FINDING THE FOOD ENTRAINABLE OSCILLATOR:

Isolating the Circadian Pacemaker of Food Anticipation

by

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Abstract

Animals under temporally restricted meal schedules exhibit food anticipatory activity (FAA). Previous investigations conclude that FAA is regulated by a central network of circadian mechanisms, the food entrainable oscillator (FEO), whose neural correlates remain unknown. This study used an intrinsic property of circadian pacemakers, the ability to continue oscillating after removal of the zeitgeber (cue), to isolate the circadian components of food anticipation. It was hypothesized that observation of anticipatory activation in central nuclei after food entrainment will elucidate the neural structures responsible for maintaining circadian oscillations of the FEO.

A second study hypothesized that ghrelin, an orexigenic hormone produced in the stomach whose secretion rises pre-prandially and quickly subsides after the presentation of a meal, serves as an important signal for the entrainment and expression of FAA. Mice expressing a genetic knock out of the ghrelin receptor gene were compared to their wild-type counterparts in order to examine this process and its neural correlates.

Keywords:

Circadian Rhythms; Clock Genes; Food Anticipatory Activity (FAA); Food Entrainable Oscillator (FEO); Ghrelin Receptor (GHS-R); Light/Dark Cycle (L:D); Light Entrainable Oscillator (LEO); Meal Anticipation; Restricted Feeding Schedule (RF); Zeitgeber

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Abbreviations:

5-HT, serotonin, AP, area postrema, ARC, arcuate nucleus, BLA, basolateral amygdala, BMAL, brain and muscle Arnt-like protein-1, BNST, bed nucleus of stria terminalis, CeA, central nucleus of amygdala, CKI ϵ , casein kinase I ϵ , CLOCK, “circadian locomotor output cycles kaput” protein, CRY, Cryptochrome, DA, dopamine, DR, dorsal raphe, E-box, noncanonical 5’-CACGTT-3’ E-box enhancer in the clock gene promoters, EPN, entopeduncular nucleus, GHSR, ghrelin receptor, GHT, geniculo-hypothalamic tract, GLU, glutamate, KO, knock out, LH, lateral hypothalamus, LRE, light-responsive elements in the clock gene promoters, MPT, Mesopontine Tegmental area, NA, noradrenaline, NacB, nucleus accumbens, NTS, nucleus of the tractus solaris, PBN, parabrachial complex, PER, Period protein, PPT, pedunculopontine tegmental area, PVN, paraventricular hypothalamus, RHT, retino-hypothalamic tract, RORE, retinoic acid-related orphan receptor response element, REV-ERB α , nuclear receptor subfamily 1 group D member 1 transcriptional repressor, R α , REV-ERB α mRNA, ROI, region of interest, SNR, substantia nigra pars reticularis, STN, subthalamic nucleus, VLM, ventrolateral medulla, VTA, ventral Tegmental area. WT, Wild-type,

1 Introduction

While completing his doctoral thesis on spontaneous activity rhythms in rats, Curt P. Richter observed that, when presented with a 25 minute meal, instead of typical *ad libitum* access, rats showed a distinct peak in their locomotor activity in the hours preceding the presentation of food (1922). This behaviour was revisited by Bolles and Stokes (1965), confirming the ability of mammals to anticipate a meal when it is presented in a timed and temporally restricted manner. This pre-prandial peak in locomotor activity is now referred to as food anticipatory activity (FAA) and is generally observed within a two hour period preceding the anticipated meal.

Food availability in the environment is often a function of specifically timed daily events, the opening of flowers, the temperature dependent activity of cold-blooded species or insects, and for higher organisms, especially in the case of predatory animals or scavengers, dependence on the actions or presence of animals lower in the food chain. This effect is even created artificially by humans in cases where outside dining leads to food wastes that are discarded on the ground or another accessible location. It is no surprise then that the ability of higher organisms like bees, birds, fish, and mammals to exploit spatial and temporal availability of foods has been highly documented (Mistlberger, 1994). For humans, there are also cultural pressures which determine both food availability and consumption. Modern patterns of food consumption vary from culture to culture, driven by social conventions, but one could argue these conventions are themselves shaped by internal biological mechanisms created and fine-tuned by evolutionary pressures. All mammals, and humans are no exception, exhibit circadian (daily) patterns of behaviour and physiology that fit roughly to a twenty-four hour cycle and have evolved endogenous molecular clocks to maintain these processes. Understanding central and peripheral mechanisms of the multiple clocks driving behaviour and physiology will be import when considering the impact of meal timing on cognitive and metabolic function. Furthermore, a concrete understanding of these clocks allows us to better assess disease pathologies with underlying circadian components;

diseases which may be alleviated by simple behavioural modulation, or even pharmacological manipulation of the circadian system.

Upon the discovery of an autonomous circadian clockwork mechanism in the anterior hypothalamus, namely in the suprachiasmatic nucleus (SCN), it became clear that clock-driven functions throughout the brain and body are controlled by a master pacemaker (Moore & Eichler, 1972; Stephan & Zucker, 1979). As research progressed, it became apparent that this master clock in the SCN was informed and controlled by the most predictable and pervasive stimulus in the environment: the sun. Investigations concerning FAA focused on its characterization, assuming that it was controlled by the same structure (the SCN) or in the same manner as light-directed behaviours. This idea gained support with the observation that rodents with bilateral lesions of the SCN did not show the typical rhythmic patterns of locomotor activity, food or water intake when provided *ad libitum* access to food (Nagai, Nishio, Nakagawa, Nakamura, & Fukuda, 1978; Stephan & Zucker, 1972a). However, this assumption proved false. Rats with similar bilateral lesions of the SCN were able to entrain to a fixed meal and demonstrated near 24 hour rhythmicity of locomotor activity with the same stable phase and amplitude when fasted for a 3 day period (Stephan, Swann, & Sisk, 1979a). Furthermore, gross oscillations of multi-neuronal activity within the SCN is not entrained by a restricted feeding schedule, and this represents an atypical dissociation between the rhythmicity of the SCN and locomotor activity, as they are typically highly correlated (Inouye, 1982a).

With the discovery that FAA did not require the SCN for entrainment of rhythmic behavioural expression, it was initially proposed that this may be the result of an hourglass mechanism. In this case, expression of anticipatory activity was thought to be the result of an “hourglass” interval timer, mechanism with the expression of FAA increasing proportionally to the amount of time since the last period of food availability (Stephan, et al., 1979a). This postulation was quickly replaced by a more parsimonious theory with FAA’s circadian

beginnings; it must be the result of the entrainment of an endogenous circadian oscillator (Stephan, Swann, & Sisk, 1979b), one that resides outside of the SCN.

The current investigation aimed to locate and characterize the oscillatory system responsible for FAA, heretofore referred to as the food-entrainable oscillator (FEO). In order to undertake such a task required the isolation of the circadian aspects of this complex behaviour, accounting not only for the effects of the homeostatic metabolic demand but also the appetitive and affective motivational drives that interact and converge to produce FAA. In order to resolve this issue, this study investigated the neural correlates of the FEO under conditions that minimize the secondary effects of restricted feeding, those that do not rely on anticipation of food, but rather activate in response to a lack of food. This was accomplished by investigating animals that have previously entrained to a daily meal (the FEO has been set), but are currently receiving *ad libitum* feeding and are not expressing FAA. It was hypothesized that returning animals to *ad libitum* feeding will not disrupt the phase or function of the FEO, since fasting would normally reinstate FAA at the time previously set, and this will be exploited to investigate specific brain areas for anticipatory activation using cFOS protein expression as a marker. A second study aimed at characterizing the FEO investigated the role of ghrelin, a circulating hormone released from the stomach, in the entrainment of the FEO and the expression of FAA. In this case it was hypothesized that ghrelin is an important peripheral cue, necessary for FAA and this was resolved using ghrelin receptor knock out mice.

1.1 Molecular Clocks

Molecular clocks can be found in various cells types and tissues throughout the body where rhythmic functioning helps to coordinate specific tasks, like hormonal secretion or neurotransmission while conserving resources and energy. Each molecular clock is driven by a complex system of auto-regulating transcriptional and translational feedback loops with positive and negative elements (Ko & Takahashi, 2006) (see **Figure 1**). While rhythmic, oscillations

Figure 1: Model of the mammalian molecular clock. A) diagram depicting interactions between multiple transcription/translational feedback loops. Abbreviations: BMAL, brain and muscle Arnt-like protein-1; CKI ϵ , casein kinase I ϵ ; CLOCK, “circadian locomotor output cycles kaput” protein; CRY, Cryptochrome; E-box, noncanonical 5'-CACGTT-3' E-box enhancer in the clock gene promoters; LRE, light-responsive elements in the clock gene promoters; PER, Period protein; RORE, retinoic acid-related orphan receptor response element; REV-ERB α , nuclear receptor subfamily 1 group D member 1 transcriptional repressor; R α , REV-ERB α mRNA. B) The phase of this molecular clock at any given time can be assessed by assaying gene or protein expression. This figure represents the expression profiles of the Per and Cry proteins as well as Bmal and Clock within the mammalian SCN in relation to the light:dark cycle.

created by these clocks do not necessarily fit perfectly to a circadian (24 hr) timeframe (Shearman et al., 2000). They require external cues, or zeitgebers, to reset and maintain a precisely coordinated system. Without zeitgebers, these oscillators enter a free run state that is stable within an organism but variable between individuals in a population. The length of a single cycle during free run, τ (Tau), in an individual is related to the biochemical interactions that maintain the oscillations of transcription and translation events in the absence of a resetting mechanism. Even slight allelic variations can affect the rate of activity and/or affinity of any one of these clock proteins, ultimately resulting in minute variations in the frequency of oscillatory mechanisms. Similarly, these same genetic variations lead to individual clocks that differ in their susceptibility to resetting stimuli. The specific mechanism of action that each zeitgeber may use to interact directly with the molecular clock varies in its site of action. For example, it can reduce the transcription of one clock gene or increase the transcription of another. Other zeitgebers are directly responsible for the physical degradation of clock proteins. In each case, these resetting stimuli interact with the clockwork to transiently shorten or lengthen τ resulting in phase advances or delays in the overall oscillation, eventually synchronizing with the presentation time of the zeitgeber itself, referred to as zeitgeber time zero (ZTO). This process occurs not only within the master circadian clock but also within peripheral oscillators like those found in the stomach and liver. In each tissue type, these clocks are affected by specific zeitgebers that are relevant to the production of clock controlled genes necessary for the rhythmic functions in these tissues.

Not only do the specific zeitgebers that entrain clock processes differ amongst tissues, but there is differential expression of clock controlled genes. Many proteins in these molecular clocks are coupled to the transcription and translation of clock genes necessary for maintaining oscillations as well as the expression of secondary proteins responsible for the proper functioning of the tissue as a whole (for a recent review see Bozek et al., 2009). For example, Per1 is a transcription factor for the gene of the renal epithelial sodium channel in murine kidneys (Gumz

et al., 2009), and entrainment of these clocks directly influences renal activity in a circadian manner. In the liver, both the time of feeding and intrinsic molecular clocks have been shown to directly regulate the transcription of hundreds of hepatic genes (Vollmers et al., 2009).

