ZT13	ZT17	-4h	Rat	6	ICC	Verwey 2007
	ZT4	3h	ZT11	ZT17	-6h	Rat	4	ICC	Segall 2008*
	ZT16	2h	ZT13	ZT5	+7h	Rat	6	ICC	Verwey 2008

Structure Gene/PRT	Meal Onset	Meal Duration or %TDI	Peak Ad lib	PeakRF	Phase Change	Species	Number of time points	Technique	Reference
	variable D	2h	ZT13	No rhythm	Su	Rat	4	ICC	Verwey 2011*
	variable N	2h	ZT13	ZT13	No Δ	Rat	4	ICC	Verwey 2011*
	variable all day	2h	ZT13	ZT13	No Δ	Rat	4	ICC	Verwey 2011*
	ZT4	2h	ZT9	ZT17	12h	Rat	6	ICC	Verwey 2011b
CEA									
PER1	ZT5	2h	ZT12	ZT12	No Δ	Rat	6	ICC	Angeles-Castellanos 2007
	ZT6	3h	No rhythm	No rhythm	No Δ	Rat	4	ICC	Angeles-Castellanos 2008*
	ZT6	66%	ZT12	ZT16	-4h	Mouse	6	ICC	Feillet 2008
PER2	ZT4	3h	ZT13	ZT19	-6h	Rat	4	ICC	Waddington-Lamont 2007
	ZT4	2h	ZT13	ZT17	-4h	Rat	6	ICC	Verwey 2007
	ZT16	2h	ZT13	ZT1	12h	Rat	6	ICC	Verwey 2008
	ZT4	3h	ZT11	ZT18	-7h	Rat	4	ICC	Segall 2008*
	ZT6	66%	ZT12	ZT16	-4h	Mouse	6	ICC	Feillet 2008*
	variable D	2h	ZT13	No rhythm	Suppressed	Rat	4	ICC	Verwey 2011*
	variable N	2h	ZT13	No rhythm	Suppressed	Rat	4	ICC	Verwey 2011*
	variable all day	2h	ZT13	ZT19	-6h	Rat	4	ICC	Verwey 2011*
	ZT4	2h	ZT13	ZT17	-4h	Rat	6	ICC	Verwey 2011b
BLA									
PER1	ZT5	2h	No rhythm	ZT6	Induced	Rat	6	ICC	Angeles-Castellanos 2007
	ZT6	2h	No rhythm	No rhythm	No Δ	Rat	6	ICC	Angeles-Castellanos 2008

Structure Gene/PRT	Meal Onset	Meal Duration or %TDI	Peak Ad lib	PeakRF	Phase Change	Species	Number of time points	Technique	Reference
PER2	ZT4	3h	ZT1	ZT19	-5h	Rat	4	ICC	Waddington-Lamont 2007
	ZT4	2h	ZT1	ZT17	-7h	Rat	6	ICC	Verwey 2007
	ZT6	66%	ZT0	ZT4 and 20	-4h	Mouse	6	ICC	Feillet 2008
	ZT4	3h	ZT23	ZT17	+6h	Rat	4	ICC	Segall 2008*
	ZT16	2h	ZT1	ZT1	No Δ	Rat	6	ICC	Verwey 2008
	variable D	2h	ZT19-1	No rhythm	No Δ	Rat	4	ICC	Verwey 2011*
	variable N	2h	ZT19-1	ZT19-1	No Δ	Rat	4	ICC	Verwey 2011*
	variable all day	2h	ZT19-1	ZT19-1	No Δ	Rat	4	ICC	Verwey 2011*
	ZT4	2h	ZT1	ZT17	-16h	Rat	6	ICC	Verwey 2011b
			ZT1	ZT21	-20h	Rat	6	ICC	Verwey 2011b
Structure Gene/PRT	Meal Onset	Meal Duration or %TDI	Peak Ad lib	Peak RF	Phase Change	Species	Number of time points	Technique	Reference
ARC									
per1	ZT5	4h	ZT15	ZT3	12h	Mouse	6	In situ	Moriya 2009
	ZT6	2h	No rhythm	ZT6	Induced	Rat	8	In situ	Minana-Solis 2009
per2	ZT5	4h	ZT17	ZT11	+6h	Mouse	6	In situ	Moriya 2009
	ZT6	2h	No rhythm	No rhythm	No Δ	Rat	8	In situ	Minana-Solis 2009
bmal	ZT5	4h	ZT3	ZT19	-6h	Mouse	6	In situ	Moriya 2009
	ZT6	2h	ZT0	ZT18	-4h	Rat	8	In situ	Minana-Solis 2009
PER1	ZT6	2h	ZT18-0	ZT12	+6h	Rat	6	ICC	Angeles-Castellanos 2008
	ZT6	66%	ZT12	ZT12	No Δ	Mouse	6	ICC	Feillet 2008*