Clock controlled proteins are expressed in a rhythmic manner that is directly related to the phase (timing) and amplitude (strength) of the oscillations of the positive and negative elements in the molecular clocks, i.e. the nuclear concentration of clock proteins at any given time. This represents a direct mechanism of interaction between the ticking of the molecular clocks found in each individual cell and the circadian rhythms of tissue, organ, and central nervous system function. Furthermore, the existence of variants of these positive and negative elements (PER1, 2 and 3 and CRY 1 and 2) allows for variation in the molecular clock sub-types that are present throughout the body. It might seem obvious that the molecular clocks in the heart and the stomach should differ in the composition of their clock “hands,” but there are even differences in closely related areas of the forebrain that represent the subtle differences in the function of these areas. For example, the dorsomedial hypothalamic nucleus (DMH) expresses only PER1 while the ventromedial hypothalamic nucleus (VMH) expresses only PER2, and the arcuate nucleus (ARC) expresses both (Feillet, Mendoza, Albrecht, Pevet, & Challet, 2008). This allows for variation in the exact phenotype of clock controlled genes that are expressed rhythmically within a given nucleus, but it also represents diversity in the exact zeitgebers that are able to interact with the clocks found in a given population of cells. In the SCN, variations of expression are even more complex. A recent investigation, using fluorescent clock gene reporters to visualize expression in real-time and with enough resolution to examine individual cells, found interspersed sub-populations of cells within the SCN which expressed only *mPer1*, *mPer2*, or both (Cheng et al., 2009). This indicates compartmentalization of function throughout the nucleus restricted to phenotypically distinct but heterogeneously distributed cells. Furthermore, this same study found that outside the hypothalamus, *mPer1* was distributed broadly in all cell types while

mPer2 was found almost exclusively in glial populations and also progenitor cells of the hippocampal dentate gyrus (Cheng, et al., 2009).

To discuss the putative FEO, one must first understand the mechanisms underlying the circadian network responsible for entrainment to light. All past models of the FEO used the light entrainable oscillator (LEO) in the SCN as a template, and researchers are now beginning to elucidate how different these two clocks may be in both in the mechanism of resetting by zeitgeber input and in the substrates of their neural networks. With each new discovery, researchers are looking for a novel way in which food, or some feeding-related cue, interacts with the known molecular clockwork to entrain the putative molecular clocks found within the putative FEO. A basic understanding of the fundamental nature of these clocks and their ability to continue oscillating in the absence of a potent zeitgeber will be a key factor in this investigation.

1.2 The Suprachiasmatic Nucleus as a Model of Integrated Clock Systems

The primary circadian pacemaker of the central nervous system of mammals is found in the SCN of the anterior hypothalamus. Efferent projections from, as well as humoral signals produced by, this nucleus have the ability to entrain circadian oscillators in other parts of the brain including the cerebral cortex and the pineal gland as well as several peripheral oscillators in the lungs, liver, kidney, and heart (Ruud M. Buijs & Kalsbeek, 2001; Hastings, Reddy, & Maywood, 2003; Reppert & Weaver, 2002). The SCN thereby modulates neuronal activity in brain areas essential for the expression of rhythmic behaviours such as arousal, body temperature, and locomotor activity (Turek, 1985). This overarching modulation of circadian processes throughout the body is described as a hierarchy with the SCN taking the seat of dominance.

In rodents, this collection of small, densely packed neurons has historically been subdivided into two sub-nuclei: a dorsomedial shell and a ventrolateral core, each with approximately 10,000 neurons. This differentiation is based on retinal innervation patterns and distinctive cell phenotypes (Moore, 1996). A recent review has highlighted that this gross

anatomical division of cell types and projections is becoming increasingly less appropriate as investigations of the SCN reveal heterogeneities in innervation patterns and neuropeptide expression which are spatially diffuse but also temporally affected in a circadian manner (Morin, 2007). For the purposes of our model, it will suffice to use these subdivisions while making explicit statements as to the phenotype of each cell population when appropriate. Each sub-nucleus maintains rhythmic oscillations of activity and individual cells within this network exhibit cellular, biochemical, and genetic processes entrained to zeitgebers presented through neuronal inputs, specifically light-derived signals to the ventrolateral core, and core –derived signals to the dorsomedial shell of the SCN.

Recently, it has been proposed that extracellular release of distinct neuropeptides from the core plays an important role in the intra-SCN communication and ultimate synchronization of all the molecular clocks found within this nucleus (Gonze, Bernard, Waltermann, Kramer, & Herzog, 2005). It is the summative effect of these gross oscillations that coordinates the precise and stable rhythmic output of the shell in order to direct physiological and behavioural outcomes. Importantly, the translation and expression of these neuropeptides themselves are directly influenced by the transcriptional loops of the molecular clocks tuned by light. Thereby creating a mechanism through which the signals of thousands of individual clocks are integrated into a single rhythmic output that is able to coordinate complex behaviours (Jin et al., 1999).

While neuropeptides are important for intra-SCN coupling and the synchrony of these nuclei, co-expression of classical neurotransmitters also suggest a role in the afferent inputs and efferent outputs of the system. So far, three major afferent pathways have been elucidated for the SCN: the retinohypothalamic tract (RHT) (Moore & Lenn, 1972; Rusak & Boulos, 1981), the geniculohypothalamic tract (GHT) (D. X. Zhang & Rusak, 1989), and projections from the dorsal raphe nucleus (DR) (Moga & Moore, 1997). Each pathway has its own unique combination of neurotransmitters and receptors that reflect, at least in part, the function of the inputs whether excitatory, inhibitory, or neuromodulative.

The RHT is comprised of a monosynaptic glutamatergic connection directly from light sensitive ganglia of the retina. In this way, photic information is relayed directly to the core of the SCN, bypassing the optic chiasm. Experimentally, it has been shown that transection of all visual pathways leaving the optic chiasm produce functional blindness, but do not affect entrainment of circadian rhythms (Stephan & Zucker, 1972b). Conversely, if the retinohypothalamic tract is severed, sight is spared, but light no longer acts as a zeitgeber to the circadian system (Stephan & Zucker, 1972a). Photic information is the strongest zeitgeber of the circadian system in mammals, therefore it is logical that it follows the most direct pathway (Rusak & Boulos, 1981).

The geniculohypothalamic tract (GHT) provides another indirect pathway of photic information. It travels from the ganglia of the retina through the intergeniculate leaflet (IGL) of the lateral geniculate complex, where neuropeptide Y- (NPY) and enkaphalin- (ENK) immunoreactive cells project to the SCN (Card & Moore, 1989; Morin & Blanchard, 1995). Unlike the RHT, it is influenced by other non-photoc zeitgebers, such as physical activity, which modulate the activation of the IGL (Reghunandanan & Reghunandanan, 2006). This particular pathway has been proposed as a site of integration for photic and non-photoc zeitgebers and is especially important to consider when addressing how the rhythms of a putative FEO may be able to seize control of the hypothalamus in times of caloric and metabolic challenge.

The light/dark (LD) cycles of the environment directly influence circadian oscillations, and a vast amount of experimental manipulations, including ablation studies as well as artificial lighting scenarios, confirm the presence of a master LEO within the SCN (Mistlberger, 2005). There are many other non-photoc stimuli that can subtly influence the rhythmic behaviour and physiology. Reported stimuli include physical activity, body temperature, corticosteroid levels, and feeding (Van Someren & Riemersma-Van Der Lek, 2007).

Finally, reciprocal connections exist between the dorsal raphé nucleus of the midbrain (DR) and the SCN. The DR also receives afferents from the retina and acts as a secondary, indirect pathway for photic information. It is hypothesized that serotonergic innervation of the

SCN from the DR can modulate the glutamatergic inputs from the retinohypothalamic tract, altering activation based on the arousal state of the animal, as measured by cFos expression and extracellular glutamate in the SCN itself (Fite, Wu, & Bellemer, 2005; Selim, Glass, Hauser, & Rea, 1993), and acting as a synergistic feedback mechanism under serotonergic control. Specifically, activation of the 5-HT_{2C} subtype of serotonin receptors within the SCN has been shown to increase PER1 expression and modulate cFos protein expression in a phase dependant manner (Varcoe & Kennaway, 2008).

Activation of cells in the core region of the SCN, caused by the net photic input from the three main pathways, directly interacts with the molecular clockwork. Cellular activation phosphorylates the cyclic-adenosine monophosphate (cAMP) element-binding protein (CREB), which then translocates to the nucleus and binds to cAMP responsive elements in the promoters of mammalian Period genes (Ding, Faiman, Hurst, Kuriashkina, & Gillette, 1997; Ginty et al., 1993; Obrietan, Impey, Smith, Athos, & Storm, 1999; Travnickova-Bendova, Cermakian, Reppert, & Sassone-Corsi, 2002). The elevated transcription of *mPer1* and/or *mPer2* and the resultant elevation in protein levels is believed to reset the clockwork mechanisms each day but also allow for shifting of circadian phase when light is presented during the dark phase (Yan & Silver, 2004). The clockwork mechanism of the SCN is not only important for maintaining a stable rhythm, but its unique properties as both a pacemaker and a pseudo-sensory cortex (integrating light information and previous circadian phase) enable it to reset and entrain rhythms to novel light cycles.

The question remains as to how exactly these internal oscillations can create rhythms in hormone secretion, locomotor activity, body temperature, sleep/wake cycles, etc. The key to this system is its reactivity to light-derived signals and the interconnectivity of the SCN neurons. Not only are they highly innervated by intra-SCN γ -aminobutyric acid (GABA)-ergic projections thought to help stabilize and synchronize internal rhythms, but efferent GABAergic projections have also been localized to interneurons in the paraventricular hypothalamus (PVN), dorsomedial

hypothalamus (DMH), and sub paraventricular zone (sPVZ) (R. M. Buijs, Hou, Shinn, & Renaud, 1994). Each hypothalamic nucleus integrates this circadian signal with the appropriate homeostatic and humoral signals and conveys them to the structures responsible for the different functions under circadian modulation (Saper, Lu, Chou, & Gooley, 2005). The suppressing effect of SCN efferent projections ultimately interacts with the entire hypothalamus and explains earlier electrophysiological evidence that light increases the activity of SCN cells *in vivo* while simultaneously suppressing the neuronal activity of surrounding ventral, caudal and posterior hypothalamic sites. These sites including the anterior hypothalamus (AHA), ventromedial hypothalamus (VMH), lateral hypothalamus (LH), DMH, and PVN (Kubota, Inouye, & Kawamura, 1981). A secondary and much smaller population of axonal projections connects directly to endocrine neurons, like those responsible for melatonin secretion in the pineal gland, while yet others target the autonomic nervous system. These are descending projections to the preganglionic parasympathetic neurons of the brainstem and the sympathetic neurons of the spinal cord. This has been demonstrated with tracing experiments both from peripheral glands and organs as well as by anterograde labeling of SCN efferents themselves (Bartness, Song, & Demas, 2001; Ruud M. Buijs & Kalsbeek, 2001; R. M. Buijs et al., 1999; Kalsbeek, Fliers, Franke, Wortel, & Buijs, 2000; Teclemariam-Mesbah, Ter Horst, Postema, Wortel, & Buijs, 1999). Select efferent neurons have been shown to release glutamate as a primary transmitter rather than GABA (Csaki, Kocsis, Halasz, & Kiss, 2000; Cui, Coderre, & Renaud, 2001; Hermes, Buijs, & Renaud, 1996; Hermes, Coderre, Buijs, & Renaud, 1996). It has been proposed that this may allow simultaneous activation of one branch of the autonomic nervous system while simultaneously inhibiting the other, although this has only been shown experimentally for the sympathetic nervous system (Kalsbeek et al., 2006).

SCN communication of circadian rhythms is not limited to direct synaptic connections. A number of diffusible signals that carry rhythmic information into the brain are sufficient to entrain certain behavioural rhythms, most notably locomotor activity. Vasopressin (AVP) and gastrin

releasing peptide (GRP) are the two main humoral factors released in a circadian manner by SCN cells. However, there are others released in smaller quantities like tumor necrosis factor alpha (TNF- α), prokineticin-2 (PK2), and cardiotrophin-like cytokine (CLC) that are implicated in suppression of locomotor activity (Cheng et al., 2002; Kramer et al., 2001; Kraves & Weitz, 2006) and neuromedin S which suppresses feeding (Ida et al., 2005; Mori et al., 2005). In certain cases, these hormones may be released locally at axon terminals, such as those found for AVP in the PVN (Kalsbeek, Buijs, van Heerikhuize, Arts, & van der Woude, 1992), or they may be released as broadly diffusible peptides, able to synchronize some behavioural rhythms even in the absence of neuronal connections (Silver, LeSauter, Tresco, & Lehman, 1996). These two types of signals, axonal and hormonal, should not be considered as separate in function as there are at least two examples where neuronal and endocrine signals converge, producing both a release of a peripheral endocrine hormones and an modulation of their sensitivity in target tissues (Kalsbeek, et al., 2006).

1.3 The Food Entrainable Oscillator: Location Unknown

It has been proposed and experimentally demonstrated that there is a second circadian system that controls the rhythmic entrainment of molecular, cellular, and tissue processes as well as behavioural manifestations of food intake (Mistlberger, 1994). The currently held theory describes this system as a parallel to the light entrained system in that it is under the control of a second hierarchical pacemaker, dubbed the FEO. This clock is subject to all of the standards of other canonical circadian pacemakers: it is only able to operate within an approximate twenty four hour period (Bolles & Stokes, 1965; Stephan, et al., 1979a, 1979b), it requires a daily zeitgeber for entrainment, it maintains a precisely timed system exhibiting oscillations that persist during several days of food deprivation (Boulos, Rosenwasser, & Terman, 1980; Clarke & Coleman, 1986; Rosenwasser, Pelchat, & Adler, 1984), it displays transient expression for many cycles after a change in feeding time (Stephan, 1984), and it influences many peripheral

oscillators found throughout the body (Mistlberger, 1994). Since zeitgeber information will not necessarily reach every peripheral clock, there is a necessity for hierarchical organization of information processing from a master oscillator to central and peripheral cellular clock networks. This organizes the light entrained system in parallel (reviewed in Hirota & Fukada, 2004), integrating light-derived signals when appropriate and it has been proposed that the FEO works in much the same manner even though there is mounting evidence that this is not strictly the case.

While light is not directly available as a zeitgeber for peripheral clocks forcing the necessity for hierarchical signaling, it is distinctly possible that food-derived signals are able to interact with peripheral clockworks important for food anticipation like those found in the liver and stomach without the need for a central pacemaker to integrate and provide this information. These peripheral oscillators, as shown by expression profiles of both *CLOCK* in the liver (Horikawa, Minami, Iijima, Akiyama, & Shibata), and *PER1* in the liver, stomach, and colon (Davidson, Poole, Yamazaki, & Menaker, 2003) can be influenced independently of the SCN. They require only the presentation of a meal in a daily timed manner but reset to normal night time phases immediately after reintroduction to *ad libitum* feeding; it is at this point they re-entrain to light-derived signals from the SCN. It is likely that under conditions of timed food restriction, some peripheral clocks may be receiving circadian phase information from a putative FEO as opposed to the SCN, whereas others are being directly informed by prescient zeitgebers that are acting directly on the clocks themselves, or perhaps there is an interaction between these two signals. The former case remains to be empirically shown, and may remain that way until the FEO is located. In the second case, it has been demonstrated that each peripheral clock is presented with differing zeitgebers that depend not only on sensitivity of the particular “hands” of that clock but also the signals produced and received by tissue within which the clocks resides.

In contrast to the transcriptional outputs of these peripheral clocks, the entrainment of the central food entrained clockwork can be maintained for a much longer time, free-running but continuing to oscillate in the absence of a zeitgeber (*ad libitum* feeding) and will quickly resume

in the same phase as previously set (Mistlberger, 1994, 2009). This remains one of the strongest arguments for the existence of a putative FEO. While current knowledge strongly suggests that FAA is under the control of a circadian pacemaker, experiments using genetic ablation of known clock genes have so far failed to abolish its expression (Pendergast et al., 2009; Storch & Weitz, 2009a); a recent review attempts to explain these findings in light of confounding experimental effects, methodological differences, and the pitfalls of behavioural phenotyping (Challet, Mendoza, Dardente, & Pevet, 2009). Similarly, attempts to locate the FEO by abolishing FAA through targeted central nervous system ablation using electrolytic, aspirative, or neurotoxic lesions continue to yield negative results (Davidson, 2009), except for a select number of experiments involving the DMH, whose findings are currently under debate in the literature and will be discussed further in section 1.4.5. In light of the sheer number of failed attempts to locate a central FEO, it has been proposed that the clockwork mechanism of the FEO resides in the periphery, possibly within the digestive system (Davidson, et al., 2003; C. Escobar, Cailotto, Angeles-Castellanos, Delgado, & Buijs, 2009; Lesauter, Hoque, Weintraub, Pfaff, & Silver, 2009). While it would seem parsimonious to believe that the site of control should be closely tied with the inputs of the systems, this is probably not the case. Early experiments tasked with finding the site of the FEO attempted to silence communication between the digestive system and the central nervous system by severing vagal nerve afferents (Comperatore & Stephan, 1990) or through capsaicin-induced deafferentation of non-vagal viscera (Davidson & Stephan, 1998), the two major axonal connections between brain and gut; neither were successful in abolishing FAA. Under these conditions, the peripheral oscillators, like those found in the liver, gut and stomach, are able to entrain to various aspects of food consumption but not relay these signals to the central nervous system through axonal transmission and neurotransmitter release. It is possible that humoral signals from these peripheral sites are able to convey rhythmic information to the CNS however the quick resetting of peripheral oscillators under SCN control after a return to ad libitum feeding make them an unlikely candidate for FEO pacemaker function. Therefore, even if

the molecular zeitgeber required for entrainment of the central FEO are produced in the form of humoral signals controlled by oscillators in the digestive system, it would appear that the site of integration, modulation, and maintenance of behaviourally relevant rhythms lies within the brain.

The experiments performed to date have lead several groups to theorize that the FEO may in fact be a distributed system of central oscillators, unlike the spatially concerted system found in the SCN (Carneiro & Araujo, 2009; Verwey & Amir, 2009). This distributed system would contain redundant lines of communication, preventing single nuclei ablation from completely abolishing FAA and anticipatory physiological responses. While there is mounting evidence that central and peripheral oscillators exhibit rhythmicity during restricted feeding schedules (C. Escobar, et al., 2009), there is currently no evidence that these loci are connected in a manner consistent with function as a pacemaker; amplifying and preserving food entrained molecular clocks in the absence of a potent zeitgeber. Indeed, one of the key differences between the light-entrained circadian rhythms and the food entrainable system relies on a fundamental understanding of the sensory mechanisms that provide zeitgeber input. The light entrained system relies on the convergence of light-derived signals solely originating within the retinal ganglion cells; whereas the food entrained system relies on the integration of food-derived cues that act at a myriad of independent peripheral and neural sites throughout various independent mechanisms. Once these independent sites have processed the cues and integrated them into internal clockwork mechanisms, the independent clocks must communicate with each other to produce an integrated network of coordinated and self-sustaining oscillations that have the ability to produce behaviourally relevant outputs (see **Figure 2**).

Our current investigation may provide the first such evidence by looking not only at a single locus, but rather at multiple sites where food entrainable oscillations have been described throughout the brain; determining if there is indeed an entire system that continues to activate rhythmically, independent of the light entrained system after a release into *ad libitum* feeding.

Figure 2: Comparison of Hierarchical SCN Pacemaker to Distributed Pacemaker in FEO system. In the light entrained system, zeitgeber information enters a concerted pacemaker found within the SCN, which then projects circadian signals throughout the brain and body. In the food-entrained system, zeitgeber information independently enters several central and peripheral clocks, entraining them to the stimulus. Uncharacterized interactions between a small subset of these clocks are presumed to maintain pacemaker function of the FEO system. Abbreviations: DR, dorsal raphe; GHT, geniculo-hypothalamic tract; RHT, retino-hypothalamic tract.

1.4 Mechanisms Driving Food Anticipatory Activity

Food anticipatory locomotor activity is currently the most popular measure used to quantify anticipation to a daily timed meal. While it is not the only output of the food entrainable clock, it is the easiest to measure without undue external influence. Spontaneous locomotor activity can be quantified without the presence of an investigator or the transfer of the animals to specialized recording boxes during the time of anticipation. Both manipulations could have deleterious effects on the capacity to observe differences due to the anticipation of a meal, magnifying generalized differences in arousal state from handling, novelty, anxiety, or learning and memory differences distorted by factors like cue-induced classical conditioning or time-place conditioning (Balsam, Sanchez-Castillo, Taylor, Van Volkinburg, & Ward, 2009; Lukoyanov, Pereira, Mesquita, & Andrade, 2002).

Given that the measured output of the clock is home cage locomotor activity, it is necessary to understand all of the mechanisms that affect this activity, especially the neural correlates directly engaged in anticipation of a meal. We will discuss three separate systems that represent loci for the integration of specific components of food anticipatory activity with signals from the FEO and then discuss the interactions between these systems that ultimately converge in the hypothalamus to produce behavioural output. We will then review the interactions capable of affecting the strength of FAA expressed via outputs of both the motor and arousal systems.

It is proposed that emotions and their motivational aspects have evolved in order to drive behaviours with beneficial outcomes and avoid behaviours that potentially diminish survival (Cardinal, Parkinson, Hall, & Everitt, 2002). In relation to food, tasting and/or smelling physiological beneficial foods may elicit a feeling of pleasure, while tasting potentially toxic foods elicits negative emotions. This response does have an innate component, but can be overridden by an associative component (Wardle & Cooke, 2008). The phenomenon of taste aversion is an appropriate and well-known example of the impinging of associative processes.

The distinct neural substrate of this affective component has been characterized, differing slightly in the inputs of both smell and taste information, but quickly converging to areas that relate directly to the emotional and motivational aspects of food, areas that interact directly with the homeostatic mechanisms found in the hypothalamus. We will briefly outline these areas as laid out by Dr. Norgren in his chapter discussing the gustatory system within *The rat nervous system* (Norgren, 1995), pausing where appropriate to indicate any relevant evidence for a circadian component of function, and follow the signals produced by food consumption into the limbic system and associated cortical regions.

1.4.1 Following Food into the Central Nervous System: The Brainstem

Facial, glossopharyngeal, and vagal nerves forming synapses with receptor cells of the taste buds conduct taste information to the hindbrain and innervate the rostral part of the nucleus of the tractus solitaries (NTS). In this nucleus, visceral information from the gastrointestinal system, via the vagal nerves, and circulating macronutrient information, via the area postrema (AP), is integrated. Examination of cellular activation within the NTS will determine if any anticipatory visceral information is entering the brain, potentially acting as a connection between peripheral and central oscillators to inform the FEO under restricted feeding conditions.