Structure Gene/PRT	Meal Onset	Meal Duration or %TDI	Peak Ad lib	PeakRF	Phase Change	Species	Number of time points	Technique	Reference
PER2	ZT6	66%	ZT16	ZT8	+8h	Mouse	6	ICC	Feillet 2008*
Striatum									
per1	ZT4	5h	No rhythm	ZT7	Induced	Mouse	2	In situ	Wakamatsu 2001
per2	ZT4	5h	No rhythm	ZT7	Induced	Mouse	2	In situ	Wakamatsu 2001
	ZT4	4h	ZT13-19	ZT7	+6h	Mouse	4	ICC	Mieda 2006
PER1	ZT6	66%	ZT1	ZT20-1		Mouse	6	ICC	Feillet 2008
PER2	ZT6	66%	No staining	No staining	No Δ	Mouse	6	ICC	Feillet 2008
	variable D	2h	ZT19-1	ZT7	12	Rat	4	ICC	Verwey 2011*
	variable N	2h	ZT19-1	ZT19	No Δ	Rat	4	ICC	Verwey 2011*
	variable all day	2h	ZT19-1	ZT19	No Δ	Rat	4	ICC	Verwey 2011*
caudate put									
PER2	ZT4	2h	early Sub N	Mid S. day	+6h	Rat	every	Luc	Natsubori 2013
Hippocampus									
per1	ZT4	5h	ZT15	ZT7	+8h	Mouse	2	In situ	Wakamatsu 2001
per2	ZT4	5h	ZT15	ZT7	+8h	Mouse	2	In situ	Wakamatsu 2001
PER1	ZT5	2h	ZT18	ZT12	+6h	Rat	6	ICC	Angeles-Castellanos 2007
dentate gyrus									
PER1	ZT6	66%	ZT20	ZT20	No Δ	Mouse	6	ICC	Feillet 2008*

Structure Gene/PRT	Meal Onset	Meal Duration or %TDI	Peak Ad lib	PeakRF	Phase Change	Species	Number of time points	Technique	Reference
PER2	ZT4	3h	ZT1	ZT19	+5h	Rat	4	ICC	Waddington-Lamont 2007
	ZT4	2h	ZT1	ZT1 and 13	No Δ/12h	Rat	6	ICC	Verwey 2007
	ZT6	66%	ZT20	ZT20	No Δ	Mouse	6	ICC	Feillet 2008*
	ZT4	3h	ZT23	ZT17	+6h	Rat	4	ICC	Segall 2008*
	ZT16	2h	ZT1	ZT1	No Δ	Rat	6	ICC	Verwey 2008
	variable D	2h	ZT19-1	No rhythm	Suppressed	Rat	4	ICC	Verwey 2011*
	variable N	2h	ZT19-1	ZT19-1	No Δ	Rat	4	ICC	Verwey 2011*
	variable all day	2h	ZT19-1	ZT19	No Δ	Rat	4	ICC	Verwey 2011*
	ZT4	2h	ZT1	ZT1	No Δ	Rat	6	ICC	Verwey 2011b
CA1									
PER1	ZT6	66%	ZT0	ZT20	+4h	Mouse	6	ICC	Feillet 2008*
PER2	ZT6	66%	ZT20	ZT20	No Δ	Mouse	6	ICC	Feillet 2008*
Cortex									
per1	ZT4	5h	ZT15	ZT7	+8h	Mouse	6	In situ	Wakamatsu 2001
per2	ZT4	5h	ZT11	ZT7	+4h	Mouse	6	In situ	Wakamatsu 2001
	ZT4	4h	ZT13-19	ZT7	+6h	Mouse	4	In situ	Mieda 2006
parietal									
per1	ZT5	4h	ZT11	ZT3	+8h	Mouse	6	In situ	Moriya 2009
per2	ZT5	4h	ZT15	ZT3-11	12h	Mouse	6	In situ	Moriya 2009
bmal1	ZT5	4h	ZT3	ZT19	-16h	Mouse	6	In situ	Moriya 2009

Structure Gene/PRT	Meal Onset	Meal Duration or %TDI	Peak Ad lib	PeakRF	Phase Change	Species	Number of time points	Technique	Reference
PER2	ZT4	2h	early S Night	late S day	~+4h	Rat	every	luc	Natsubori 2013
prefrontal									
PER1	ZT5	2h	ZT6	ZT12	-6h	Rat	6	ICC	Angeles-Castellanos 2007
	ZT6	2h	ZT6	ZT12	-6h	Rat	6	ICC	Angeles-Castellanos 2008
piriform									
per1	ZT4	5h	No rhythm	ZT7	Induced	Mouse	2	In situ	Wakamatsu 2001
per2	ZT4	5h	No rhythm	ZT7	Induced	Mouse	2	In situ	Wakamatsu 2001
PER1	ZT6	66%	ZT0 and 16	ZT12 and 20	~12h	Mouse	6	ICC	Feillet 2008*
PER2	ZT6	66%	No staining	No staining	No Δ	Mouse	6	ICC	Feillet 2008*
PVT									
PER1	ZT0	5g chocloate	ZT20	ZT0	-4h	Rat	6	ICC	Mendoza 2005*
	ZT5	2h	No rhtyhm	ZT4-7	Induced	Rat	6	ICC	Angeles-Castellanos 2007
	ZT6	2h	ZT0	ZT6	-6h	Rat	6	ICC	Angeles-Castellanos 2008
	ZT6	66%	ZT16	ZT16	No Δ	Mouse	6	ICC	Feillet 2008*
PER2	ZT6	66%	ZT16	ZT12	+4h	Mouse	6	ICC	Feillet 2008*
PVH									
per1	ZT6	2h	ZT15	ZT12	+3h	Rat	8	In situ	Minana-Solis 2009
	ZT3	3h	ZT11	ZT2	+9h	Rat	2	In situ	Girotti 2009
	ZT4	5h	No rhythm	ZT7	Induced	Mouse	2	In situ	Wakamatsu 2001
per2	ZT6	2h	ZT18	ZT12	+6h	Rat	8	In situ	Minana-Solis 2009
	ZT3	3h	ZT11	ZT11	No Δ	Rat	2	In situ	Girotti 2009
	ZT4	5h	ZT15	ZT7	+8h	Mouse	2	In situ	Wakamatsu 2001