Processing of taste, gastrointestinal, and circulating signals from the NTS leads to parallel glutamatergic outputs, transmitting both taste and nutritional information in concert to the parabrachial nucleus (PB). Lesioning of PB by both electrolytic and neurochemical means has been shown to severely attenuate and, in some cases, abolish FAA in rats, whereas lesioning of the AP had no effect (Davidson, Cappendijk, & Stephan, 2000; Gooley, Schomer, & Saper, 2006). While it was originally dismissed as a site of the FEO because clock gene expression is not reported in the PB, it has recently been shown that proximal neurons of the mesencephalic trigeminal nucleus do express clock gene rhythmicity which may interact with the PB to modulate function (Hiler, Bhattacharjee, Yamazaki, Tei, & Geusz, 2008). Interestingly, an investigation

characterizing neuronal activation of brainstem nuclei after a scheduled meal showed increased cFos expression in the AP, NTS and PB. Since there was no increase in activation observed after a single fasting-refeeding bout and activation was not increased during a fasting period presented several days after entrainment, there is either a circadian interaction within, or anticipatory input into these nuclei modulating sensitivity to incoming visceral signals (Angeles-Castellanos, Mendoza, Diaz-Munoz, & Escobar, 2005). The PB therefore represents an important point of synaptic zeitgeber input into the CNS, analogous to the light sensing retinal ganglia, and its role in circadian anticipation of food needs to be further investigated.

1.4.2 The Affective Component of Food Anticipation

Signals from the brainstem, carrying both taste and nutritional information, ascend towards the limbic and cortical systems, first innervating the ventral posteromedial thalamic nucleus (VPMpc) followed by the gustatory region of the insular cortex (IC). While information reaching the IC travels directly as well as indirectly, via the prefrontal cortex (PFC), to the nucleus accumbens (NAcb), each of these regions (the PB, VPMpc and IC) also project to the amygdala for emotional processing. Visual food related cues also have inputs to the thalamus, while feedback from both the arousal system and the associative centers in the hippocampus and cortex modulate the thalamic integration of all three sensory cues before signaling the amygdala and the IC.

There are two prime locations within the basal forebrain that have drawn significant attention in regard to food anticipatory behaviours: the amygdala, including subnuclei in the basolateral (BLA) and central nucleus of the amygdala (CeA), and the oval nucleus of the bed nucleus of the striatus terminalis (BNSTov), where outputs of the amygdala are integrated before projecting to the nucleus accumbens, paraventricular nucleus and lateral hypothalamus. It was first reported in 2004 that the BNSTov exhibited oscillations of Per2 proteins in synchrony with the SCN. This is likely, given that this area is known to modulate both physiological and

motivational processes including neuroendocrine, autonomic, and behavioural responses to stress, drugs of abuse, ingestive behaviours and reproductive/maternal behaviours, many of which are under circadian control (Amir, Lamont, Robinson, & Stewart, 2004). More recently, it has been shown that restricted feeding schedules (RFS) are also able to entrain Per2 rhythms in this region even under normal lighting conditions (Waddington Lamont, Robinson, Stewart, & Amir, 2005). Similarly, two subdivisions of the amygdala are particularly involved in the control of motivational and emotional states produced by food: the BLA and the CeA (Cardinal, et al., 2002; Everitt, Cardinal, Parkinson, & Robbins, 2003). These areas have been shown to have a circadian component with oscillations of Per2 protein expression. While the CeA is normally entrained to the light cycle and oscillates in synchrony with both the SCN and the BNSTov, the BLA and dentate gyrus of the hippocampus exhibit opposite rhythms (Waddington Lamont, et al., 2005). It has been experimentally demonstrated that this synchrony is modified by rhythms of glucocorticoids, with adrenalectomy and thyroidectomy but not pinealectomy, resulting in a shift of Per2 rhythms in both the CeA and the BNSTov but not the BLA or DG (Amir, Harbour, & Robinson, 2006; Amir & Robinson, 2006; Segall, Perrin, Walker, Stewart, & Amir, 2006). The desynchronization of all these nuclei, including the BLA and DG under constant light conditions can be rescued by restricted feeding paradigms, with peaks in Per2 expression exactly 12 hours after the presentation of food (Verwey, Khoja, Stewart, & Amir, 2007; Waddington Lamont et al., 2007).

These areas therefore represent emotional centers responsive to glucocorticoids (and potentially other food derived cues) with an oscillatory mechanism that confers the ability to anticipate a daily meal. Whether these food-entrained rhythms remain entrained to the previous feeding schedule under *ad lib* conditions remains to be investigated. While this study will not explicitly examine clock rhythms *per se*, it will help us to understand the participation of these

areas in FAA and tell us if they remain activated at the time of anticipation after removal of the RFS, giving us an indirect insight into the state of their internal oscillators.

1.4.3 Appetitive Component of Food Anticipation

Following processing based on the motivational and emotional components of consumption; these signals exit the amygdala in four distinct pathways. Due to the ever-increasing levels of complexity that begins to develop in the divergent paths of food-derived signals, the entire system has been summarized in **Figure 3**. Two of the efferent pathways represent inputs into separate nuclei of the mesolimbic reward pathway, while the third and fourth make connections with the arousal network via the lateral hypothalamus and the dorsal raphe. The former two connections represent the most important sites of appetitive modulation of anticipatory behaviour: the nucleus accumbens (NAcb) in the ventral striatum and reciprocal connections to the ventral tegmental area (VTA) of the midbrain.

The mesolimbic dopaminergic pathway originates in the VTA and projects, via the medial forebrain bundle, to the NAcb, the amygdala, the hippocampus, the PFC and other rostral structures (Ikemoto, 2007). This system comprises the neural substrate for reward associated with natural stimuli, like food, and is critical for the expression of appetitive behaviours (Kelley, 2004). The NAcb is generally considered the interface between limbic and motor systems and is relevant for reward-related behaviours (Mogenson, Jones, & Yim, 1980). It can be subdivided into two separate nuclei: a dorsolateral core (NAcbC) and a ventromedial shell (NAcbSh), each with their own specific role in the reward pathway. The NAcbC, following interactions with cortical structures, projects to the motor systems in the substantia nigra (SNc) and subthalamic nuclei, via the dorsal pallidum (Heimer, Zahm, Churchill, Kalivas, & Wohltmann, 1991). This acts as an output of anticipatory locomotor activity and represents a main route for the finely tuned generation of appropriate patterns of food-seeking locomotor behaviour. The NAcbSh interacts with the limbic system processing reciprocal connections to the VTA, amygdala, and the

Figure 3: Limbic interactions modulating affective and appetitive drives. Schematic diagram demonstrating the participation of the nucleus accumbens, ventral pallidum, amygdala and cortical association areas in the neural control of food consumption (rounded rectangles). Dotted arrows indicate behavioural feedback by autonomic adjustment of the amygdala and motor associated cortices. Abbreviations: 5-HT, serotonin; AP, area postrema; ARC, arcuate nucleus; BLA, basolateral amygdala; BNST, bed nucleus of stria terminalis; CeA, central nucleus of amygdala; DA, dopamine; EPN, entopeduncular nucleus; GLU, glutamate; LH, lateral hypothalamus; MPT, Mesopontine Tegmental area; NA, noradrenaline; Nacb, nucleus accumbens; NTS, nucleus of the tractus solaris; PBN, parabrachial complex; PPT, pedunculopontine tegmental area; PVN; paraventricular hypothalamus; SNR, substantia nigra pars reticularis; STN, subthalamic nucleus; VLM, ventrolateral medulla; VTA, ventral tegmental area.

lateral hypothalamus (Heimer, et al., 1991) as well as direct input from the serotonergic and noradrenergic systems in the brainstem, and glutamatergic inputs from the hippocampus and the neocortex (Berthoud, 2007), ultimately producing modulatory control of locomotor behaviours via the ventral pallidum (Heimer, et al., 1991).

While the NAcB may be considered the site of genesis for the appetitive and subsequent motor components of reward, the VTA maintains the role of an integrator of homeostatic and motivational drives. Dopaminergic output from this nucleus interacts with the arousal and attention system via the LH, “promoting” appetitive behaviours by altering the salience of cues, increasing attention and arousal towards them while determining relevance according to the current homeostatic states driven by physiological need. In order to accomplish this function, the VTA integrates projections from the PFC, the hypothalamus, the amygdala and the NAcB while generating signals that are sensitive to hormonal cues relevant to both hunger and satiety (Berthoud, 2007). The application of ghrelin, an orexigenic hormone produced in the stomach, directly into the VTA has been shown to increase the activity and synaptic organization of dopaminergic neurons (Abizaid et al., 2006) and has been shown to massively increase food intake (Naleid, Grace, Cummings, & Levine, 2005). Conversely, leptin, a satiety signal, reduces firing rate in dopaminergic VTA neurons as well as food intake (Hommel et al., 2006). An insulin receptor has also been localized in the VTA (Pardini et al., 2006) with distinct interactions between receptor activation and cocaine- and opiate-regulated behaviours (Iniguez et al., 2008; Russo et al., 2007). There is a possibility that the preprandial anticipatory nadir of insulin observed during RFS and the post-prandial peak of insulin after feeding (Diaz-Munoz, Vazquez-Martinez, Aguilar-Roblero, & Escobar, 2000) may also modulate the firing rate of the VTA.

While both PER1 and BMAL expression maintain circadian oscillations within the NAcB, they are both constitutively expressed in the rat VTA. Since both regions express rhythmicity in electrical activity, dopamine synthesis and metabolism, tyrosine hydroxylase

activity, and cFos expression, it has been suggested that these rhythms are driven by efferent projections from nuclei with internal clock gene oscillations such as the NAcB or even the SCN, and it will be interesting to see if the VTA is also subject to modulation by the FEO (Webb et al., 2009). It has been suggested that this may be driven by efferent projections from nuclei with internal clock gene oscillations such as the NAcB or even the SCN, and it will be interesting to see if the VTA is subject to modulation by the FEO.

The NAcB is important for both the emotional “liking”, i.e. pleasurable aspects of food consumption and the motivational “wanting”, ie. increased salience of a food-related reward (Berridge, 1996). Two separate neurotransmitters are implicated in the differential roles played by the NAcB. Opiates play a crucial role in mediating the pleasurable nature of rewarding stimuli. Morphine injected directly into the NAcB increases the number of positive affective reactions to the consumption of a palatable diet (namely one with high sugar and high fat content) (Pecina & Berridge, 2000). Similarly, local injection of DAMGO, a μ -opioid receptor agonist, elicited voracious food intake (Kelley et al., 2002; Will, Franzblau, & Kelley, 2003; M. Zhang & Kelley, 2000). Conversely, the injection of a μ -opioid antagonist reduces sucrose drinking (Kelley, Bless, & Swanson, 1996). Using cFos to visualize the neuronal activation caused by the application of morphine revealed the site of this effect to be in the NAcB_{SH} but near the border shared with the NAcB_{CO} (Pecina & Berridge, 2000).

While there is very little evidence that dopamine release within the mesolimbic pathway is involved in the perceptual and pleasurable aspects of food consumption, there is mounting evidence implicating dopamine release within this system in motivating food seeking behaviors and in particular, increasing salience of rewarding stimuli, i.e. craving (Berridge, 2007). In accordance with this, there are several metabolic hormones that modulate the release of dopamine within this circuit in order to influence feeding behaviours (Narayanan, Guarnieri, & DiLeone, 2010). These represent two different functions, one emotional and one motivational, with

different neural substrates contained within the same nucleus. Interactions occurring between the dopaminergic and opioid systems and the implications for food anticipatory behaviour has been shown experimentally. An investigation examining cFos expression in the NAcb during restricted feeding demonstrated a role for the core in the anticipatory locomotor behaviour of a meal while the shell was activated due to the appetitive nature of food consumption (Mendoza, Angeles-Castellanos, & Escobar, 2005b). Furthermore, dopamine activity within the nucleus accumbens appears to mediate increases in locomotor activity normally observed in rats during food deprivation (Salamone, 1988) and dopamine activity is increased by rewarding stimuli including food (Blackburn, Phillips, Jakubovic, & Fibiger, 1986; Radhakishun, van Ree, & Westerink, 1988). A manipulation that typically increases food intake, the application of a GABA_A agonist, muscimol, into the NAcbSh, was shown to increase cFos expression in hypothalamic orexin neurons and arcuate orexigenic neuropeptide Y (NPY) neurons while suppressing anorexigenic proopiomelanocortin (POMC) neurons. There is therefore a mechanism by which the limbic system, specifically the NAcbSh, is able to modulate metabolic signals in the hypothalamus to increase food consumption (Zheng et al., 2003).

There is evidence of a rhythmic component to these behaviours. Arrhythmic SCN-ablated rats, whose activity in the limbic system is rescued using methamphetamines, show rhythmic behaviour that is food- but not light-entrainable (Honma, Honma, & Hiroshige, 1989). Taken together, this implies that the mesolimbic dopamine signal in the NAcb may represent another input into the entrainment pathway of the FEO or part of the timing mechanism itself. Counter to these implications, neither the ablation of the NAcb, the hippocampus, or blocking dopamine by pharmacological means was able to alter anticipation to feeding time (Mistlberger & Mumby, 1992). As discussed earlier, these types of manipulations represent a problem when considering the theory of a distributed FEO system. The observation of a single component, locomotor activity, in a complex behaviour such as FAA even after these types of manipulations, is not

strong enough evidence to eliminate them from the system because they may represent only one pathway in a multitude of redundant connections.

1.4.4 Cortical Loops: Reinforcing Food-Derived Cues and Shaping Locomotor Outputs

As with all cortical structures, the insular and prefrontal cortices represent the location of the highest order cognitive processing and shaping of behaviour. Along with the hippocampus, they are implicated in providing the plastic axonal pathways necessary for the associative learning of multiple food-induced cues including sensory (ie. taste, sight, and smell) and gustatory (ie. nutritional content, visceral function, metabolic status) (Groenewegen, Uylings, & H.B.M. Uylings, 2000). Once consolidated, these cues are retrieved with the help of the hippocampus. Glutamatergic signals from these regions are being continually looped in parallel through the amygdala and the NAcSh and back to the cortex by way of the ventral pallidum and the thalamus (see **Figure 3**). Any cue-derived signals amplified from arousal centers, concordant with a positive emotional state processed in the amygdala and an appropriate level of “wanting” created by the dopamine-gated NAcSH will be reinforced within these cortical structures (Berthoud, 2007).

Both the prefrontal cortex and the hippocampus have demonstrable internal oscillations of circadian molecular clocks. An investigation of Per1 and Per2 rhythms in these regions reveals that in naïve animals these rhythms are correlated with the nocturnal active phase. When presented with a restricted feeding schedule however, these rhythms quickly change, here within six days, and their peak expression is now shifted to the time of the anticipated meal (Wakamatsu et al., 2001). This demonstrates independence from SCN-derived rhythms under conditions of food restriction and an ability to entrain to the multitude of food-derived cues available to the hippocampal and cortical nuclei.

While receiving and processing the myriad of cues directly related to a particular stimulus, the PFC also generates signals that travel dorsally along the cortex towards the premotor- and sensory-motor cortices, converging on the primary motor cortex and then traveling down through the midbrain, brainstem and into the spinal cord (see **Figure 3**). The behavioural sequences generated by this signal stream are continually shaped by feedback from striatal motor pathways, passing through the dorsal core of the nucleus accumbens and dorsal pallidum paralleling the looping seen for the cues themselves in the ventral stream, ultimately producing coordinated, goal-directed, ingestive behaviour (Berthoud, 2007).

1.4.5 Homeostatic Component of Food Anticipation

If the VTA can be considered a site for the integration of endocrine signals into the mesolimbic reward pathway, then the hypothalamus is the site of integration for all homeostatic signals, including plasma-borne macronutrients, endocrine, and visceral information relating to the metabolic and nutritional status of the animal. The hypothalamus integrates these with signals from the mesolimbic, cortical, and arousal pathways, ensuring that all related activation occurs at the appropriate time in relation to homeostatic set points of physiological need. This is achieved through various axonal and neuropeptide signals produced within the mediobasal hypothalamus, which are capable of modulating food consumption through a variety of mechanisms and divergent pathways (see **Figure 4**) (for review see Sánchez-Lasheras, Christine Köner, & Brüning, 2010; M. W. Schwartz, Woods, Porte, Seeley, & Baskin, 2000; Valassi, Scacchi, & Cavagnini, 2008)**Error! Reference source not found..** The mediobasal hypothalamus is known to be the site of circadian integration and modulation of behaviour related to light, making it a prime candidate for the integrative pathways necessary to convey signals of the FEO system as well.

The arcuate nucleus (ARC) receives visceral inputs via the NTS, but it is also extremely sensitive to circulating signals. It is located at the ventral surface surrounding the third ventricle. This location allows this nucleus to be one of the most accessible brain regions for circulating

Figure 4: The primary hypothalamic nuclei, neuropeptides, and pathways involved in the regulation of appetite. Circulating hormones act directly on the arcuate nucleus informing nuclei downstream to modulate food consumption based on homeostatic set points of metabolism. Orexigenic pathways are presented on the left and anorexigenic pathways on the right for clarity only, this function is not lateralized in the human brain. Also note that circulating glucose levels in portal blood and cerebrospinal fluid interacts with glucose sensing neurons of the VMH. Dotted lines represent inhibitory signals. Abbreviations: AA, amino acids; AGRP, agouti-related peptide; ARC, arcuate nucleus; α MSH, alpha melanocyte stimulating hormone; BDNF, brain-derived neurotrophic factor; CART, cocaine-amphetamine regulated transcript; CB1, endocannabinoid receptor 1; CRH, corticotropin releasing hormone; DMH, dorsomedial hypothalamus; FFA, free fatty acids; LH, lateral hypothalamus; MCH, melanin concentrating hormone; NPY, neuropeptide Y; POMC, pro-opiomelanocortin; PVN, paraventricular hypothalamus; T3, triiodothyronine; TRH, thyrotropin releasing hormone; VMH, ventromedial hypothalamus.

hormones, given its proximity to cerebrospinal fluids and portal vessels outside of the blood brain barrier (for review see Rodríguez, Blázquez, & Guerra, 2010). This access is important, as the ARC represents the most important hypothalamic nucleus for the control of food consumption. The ARC is composed of two distinct populations of interconnected neurons characterized by differing expression of neuropeptides, axonal connections, and function. The neuropeptide-Y (NPY)/agouti related peptide (AGRP) expressing cells are responsible for sending orexigenic signals encouraging food intake via the PVN, VMH, DMH and LH while the pro-opiomelanocortin (POMC)/cocaine- amphetamine-regulated transcript (CART) expressing cells send anorexigenic alpha-melanocyte stimulating hormone (α -MSH) signals of satiety to the PVN (Bouret, Draper, & Simerly, 2004). These distinct neuronal populations, while homogeneously distributed within the ARC, can be considered as two sub-nuclei with opposing function. Since our investigation is primarily concerned with the orexigenic signals produced by the NPY/AGRP cells and their role in the anticipation of a meal, this study will focus on the inputs and subsequent outputs of these cells. As previously mentioned the ARC receives neuronal signals of hunger and satiety and is also sensitive to circulating hormonal cues. Ghrelin activates arcuate nucleus NPY/AGRP neurons when injected peripherally or ICV (Kamegai et al., 2001; Shintani et al., 2001) and has also been shown to inhibit POMC/CART cells. This occurs in direct opposition to the effect seen for the adipose tissue-derived signal, leptin, which increases POMC and CART expression while reducing that of NPY and AGRP (M. W. Schwartz, et al., 2000). Similar to leptin's anorexigenic properties, insulin has also been shown to reduce NPY gene expression in the ARC (M. W. Schwartz et al., 1992). However, insulin has differential effects, increasing AGRP expression (Xu et al., 2005). Because leptin and insulin signals are not always co-comitant, where leptin levels increase with adiposity, and insulin levels increase due to high plasma glucose concentrations, their functions as signals of long- and short-term satiety have differential effects.

NPY is a powerful appetite enhancer. Central administration of NPY has been shown to inhibit thermogenesis, enhance food intake, and promote adipogenesis (Williams, Cai, Elliott, & Harrold, 2004). There is a differential function of NPY in hypothalamic targets; activation of NPY receptors in the perifornical hypothalamus (PFA) increases food consumption whereas NPY injected into the PVN decreases post-prandial body temperature (Currie & Coscina, 1995). AGRP exerts its effect through antagonism of melanocortin receptors (Ollmann et al., 1997) with interactions mediated by opioid and orexin receptors (M. W. Schwartz, et al., 2000).

The ARC is also a prime potential location for the FEO. Its role in food anticipation, propagating hunger- and satiety-related signals to the rest of the hypothalamus through neuropeptide release, may be under direct circadian control via clock gene expression within ARC cells. Using *in situ* hybridization, it was shown that ARC can become entrained to a restricted feeding schedule with a *Per1* peak in anticipation of a meal and a *Bmal1* peak exactly twelve hours afterwards (Minana-Solis et al., 2009). It is also likely that this change is mediated not by the presentation of food but rather by the hypo-caloric conditions occurring pre-prandially. Hypo-caloric daytime feeding (only 66% of normal food intake) increased *Per1* expression over the entire cycle and phase advanced *Per2* rhythms with a peak 1-2 hours after feeding; therefore *Per1* peaks in anticipation, whereas *Per2* peaks after consumption of a hypo-caloric meal (Feillet, et al., 2008). It remains to be shown whether these clock genes can continue to oscillate without the timed presentation of a meal.

The VMH is a major center for detection of energy homeostasis in the brain. It is a converging pathway for a number of metabolic cues relaying nutritional state. These include signals of a hyper-caloric state and/or satiety, such as blood glucose (Mobbs, Kow, & Yang, 2001; Song & Routh, 2005), alpha-melanocyte stimulating hormone (α MSH) (Fu & van den Pol), insulin (Canabal et al.), polypeptide YY (Huang et al.), cholecystokinin (Barnes, Whistler, Hughes, Woodruff, & Hunter), and leptin (Dhillon et al.) as well as several orexigenic and/or hypo-caloric state indicators like NPY and dopamine (Davidowa, Li, & Plagemann), AGRP (Fu

& van den Pol), orexins A and B (Muroya et al.), norepinephrine (Lee et al.), and ghrelin (Chen et al., 2005; Yanagida et al., 2008).