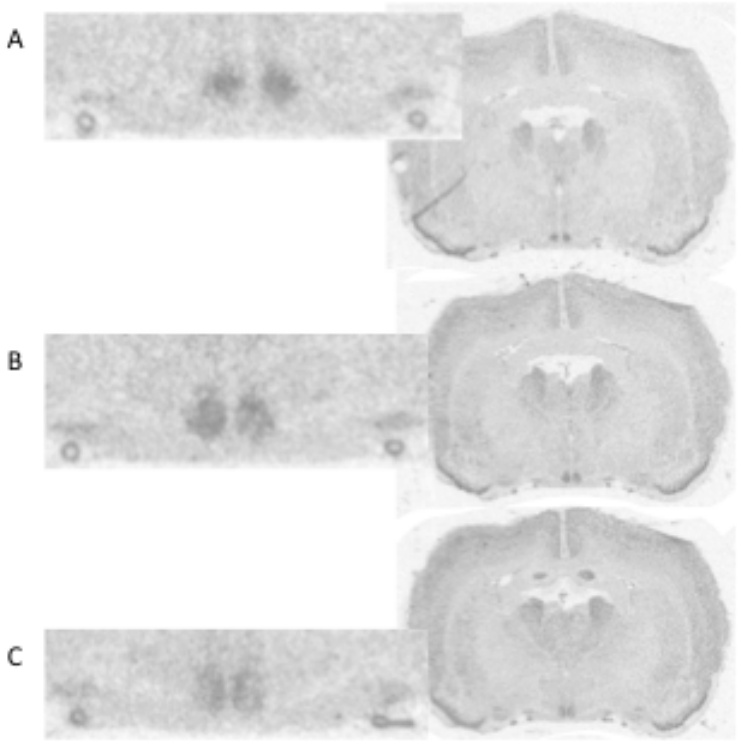
Structure Gene/PRT	Meal Onset	Meal Duration or %TDI	Peak Ad lib	PeakRF	Phase Change	Species	Number of time points	Technique	Reference
bmal1	ZT6	2h	ZT3 and 21	No rhythm	Supressed	Rat	8	In situ	Minana-Solis 2009
	ZT3	3h	ZT2	ZT11	-8h	Rat	2	In situ	Girotti 2009
PER1	ZT6	66%	No staining	No staining	No Δ	Rat	6	ICC	Feillet 2008*
	ZT6	66%	ZT16	ZT8	+8h	Mouse	6	ICC	Feillet 2008*
VMH									
per1	ZT6	2h	ZT3	ZT3	No Δ	Rat	8	In situ	Minana-Solis 2009
per2	ZT6	2h	ZT12	No rhythm	Suppressed	Rat	8	In situ	Minana-Solis 2009
bmal	ZT6	2h	ZT18	No rhythm	Suppressed	R	8	In situ	Minana-Solis 2009
PER1	ZT6	2h	No rhythm	No rhythm	No Δ	Rat		ICC	Angeles-Castellanos 2008
	ZT6	66%	ZT16	ZT8	+8H	Rat	6	ICC	Feillet 2008*
PER2	ZT6	66%	No staining	No staining	No Δ	Rat	6	ICC	Feillet 2008*
Structure Gene/PRT	Meal Onset	Meal Duration or %TDI	Peak Ad lib	Peak RF	Phase Change	Species	Number of time points	Technique	Reference
OB									
PER1	ZT0(AM or PM)		N/A	ZT1.5	N?A	Rabbit	7	ICC	Nolasco 2012
PER2	ZT4	2h	early Sub N	ZT12	~+1h	Rat	every	Luc	Natsubori 2013
Cerebellum									
per1	ZT6	66%	ZT13-14	ZT6	+7h	Mouse	4	In situ	Mendoza 2010*
per2	ZT6	66%	ZT13-14	ZT6	+7h	Mouse	4	In situ	Mendoza 2010*
reverb	ZT6	66%	ZT8-9	ZT6	+2h	Mouse	4	In situ	Mendoza 2010*
dbp	ZT6	66%	ZT8-9	ZT14	-6h	Mouse	4	In situ	Mendoza 2010*

Structure Gene/PRT	Meal Onset	Meal Duration or %TDI	Peak Ad lib	PeakRF	Phase Change	Species	Number of time points	Technique	Reference
IGL									
PER1	ZT0	5g chocolate	No staining	No staining	No Δ	Rat	6	ICC	Mendoza 2005*
N of Solitary T									
per2	ZT4	4h	ZT19	ZT7	12h	Mouse	4	In situ	Mieda 2006
Area Postrema									
per2	ZT4	4h	N	ZT7	Induced	Mouse	4	In situ	Mieda 2006
Lateral Septum									
PER1	ZT5	2h	No rhtyhm	ZT5	Induced	Rat	6	ICC	Angeles-Castellanos 2007
Perifornical Area									
PER1	ZT6	2h	No rhtyhm	ZT8	Induced	Rat	6	ICC	Angeles-Castellanos 2008
Substantia Nigra									
PER2	ZT4	2h	Late S Night	Early S Night	~+4h	Rat	every	Luc	Natsubori 2013
Anterior Pituitary									
per1	ZT3	3h	No rhtyhm	No rhtyhm	No Δ	Rat	2	In situ	Girotti 2009
per2	ZT3	3h	ZT11	ZT2	+8h	Rat	2	In situ	Girotti 2009
bmal1	ZT3	3h	ZT2	ZT11	-8h	Rat	2	In situ	Girotti 2009

Damiola 2000* Animals were fed for 12h starting at either ZT 0 or ZT12. Adlib values reflect the ZT0 fed animals and RF values reflect the ZT12 fed animals
Mendoza 2005* 5g chocolate given during projected mid subjective day (in DD) ZT0 is defined as food in Mendoza 2007* 66% of total daily intake once daily at ZT6
Feillet 2008* Fed 66% of total daily intake once daily at ZT6
Segall 2008* Rats were adrenalecotmized
Verwey 2009* variable feeding schedule 3 groups: 2h of food access per day either during light period, during dark period or light and dark period. Fed ensure during 2h food access
Mendoza 2010* Fed 66% of total daily intake once daily at ZT6
Verwey 2011* variable feeding schedule 3 groups: 2h of food access per day either during light period, during dark period or light and dark period

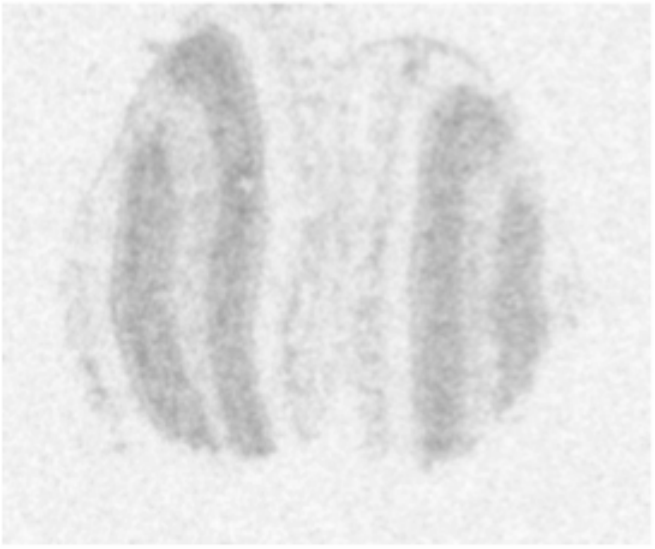
Appendix B. Representative In Situ Images for each Region of Interest

Suprachiasmatic Nucleus

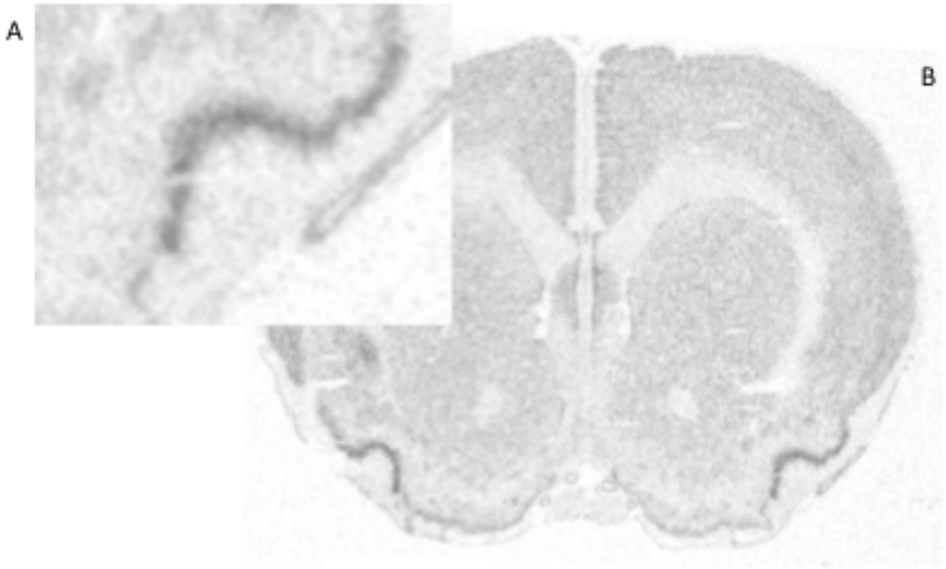


A Enlarged view of the anterior SCN and representative coronal section for the anterior SCN. B. Enlarged view of the medial SCN and representative coronal section of the medial SCN. C. Enlarged view of the posterior SCN and representative coronal section for the posterior SCN.