Several studies examined ablation of the VMH, with earlier work implicating this region as necessary for body temperature, corticosterone (Krieger, 1980), and later food anticipatory rhythms (Inouye, 1982b). However, it was quickly dismissed as the food-entrainable pacemaker when it was discovered that this effect was abolished after a sufficient recovery period (Mistlberger & Rechtschaffen, 1984). This evidence does not prevent the VMH from playing a role in food anticipation; it simply indicates that the VMH is not likely the location of the clock itself. A separate study investigating ablation of the VMH found that it was necessary not only for increases in daytime activity but also affected the phase-related changes in nocturnal activity normally seen in rats under caloric restriction (Challet, Le Maho, Pevet, Nobelis, & Malan, 1996). Similarly, colchicine disruption of the VMH was shown to significantly reduce the rhythmic amplitude of locomotor activity, body temperature, and corticosterone entrained to an L:D cycle. Animals with the same axonal transport disruption of the VMH placed in a timed restricted feeding schedule showed attenuated food anticipatory activity, all of which was in the absence of any shift in the period or acrophase of these overt behavioural rhythms. From these observations, it was proposed that the VMH acts as a site of amplification for SCN clock-driven rhythms, sending projections to hypothalamic sites that are important for the expression of rhythmic behaviours (S. Choi, Wong, Yamat, & Dallman, 1998). While there is direct evidence that the VMH is not necessary for the ultimate expression of FAA, it is still very likely to play an important role in modulating the magnitude of the activity peak in relation to metabolic status.

Recently, it was hypothesized that the VMH acts as an integral site for the processing of metabolic state within the brain, and its connections with arousal-promoting regions of the nervous system contribute to either “energy conserving” or “energy dissipating” behaviour (Ribeiro, LeSauter, Dupre, & Pfaff, 2009). It seems possible that either the daily, timed component of an external stimulus (i.e. plasma glucose levels, etc), or the intrinsic circadian

component of the FEO contributing to the production and subsequent timely release of humoral factors (i.e. ghrelin, insulin, glucocorticoids) may directly modulate the VMH at the time of food anticipation.

The dorsomedial hypothalamus (DMH) has received a lot of attention as a site necessary for the expression of food anticipatory activity. The DMH functions as a relay for circadian information from the master clock in the SCN, via the sub paraventricular zone (sPVZ), to areas involved in regulating the neuroendocrine system (PVN), thermoregulation in the medial pre-optic area (MPOA), and sleep/wake cycles of arousal in the ventrolateral pre-optic nucleus (VLPO), and the orexin cells in the lateral hypothalamus (LH), which feed directly into the serotonergic arousal system (Chou et al., 2003; Saper, 2006). In its capacity as a relay of light-entrained circadian information, it is also an integrator of hypothalamic signals of food deprivation and intake from the arcuate nucleus and ventromedial hypothalamus (Saper, et al., 2005) with corticolimbic inputs from the PFC and the BNST and brainstem inputs from the ventral medulla and parabrachial nucleus (Thompson & Swanson, 1998).

Ablation of the DMH produces hyper-phagia and obesity in rats (Bernardis & Bellinger, 1987). This is attributed to disruption of the ascending anorexigenic α -MSH terminals, originating from the POMC-positive neurons in the ARC (Jacobowitz & O'Donohue, 1978), with DMH neurons normally passing on these signal to the thyrotropin releasing hormone (TRH) neurons in the PVN with its own α -MSH fibers (Mihaly, Fekete, Legradi, & Lechan, 2001). Conversely, in several models of obesity including diet-induced mice, obese agouti mice, and melanocortin-4 receptor (Mc4r) knockout mice, there is a marked increase in orexigenic NPY mRNA expression within the DMH that is presumed to play a pivotal role in hyper-phagia (Guan, Yu, & Van der Ploeg, 1998; Kesterson, Huszar, Lynch, Simerly, & Cone, 1997). Melanocortin3 receptor knockout mice also show a lack of wakefulness and FAA that is correlated with abnormal expression of clock gene oscillations in the DMH (Sutton et al., 2008). While the DMH

is important for increasing food consumption due to the orexigenic signals it receives, it must also mitigate this increase by integrating anorexigenic signals from separate systems. Interestingly, increases in cFos expression are also observed in the DMH and PVN following a single peripheral intraperitoneal injection of ghrelin. This is presumed to be due to the direct action on NPY neurons in the arcuate nucleus. In the same study, ghrelin did not increase cFos in the VMH, NTS, or AP indicating that circulating ghrelin is not able to act on these nuclei after acute and therefore unpredictable exposure (Kobelt et al., 2008). In investigations from this research group, the DMH, ARC, PVN and the VMH all showed increases in activation but only after seven repeated injections at mid day (Blum et al., 2009).

The DMH has also been implicated in receiving and integrating food-entrained circadian signals. Whether the DMH functions as part of the FEO pacemaker remains to be determined and is debated within the literature. The first study to implicate the DMH reported that even partial ablation using neurotoxic ibotenic acid lesions of this nucleus (~70-90%) severely attenuated, and in some animals eliminated, food anticipatory rhythms of activity, sleep/wake cycles, and temperature rhythms in rats (Gooley, et al., 2006). A follow up study performed by the same laboratory used genetic techniques to show that FAA and temperature rhythms are absent in mice lacking expression clock gene *Bmal1*, and rhythmicity may be rescued by virally restoring *Bmal1* expression selectively in the DMH, but not the SCN (Fuller, Lu, & Saper, 2008). These studies indicate that the DMH contains *Bmal1*-dependent circadian oscillators that are both necessary and sufficient for the expression of FAA. Unfortunately, and despite considerable time and effort, no study to date has been able to replicate either of these two findings (Landry, Simon, Webb, & Mistlberger, 2006; Landry, Yamakawa, Webb, Mear, & Mistlberger, 2007; Moriya et al., 2009; Pendergast, et al., 2009; Storch & Weitz, 2009a). Therefore, while the DMH may be necessary for the expression of FAA, it is not sufficient. There are several key pieces of evidence for this necessity, including the presence of food-entrainable oscillations of clock genes within the intact

DMH (Mieda, Williams, Richardson, Tanaka, & Yanagisawa, 2006) and the ability to dissociate this clock from food anticipatory behaviours and peripheral clocks with the use of a diurnal feeding schedule with random meal presentation time (Verwey & Amir, 2009).

Often considered the hypothalamic site of neuroendocrine control, projections from the PVN demonstrably affect the secretion of hormones, including melatonin from the pineal gland (Larsen, Enquist, & Card, 1998; Moore & Danchenko, 2002), Thyroxine (T4) and triiodothyronine (T3) from the thyroid gland (Boelen, Wiersinga, & Fliers, 2008), and prolactin and adrenocorticotrophic hormone (ACTH) from the anterior pituitary (Jorgensen, 2007). ACTH causes glucocorticoid secretion from the adrenal cortex, and this pathway comprises what is traditionally known as the hypothalamic-pituitary-adrenal axis (HPA). Interestingly, there are reports of pre-prandial peaks in corticosterone that coincide with behavioural activation in anticipation of a daily meal (Diaz-Munoz, et al., 2000; Krieger, 1979) however this is not due to either increased corticotropin releasing hormone (CRH) expression or release from the PVN nor due to alterations in serum ACTH (Belda, Ons, Carrasco, & Armario, 2005; Pesonen, Huupponen, Rouru, & Koulu, 1992; Poulin & Timofeeva, 2008). Similarly, elevated plasma corticosterone levels following acute fasting are not accompanied by neuronal activation of PVN neurons or an increase in PVN CRF expression (Timofeeva, Picard, Duclos, Deshaies, & Richard, 2002; Timofeeva & Richard, 2001), and, at least for the case of acute fasting, this elevation is due to a reduction in the plasma clearance rate of the hormone (Kiss, Jezova, & Aguilera, 1994).

A recent study investigated the role of the HPA axis and its response to RFS; restricted feeding acted as a strong zeitgeber for clock protein expression (PER1, PER2, BMAL1) with post-prandial peaks throughout the HPA axis (Girotti, Weinberg, & Spencer, 2009). Furthermore, the post-prandial expression of PER2 within the PVN coincides with a specific induction of cFOS mRNA and protein (Angeles-Castellanos, Aguilar-Roblero, & Escobar, 2004; Poulin & Timofeeva, 2008). These food-entrained rhythms quickly established themselves independently

of the light-entrained rhythms in the SCN, and this was correlated with the expression of several functional genes (cFOS, CRH, POMC) and increases in plasma neuropeptide and glucocorticoid levels (ACTH and corticosterone), while RFS had no effect on cFos expression in either the anterior pituitary, or the adrenal cortex. It is suggested that the circadian glucocorticoid rhythms generated by the HPA under RFS are strong mediators of peripheral entrainment to the FEO (Girotti, et al., 2009), and this is confirmed by several studies showing that glucocorticoids are involved in the temporal entrainment of circadian clocks in the liver (Balsalobre et al., 2000; Oishi et al., 2005; Reddy et al., 2007).

In order to coordinate the most appropriate hormonal responses to internal and external states, the PVN must integrate afferent axonal projections as well as detect intra-hypothalamic neuropeptide signals from the ARC, the SCN, and the DMH. Anorexigenic signals like α -MSH from the ARC induce secretion of a cascade of various anorexigenic peptides, including TRH, CRH, AVP, and oxytocin (OXT) whereas orexigenic arcuate NPY and AGRP released into the PVN have the opposite effect (Valassi, et al., 2008). Perhaps the single most important factor influencing the PVN in this investigation will be neuropeptide NPY as it is critically important for appetite induction.

Several experimental observations suggest that NPY in the PVN is important for consumptive behaviours by coordinating various metabolic systems. Signals from the PVN decrease brown adipose tissue (BAT) thermogenesis and increase white adipose tissue (WAT) lipoprotein lipase activity, an enzyme important for fat storage (Billington, Briggs, Grace, & Levine, 1991; Billington, Briggs, Harker, Grace, & Levine, 1994). Direct NPY micro-injections into the PVN increase the respiratory quotient in rats, a direct index of a shift from fat to carbohydrate oxidation (Jhanwar-Uniyal, Beck, Burlet, & Leibowitz, 1990; Menendez, McGregor, Healey, Atrens, & Leibowitz, 1990). This is consistent with a selective enhancement of carbohydrate appetite and preferential consumption of a high carbohydrate diet after an acute

injection of NPY into the PVN (Stanley, Daniel, Chin, & Leibowitz, 1985) and the extremely potent effect of this arcuate-derived neuropeptide on visceral fat deposition and body weight (Stanley, Kyrkouli, Lampert, & Leibowitz, 1986).

Rats with either complete radiofrequency ablation of the PVN or ibotenic acid lesions of the LH exhibit FAA and remain entrained for at least 12 days after a return *ad libitum* feeding. Some animals with total PVN ablations failed to exhibit significant anticipatory activity but did show anticipatory food bin approaches, indicating that there may be a splitting of function downstream of the PVN, relying at least partially on signals from within this nucleus, so that ablation of this nucleus affects the appropriate expression of locomotor activity and arousal but not food anticipation *per se* (Mistlberger & Rusak, 1988).

1.4.6 Lateral Hypothalamic Modulation of Cerebral Activation: The Arousal System

We have already discussed the cortical and limbic interactions that contribute to locomotor output in anticipation of a meal, but there is one more output system of critical importance to the anticipation of food. The serotonergic system is a major modulatory mechanism of central nervous system activation. As with the direct and circadian effects of light on serotonin (5-HT) production in the DR (Cuesta, Clesse, Pevet, & Challet, 2009; Fite, et al., 2005; Silver & Lesauter, 2008; Smith, Sterniczuk, Phillips, & Antle, 2008) with modulation by both photic and non-photic stimuli discussed previously, metabolic demand and circadian signals entrained by rhythmic feeding can also increase activation of this system. It thereby performs in a timely manner to promote a state of generalized arousal that is coordinated and perfectly timed; in order to reduce the energy demands of the CNS while increasing the chances of finding food at a time that is previously predictive of availability.