Olfactory Bulb

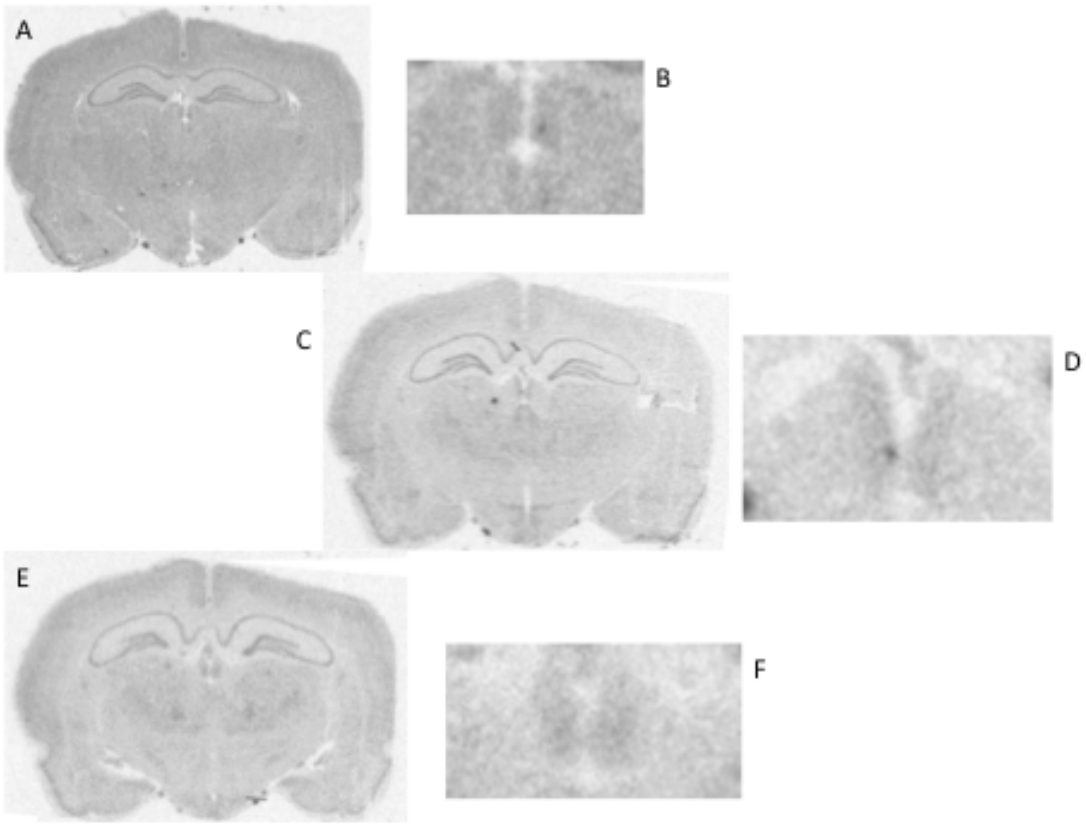


Piriform Cortex



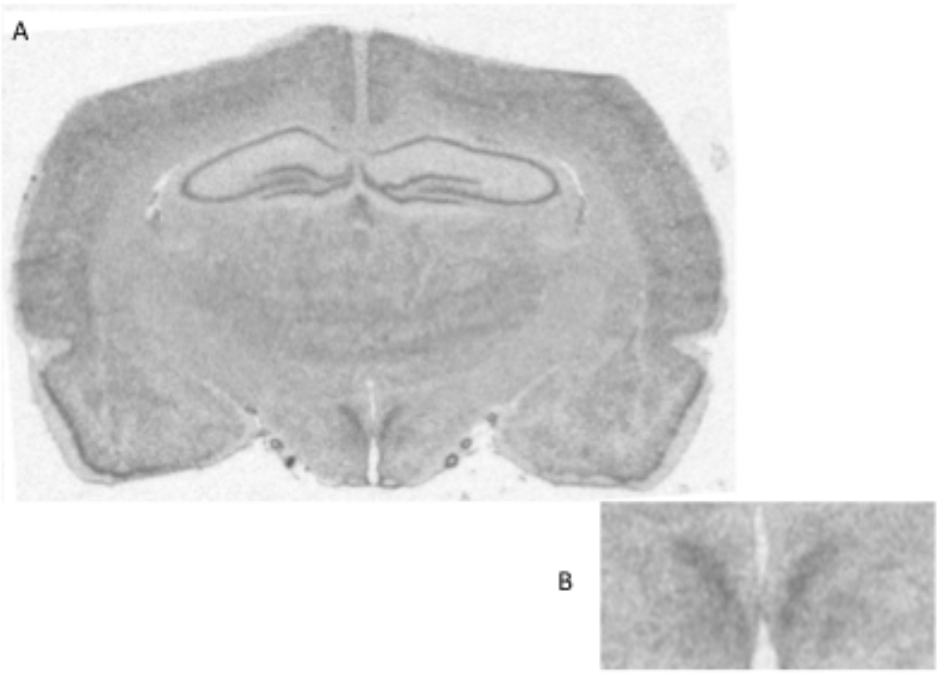
A. Enlarged view of the piriform cortex. B Representative Coronal section of the piriform cortex.