The chemical structure of serotonin was identified and synthesized almost sixty years ago (Twarog & Page, 1953), and its function as a neurotransmitter was soon characterized

(Bogdanski, Pletscher, Brodie, & Udenfriend, 1956). Its effects throughout the mammalian brain have been studied extensively following the discovery of its localization in the pituitary gland (Hyypä & Wurtman, 1973). There are also several nuclei containing serotonergic-expressing cells found within the brainstem, and these are anatomically divided into nine groups, labeled B1-9. The most important of these nuclei are the dorsal raphe (DR, B7), with efferent projections to the hypothalamus, caudate, putamen, and locus coeruleus (LC), and the median raphe (MR, B8), with projections to the hypothalamus and the hippocampus (Azmitia & Segal, 1978; Dahlstrom & Fuxe, 1964; Jacobs & Azmitia, 1992; Steinbusch & Nieuwenhuys, 1981; Steinbusch, Nieuwenhuys, Verhofstad, & Van der Kooy, 1981). With such widespread projections, specific receptor subtypes found throughout the brain determine the functional significance of these serotonergic signals in multiple neuronal systems. There are currently seven different 5-HT receptors with 14 subtypes identified, each performing distinct functions, including: actions as auto-receptors mediating serotonin release, regulation of sleep/wake cycles, control of mood and anxiety, increasing feeding and GI motility, mediation of cholinergic function, interactions with the HPA axis stress response, and cardiovascular effects including blood pressure and penile erection (Jorgensen, 2007). Although 5-HT signaling through its various receptors has been implicated in a large list of physiological and behavioural processes, it is not essential for their function. Its role is modulatory, creating tonic activation or arousal of these widespread neuronal systems in accordance with signals integrated within the serotonergic system. 5-HT not only plays an important role in generalized CNS arousal but will specifically modulate many behavioural and physiological aspects of food anticipation. Specifically, DR derived 5-HT reduces food intake and body weight by diminishing appetite and increasing energy expenditure, exactly the type of modulation that is important for food seeking (M. W. Schwartz, et al., 2000). Serotonin-induced hypophagia exerts its action by specific activation of melanocortin-4 receptors in the hypothalamus and represents a lone example of a central signal reciprocally regulating the anorexigenic melanocortin system in a manner similar to peripheral adiposity signals like leptin

(Heisler et al., 2006). Serotonin increases the rate of satiation and prolongs the state of satiety (Simansky, 1998). On the other hand, specific activation of the 5-HT_{1A} autoreceptor, using the agonist 8-OH-DPAT, reduces serotonergic activity in the brain when given systemically or directly into the DR (Dourish, 1993) and this stimulates food intake in *ad libitum* fed rats (Dourish, Hutson, & Curzon, 1985). In fasted rats (Dourish, et al., 1985; Ebenezer, 1992) or those under a timed restricted feeding schedule (Aulakh et al., 1988), this agonist inhibits food intake. Therefore, there exists a mechanism by which the metabolic state of the animal is able to influence the effects of serotonin on food intake.

The locus of this mechanism lies within the lateral hypothalamus (LH), with 8-OH-DPAT reducing extracellular 5-HT in this nucleus by acting on somatodendritic autoreceptors in satiated animals (Lorrain, Matuszewich, & Hull, 1998 1998; D. H. Schwartz, Hernandez, & Hoebel, 1990 1990). However, this effect is not seen in fasting animals. This is due in part to the availability of the serotonin precursor tryptophan, whose concentrations in the brain actually increase following starvation (Curzon, Joseph, & Knott, 1972; Schweiger, Brooks, Tuschl, & Pirke, 1989). Increased 5-HT degradation is also observed in the lateral hypothalamus of fasted rats (Kantak, Wayner, & Stein, 1978; Loullis, Felten, & Shea, 1979) and is accompanied by an increase in neuronal release of serotonin (Fuenmayor & Garcia, 1984). While the overall effect of 8-OH-DPAT is nullified in fasted and food restricted rats, this is potentially due to compensatory mechanisms within the hypothalamus that protect feeding behaviour from serotonergic inhibition during food anticipation (Voigt, Kienzle, Sohr, Rex, & Fink, 2000). This is an important process as it spares the hypothalamus from the anorexigenic arousing effects that the LH promotes due to metabolic demand and output of the FEO. Furthermore, noradrenergic (norepinephrine or NE) signals produced in the LC, with increased activation caused by serotonergic signals from the DR, bypass the effect of serotonin insensitivity due to fasting and stimulate food intake through activation of α_2 receptors in the PVN (Ramos, Meguid, Campos, & Coelho, 2005). It is by this detour that arousal signals converging on the DR are able to simultaneously increase CNS arousal

and promote feeding in a slightly paradoxical manner that is only possible under conditions of food restriction and anticipation.

While the DR in the brainstem is the site of genesis for serotonergic signals, it is the lateral hypothalamus nucleus (LH) that is the integrator of both homeostatic and mesolimbic signals, enabling higher order processing of all food pertinent stimuli to descend to the brainstem and modulate the ascending input of the monoaminergic arousal network. Like the serotonergic and noradrenergic systems of arousal, the LH also expresses a pair of specific neuropeptides responsible for conducting diffuse signals throughout the CNS, orexins A and B (OX), with at least two known receptors, OX₁R and OX₂R (Langmead et al., 2004). The orexins play an extremely important role in regulating sleep states, and a rare genetic disorder, which inactivates this gene, leads to severe narcolepsy and/or cataplexic states (Chemelli et al., 1999; Saper, 2006). Interestingly, the orexin positive cells of the LH are also implicated in the increased arousal observed due to metabolic demand. A single period of food deprivation can impair sleep/wake states, vigilance, and normative arousal caused by novel surroundings in orexin knock-out mice (Yamanaka et al., 2003). Patch clamp recordings of orexin-positive cells show increased activation upon the application of ghrelin, and decreased activation upon application of either glucose or leptin (Yamanaka, Muraki, Tsujino, Goto, & Sakurai, 2003). There is also evidence that these neuropeptides may play a role in the increased wakefulness observed in the anticipation of a timed meal; rats with LH implanted electrodes show multiunit activity that correlates with anticipatory behaviour under restricted feeding conditions, even when the SCN has been ablated (Kurumiya & Kawamura, 1991). Mice with a total knock out of the orexin gene show severely blunted FAA (Akiyama et al., 2004) and saporin ablations directed at only the orexin cells within the LH also show deficits in both food bin approach and drinking behaviour (Mistlberger, Antle, Kilduff, & Jones, 2003). However, in these mice, there have been no other behavioural or physiological deficits observed, either in circadian entrainment of behaviour under L:L, D:D, or L:D conditions, clock gene oscillations in central and peripheral clocks, food intake, or

disturbances of the typical preprandial peak in body temperature (Kaur et al., 2008; Mieda et al., 2004). This suggests that the lateral hypothalamus participates as a downstream effector of the FEO, mediating the arousal response and is not likely involved in the circadian processes of this behaviour. It does however allow us to speculate as to where these upstream circadian signals are being generated.

Throughout this thesis, efferent projections to the LH have been mentioned, including inputs from the SCN, the ARC, the PVH, and the DMH of the hypothalamus, neocortical structures, the nucleus accumbens, the amygdala, the BNST, and even the hippocampus. There are even small subpopulations of neurons projecting from the parabrachial nucleus and the dorsal raphe in the brainstem (Yoshida, McCormack, España, Crocker, & Scammell, 2006). In this way, appetitive, emotional, homeostatic, and even circadian signals pertaining to all aspects of food anticipation and consumption are able to modulate the activation of the LH and the release of arousing orexin, serotonin, and noradrenaline throughout the cortex and midbrain as well as down the spinal cord. If the LH is truly a downstream effector of the FEO then the system responsible for entrainment to food is expected to reside in nuclei with direct connections to the orexin system.

2 Experimental Paradigms

2.1 Study 1: Isolating the FEO

The current behavioural paradigm employed by researchers to investigate the effects of experimental manipulations on FAA follows this typical schedule: 1) establishment of a baseline for naïve animals with *ad libitum* feeding, 2) a fourteen day period of RFS where food is presented at the same time each day in the middle of the inactive phase (entrainment), 3) the animals are then placed back on *ad libitum* feeding for three days to abolish the expression of FAA, 4) a two day fast where FAA is again observed (Blum, et al., 2009; for review see Mistlberger, 2009; Pendergast, et al., 2009; Storch & Weitz, 2009b)(See). The purpose of this paradigm is four-fold with each change in the schedule representing a particular circadian test: step 1 verifies that all animals can properly entrain to the light:dark cycle, since perturbances in the light entrained system caused by the experimental manipulations could subsequently affect expression of FAA without disturbing the FEO; step 2 tests the ability of the animals to entrain to a daily meal; step 3 allows the animal to re-feed *ad libitum* in order to abolish the expression of FAA and return animals to a light:dark cycle driven rhythm; and step 4 tests the re-emergence of FAA under fasting conditions with no zeitgeber to ensure that the anticipatory activity observed is due to circadian processes and not a homeostatic mechanism driving behaviour in the absence of food. In this last step, it is important that FAA occurs only in anticipation of the meal and quickly falls off after the time the meal should have presented, therefore both the quantity and the temporal profile of FAA is important for assessing true circadian function. As previously discussed, an underlying principle of circadian oscillators is the ability to entrain to a zeitgeber and continue oscillating at the time previously set even once the zeitgeber has been removed. This process occurs after a return to *ad libitum* feeding, where the FEO continues to oscillate at the previous time of feeding even though FAA is not expressed, and is tested by the fasting condition

Figure 5: Food Restriction Paradigm. A, typical restricted feeding paradigm used in mice; including *ad libitum* baseline (AL), restricted feeding with food available only for a short temporal window during the light phase leading to entrainment (RF), *ad libitum* refeeding to abolish FAA (AL), and finally a two day fast to verify circadian entrainment (F). B, Groups and treatments employed in this study. Restricted feeding occurred between ZT 4 and 8 during RF with all body weight and food consumption measures taken at ZT8.

where food deprivation re-engages the FEO and rhythms of food seeking, i.e. FAA, are re-expressed.

The experiment carried out for this thesis involved no direct manipulation of the animals themselves (other than the application of a feeding schedule); it is not a comparison between two groups of animals, one control, and one experimental, under the same behavioural paradigm, rather it uses observations of the internal activation state of the animals at different time points along the typical circadian testing paradigm described above. It compared three groups of animals: one naïve, receiving *ad libitum* feeding for the entire experiment (AL) representing normal baseline conditions, a second group at the time of meal anticipation after 14 days of restricted feeding (RFS) representing the end of an entrainment schedule, and the last at the time of meal anticipation after 14 days of RFS followed by the 3 days of *ad libitum* feeding (RFAL), representing animals with extinguished FAA behaviour but an entrained FEO clock. A fourth group, one that would receive the entire RFS paradigm followed by *ad libitum* feeding, then fasting, is unnecessary in this case. The appearance of FAA after entrainment then fasting is well documented for rats and mice and there is no discernable reason why the animals employed in this study would not show true circadian entrainment given that they are un-manipulated in any way. Indeed all of our animals represented what would typically be considered a control group in a standard RFS paradigm, and this investigation was merely observational, not manipulative. Therefore, behavioural testing was not necessary to ensure entrainment was unperturbed. Furthermore, the use of a fasting group for the immunohistological investigation becomes redundant because it is assumed *a priori* that the behavioural manifestation of FAA, and the neural activation required for its appropriate expression is identical to that of an animal still under RFS with the only difference being that it is not receiving a zeitgeber to reset or shape the subsequent cycle of the FEO.