Habenula



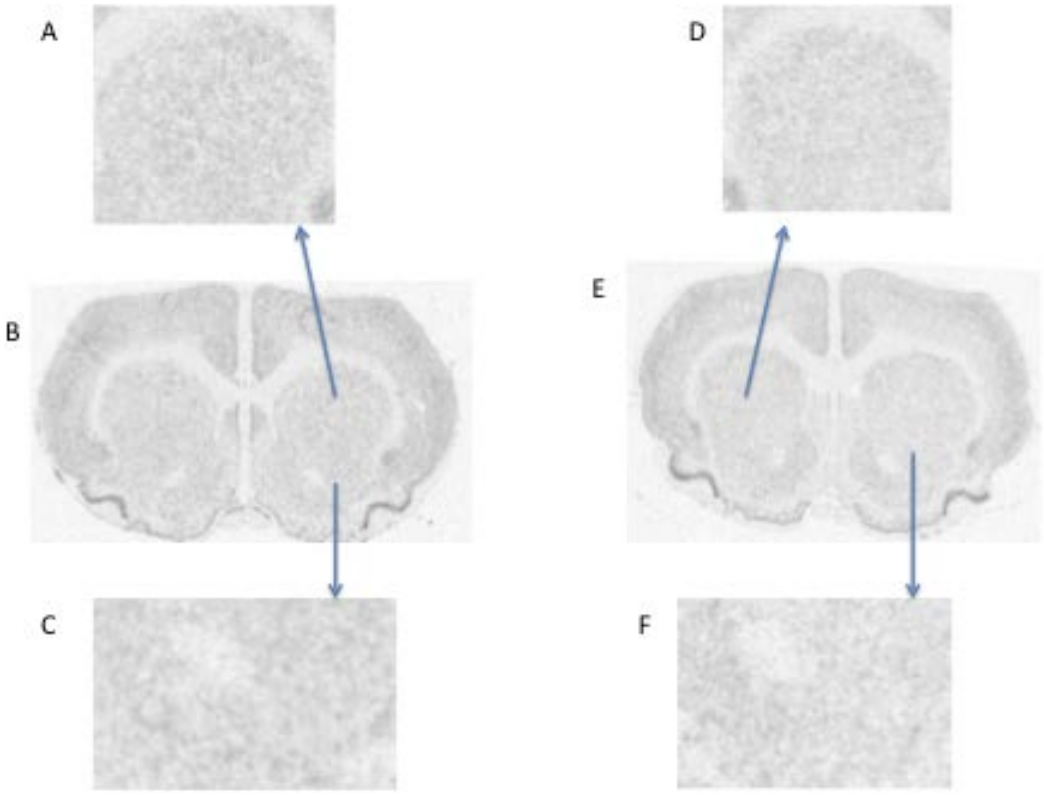
A. Representative coronal section of the anterior habenula. B Enlarged view of the anterior habenula. C Representative coronal section of the medial habenula. D Enlarged view of the medial habenula. E. Representative coronal section of the posterior habenula. F Enlarged view of the posterior habenula.

Dorsal Medial Hypothalamus



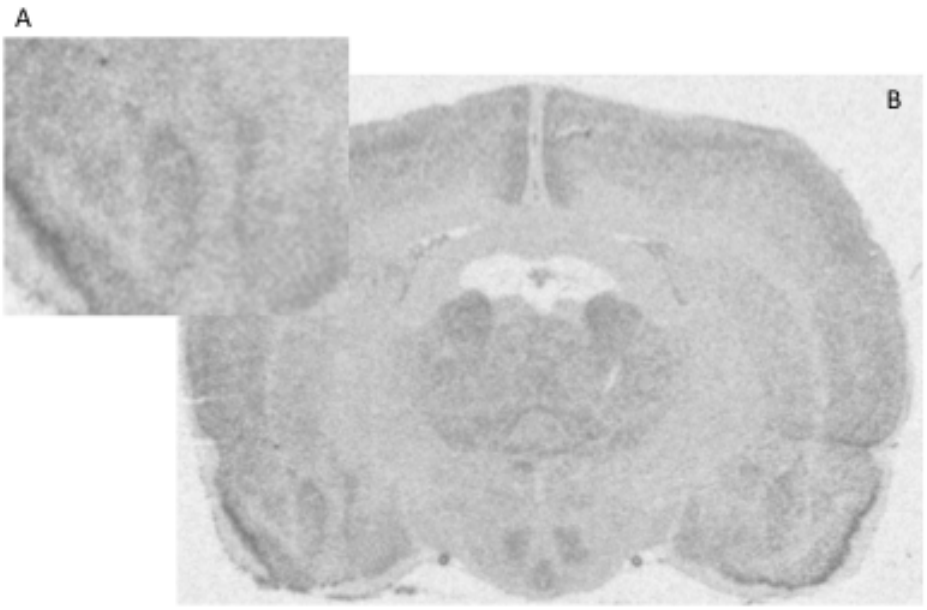
A. Representative coronal section of the dorsal medial hypothalamus. B. Enlarged view of the dorsal medial hypothalamus.

Nucleus Accumbens and Dorsal Striatum



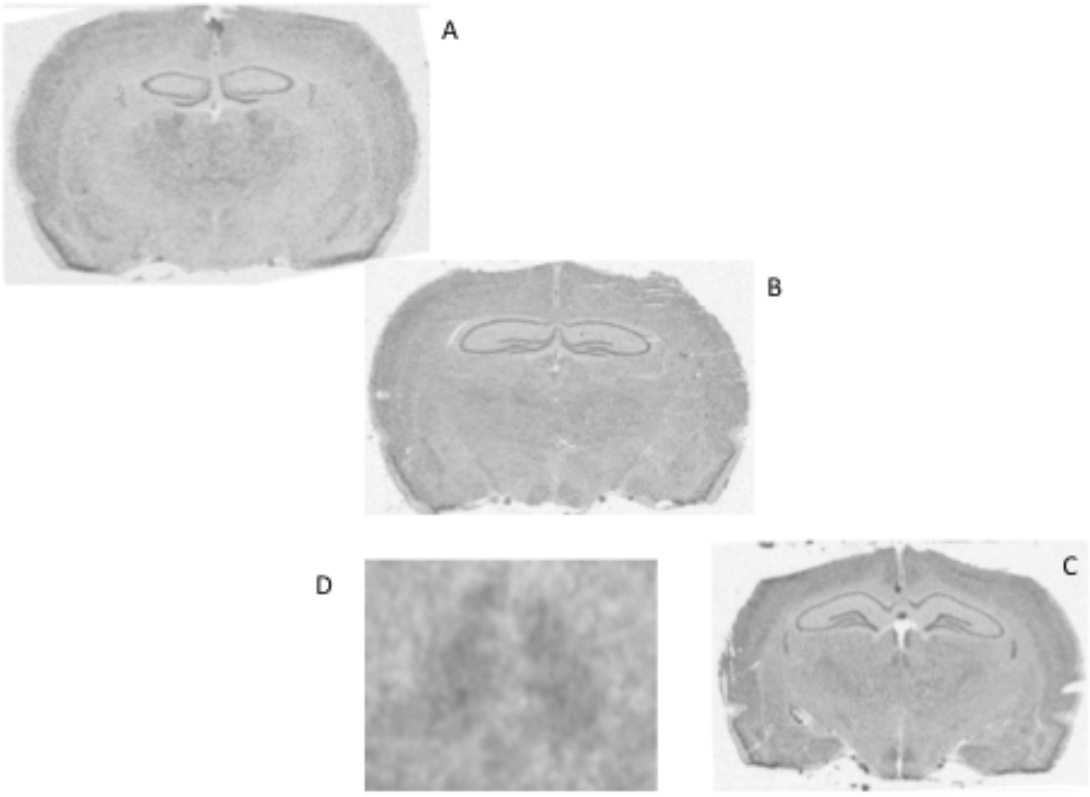
A. Enlarged view of the anterior aspect of the dorsal striatum. B. Representative coronal section of the anterior dosal striatum and nucleus accumbens. C. Enlarged view of the anterior aspect of the nucleus accumbens. D. Enlarged view of the medial aspect of the dorsal striatum. E. Representative coronal section of the medial aspect of the dorsal striatum and nucleus accumbes. F. Enlarged view of the medial aspect of the nucleus accumbens

Amygdala



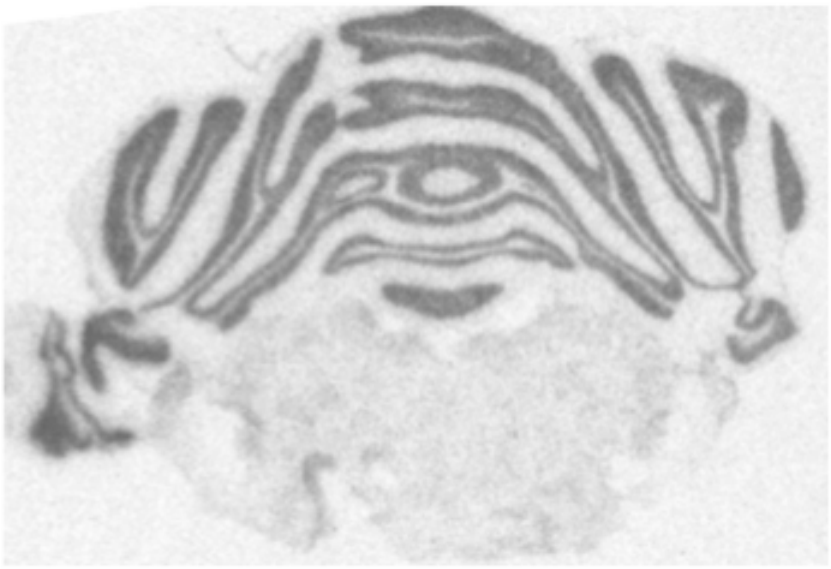
A. Enlagred view of regions of the amygdala. B. Representative coronoal section of the amygdala

Paraventricular Nucleus of the Thalamus



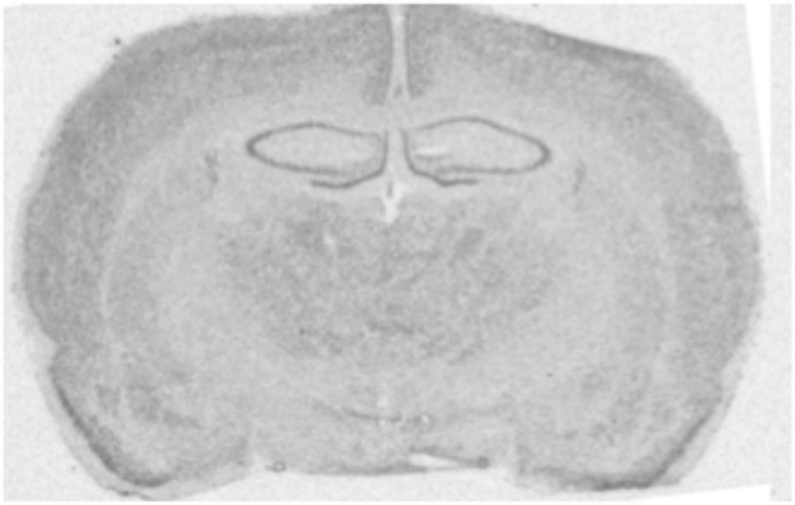
A. Representative coronal section of the anterior aspect of the paraventricular nucleus of the thalamus. B. Representative coronal section of the medial aspect of the paraventricular nucleus of the thalamus. C. Representative coronal section of the posterior aspect of the paraventricular nucleus of the thalamus. D. Englarged view of the posterior aspect of the paraventricular nucleus of the thalamus

Cerebellum



Representative coronal section of the cerebellum

Barrel Cortex



Representative coronal section of the barrel cortex

Appendix C. RT PCR Oligos

Clock Genes

Per1

Forward (5'-3-) ACACCCAGAAGGAAGAGCAA

Reverse (5'-3') GCGAGAACGCTTTGCTTTAG

Per2

Forward (5'-3') CACCCTGAAAAGAAAGTGCGA

Reverse (5'-3') CAACGCCAAGGAGCTCAAGT

Rev-erb α

Forward (5'-3') ACAGCTGACACCACCCAGATC

Reverse (5'-3') CATGGGCATAGGTGAAGATTTCT

Npas 2

Forward (5'-3') ATCTGTGACATCCAGCAGGA

Reverse (5'-3') GGACACATAGATGATGCTGC

Bmal-1

Forward (5'-3') CTTGCGGAATGTCACAGGCA

Reverse (5'-3') ACACCAGTGTTGGTTGAGAC

House Keeping Genes

Gapdh

Forward (5'-3') ATGTCGTGGAGTCTACTGGC

Reverse (5'-3') AGGATGCATTGCTGACAATC

Actin

Forward (5'-3') CTACAATGAGCTGCGTGTGG

Reverse (5'-3') CATCACAATGCCAGTGGTAC

Histone 1

Forward (5'-3') GAACGCCGACTCCCAGATC

Reverse (5'-3') CCCCTTTGGTTTGCTTGAGA

Ribosomal protein s13

Forward (5'-3') CTGATCTTCCTGAGGATCTC

Reverse (5'-3') CTCAATTAGAATCAGGCGGA

Ribosomal protein L27

Forward (5'-3') CAAGCGATCCAAGATCAAGT

Reverse (5'-3') TCCTCAAACTTGACCTTGGC

Ribosomal protein L30

Forward (5'-3') CAGACTCTGAAGATGATCAG

Reverse (5'-3') ACGCTGTGCCCAATTCAATG

Orthithine decarbosylase antizyme

Forward (5'-3') CTCTACTAGAGTTCGCAGAG

Reverse (5'-3') GCGTGTAGACCATGAAGCAA

Cyclophilin

Forward (5'-3') AGCACTGGGGAGAAAGGATT

Reverse (5'-3') ACTGCCAAGACTGAGTGGCT

Appendix D. Housekeeping Genes