True autonomous circadian oscillators maintain the ability to shift their phase in response to changes in the time of zeitgeber presentation and the FEO is no exception. However, for long shifts, greater than 4 hours in rats, transients appear for several cycles where the delay or advance of rhythms can only be accomplished by 2-4 hour steps per cycle (Stephan, et al., 1979b). Furthermore phase delays are much more readily accomplished than phase advances; this is true for the SCN but especially true for the FEO. In fact, FAA is almost impossible to phase advance, when meal presentation is shifted forward by 6 hours FAA will reset to this new mealtime by transient delays through the 18 hour time difference taking a full 6 days to accomplish the resetting (Stephan, 1984, 1992). Regardless of direction this shift is accomplished readily but not quickly, as anyone who has experiences jet lag after a long flight across multiple time zones can attest. In our experiment the use of 3 days of *ad libitum* feeding was expressly used to ensure that any and all circadian oscillators entrained to some potential aspect directly related to food consumption, like plasma glucose levels or stomach distention, but not a part of the autonomous food entrainable pacemaker driving FAA, would have enough time to transiently shift to the presumable new time of food consumption at ZT12 when the dark phase begins. Furthermore, the FEO exhibits free running oscillation with an intrinsic τ close to, but not exactly 24 hours (Mistlberger, 1994). By using 3 days of *ad libitum* feeding, i.e. three cycles of zeitgeberless free running rhythms of the FEO, this study ensured that any individual genetic variability of τ_{FEO} in our animals which should be, but is not necessarily, negligible, maintains the oscillations of the FEOs of all of our animals within an acceptable range, should they differ too greatly. For example, if by chance one animal has an FEO with $\tau_{\text{FEO}} = 23.5$ hours and another has a $\tau_{\text{FEO}} = 24.5$ hours, then even after three days of free-run the difference in their internal oscillations will be no greater than 3 hours and no further than 1.5 hours from meal time in either direction. Well within an acceptable range for comparisons of anticipatory activation of neuronal populations, especially given the relatively large temporal window of FAA expression. This paradigm is popularly used for the exact reasons noted above. This is by no means a new, or otherwise novel

manipulation and has been carefully thought out and employed by numerous researchers in the field of circadian rhythms.

By allowing the animals to have unrestricted access to food for several days post-entrainment, which abolishes the outward expression of FAA but does not perturb entrainment of the FEO, this study will isolate neural activation caused only by circadian processes and not those caused by negative metabolic status *per se*: the appetitive, affective, and homeostatic components that drive appropriate expression of FAA. This study also tested neuronal activation within these non-circadian systems and measured plasma acylated ghrelin levels as well as intrinsic oscillations of clock gene PER2 within the peripheral clock of the stomach, but only to ensure that any and all activation seen in our experimental group was due solely to circadian oscillations of the FEO.

There is only one other study of this kind in the literature (Angeles-Castellanos, et al., 2004). In that investigation, the authors chose to use three groups, two of which are similar to the ones employed in our first study: the use of an *ad libitum* fed control and a food entrained group. The third group consisted of food entrained then fasted animals. The authors of this study contend that any significant increase in anticipatory cFOS immunoreactivity in the fasting group may elucidate structures involved with the expression of FAA, and this is certainly true. The authors located several nuclei in the hypothalamus that activate during expression of food anticipatory behaviour and are reactivated by fasting. However, and this is key to understanding the decision to investigate entrained then *ad libitum* fed animals, when using the fasting paradigm, activation is elicited in all areas involved in the manifestation of FAA including the homeostatic, affective, appetitive, arousal, and motor areas of this complex behaviour (Armstrong, Coleman, & Singer, 1980; Koubi et al., 1991) thereby masking or otherwise obscuring only those areas that continue to activate due solely to circadian influences of the FEO.

The AL group represented a negative control group as they are free to consume chow according to intrinsic drives, and meal onset under these conditions is typically at the beginning of the dark cycle (ZT12). This was aligned with circadian patterns of arousal and locomotor activity set by the light-entrained SCN, however they were not constrained to this period for feeding and may have consumed chow at any time. The RFS group represented the positive control group, which were entrained to a temporally fixed meal presented each day from ZT4-8 for 14 days. The RFAL groups represented the experimental group as they received the same entrainment period as the RFS group but were released back into an *ad libitum* feeding paradigm for three days.

By using animals receiving *ad libitum* feeding as the positive control in this study, it was hypothesized that continued activation will occur only in nuclei within the circadian component of the FEO, not those responsible for homeostatic, motivational, or appetitive drives and especially not those producing behavioural output all of which are suppressed by a positive metabolic status. By moving this investigation beyond the hypothalamus, this study investigated all of the proposed nuclei involved in food entrainment and its behavioural output. Further, this study attempts to demonstrate that the experimental paradigm removes the activation of all of these systems but one: a distributed but interconnected system of central nuclei tasked with the self-sustaining oscillations necessary for a function as true circadian pacemaker.

2.1.1 Animals

This experiment was carried out using 24 CD1 adult male mice purchased from Charles River laboratories. Each animal was randomly assigned to one of three groups: *Ad libitum* fed (AL, n=8), restricted feeding schedule (RF, n=8) or restricted feeding schedule then *ad libitum* fed (RFAL, n=8). Before experimentation, all animals were single-housed in standard mouse cages and received *ad libitum* feeding of mouse chow and water in a temperature and humidity

controlled vivarium with a 12-hour Light Dark (L:D) cycle starting at 8:00 AM (Zeitgeber Time 0 (ZT0)).

All Research was conducted under the guidelines of the Canadian Council on Animal Care and approved by Carleton University's Animal Care Committee (Protocol # P07-15, see Appendix B: Ethics Protocol Approval for a digital copy of the University's protocol approval form).

2.1.2 Behaviour

Each animal was housed in a standard home cage assembly with two home cages placed within a single Superflex HC infrared locomotor activity monitoring module (Accuscan Instruments, Columbus, OH). Each module was attached to a single Superflex node (Accuscan Instruments, Columbus, OH), which in turn was attached, via USB, to a Pentium computer running Windows XP (Microsoft Corp., Redmond, WA). All 'ambulatory activity' data was continually collected in 6 minute bins over 24 hours for each day of the experiment. This was recorded and analyzed using Fusion HC v1.5 software (Accuscan Instruments, Columbus, OH). Several measures were calculated including food anticipatory activity, total daily activity, nocturnal activity, and nocturnality (defined as nocturnal activity as a percentage of total daily activity). Daily food consumption and post-condition body weight were recorded after feeding at ZT8 using an SI-2002 digital scale (Denver Instruments, Bohemia, NY). The data was divided into each feeding condition for analysis 1) entrainment, days 1 through 14 and 2) *ad libitum* feeding, days 15-17. Repeated Measures ANOVAs ($\alpha = 0.05$) and planned comparisons between experimental groups (AL vs. RF vs. RFAL) were performed using PASW statistics v17.0 (SPSS Worldwide, Chicago, IL) to analyze the effect of experimental group, time and any interaction between these two factors.

2.1.3 Tissue Collection and Processing

On the last day of behavioural recordings, between ZT3-4, animals were injected with an overdose of sodium pentobarbital and intracardially perfused with 100ml of saline (0.9%) followed by 250ml of para-formaldehyde (4%) according to the university's life sciences research center standard operating procedure P1. The animals were then decapitated and both brains and stomachs were collected.

2.1.3.1 Brains

Brains were post-fixed in 50ml of 4% paraformaldehyde overnight and then transferred to a 30% sucrose solution until sliced in a Cryotome FSE cryostat (Thermo Fisher Scientific, Waltham, MA). Brains were blocked from the olfactory bulb to the brainstem according to *The Mouse Brain in Stereotaxic Coordinates 2nd Edition* (Paxinos & Franklin, 2001), and 60µm slices of tissue were collected and placed into 4 serial sets. They were stored in a cryoprotectant solution at -20°C until processed for immunocytochemistry.

One set of serial whole brain sections was simultaneously processed for cFOS immunocytochemistry according to the following procedure. Free floating slices were washed in phosphate buffered saline (0.1M) followed by a 15 minute quenching step using hydrogen peroxide (1%) to neutralize any endogenous peroxidase activity in the tissue. After a subsequent wash, slices were incubated for 30 minutes in a blocking solution containing bovine serum albumen (1% w/v), triton-X (0.3% v/v), and PBS. This was followed directly by 48 hour incubation, with stirring, of the primary rabbit antibody raised against cFOS proteins (1:20,000, Calbiochem, LaJolla, CA) in blocking solution at 4°C (for testing and verification of this antibody see Abizaid, et al., 2006). After subsequent washing, a 1 hour incubation of the secondary antibody (1:500 donkey anti-rabbit, Jackson ImmunoResearch, West Grove, PA) in blocking solution was carried out at room temperature. This was followed by a 1 hour incubation of an avidin-biotin complex (ABC Elite Kit; Vector laboratories, Burlingame, CA, USA) in PBS

followed by a washing step. Staining was developed in 0.5% 3,3'-diaminobenzene (DAB) with 1% cobalt chloride, 1% nickel ammonium sulfate and 0.01% H₂O₂ in PBS until sections were fully developed. Sections were determined to be fully developed when staining appeared dark in colour and specific to cellular nuclei with only a hint of non-specific background staining. This was determined to be approximately 24 minutes. Sections were then washed in PBS for five minutes and stored in PBS at 4°C until mounted onto glass slides and allowed to air dry.

Mounted sections were dehydrated in serial solutions of 70%, 95% and 100% ETOH for 5 minutes each and then defatted in Clearene® (Surgipath Canada Inc., Winnipeg, MB, Canada) for 5 minutes before coverslipping with Permount (Fisher Scientific, Fairlawn, NJ) mounting medium.

2.1.3.2 Stomachs

Stomachs were post-fixed in 4% paraformaldehyde and stored in the fridge at 4°C until paraffin embedding. The gastric fundus of each stomach was dissected and bundled in gauze with an identifying label. These bundles were then dehydrated in serial steps in the following dilutions of ethanol (v/v): 60%, 70%, 80%, 95%, 100%, 100% for 90 minutes each followed by an overnight final dehydration in a fresh 100% ethanol solution. Bundles were then defatted in serial in three separate solutions of fresh Clearene® (Surgipath Canada Inc., Winnipeg, MB, Canada) for one hour each to remove organics. After removal from Clearene, bundles were placed in three serial treatments of liquid paraplast tissue embedding medium (McCormick Scientific, Richmond, IL) maintained at 58°C in a 1330 GM gravity oven (VWR Scientific, Mississauga, ON). The first two treatments were approximately 3 hours each and the final step took place overnight. After removal from the oven, each individual bundle was opened and the tissue, along with its ID tag, was embedded in a square peel-away mold #15160-215 (VWR scientific, Mississauga, ON) using fresh liquid paraplast. The blocks were allowed to harden at room temperature until slicing.

Sectioning of the embedded stomachs was carried out at 15 μm using a vertical microtome (American Optical Corp., Buffalo, NY) and slices were mounted individually onto glass slides by floating them in a heated water bath #B6990 (Baxter Scientific Products, Deerfield, IL) with distilled water and 5% (v/v) Staysure® (Surgipath, Richmond, IL).

Before processing for immunocytochemistry, it was necessary to de-wax and rehydrate the slide mounted sections. This was performed using serial treatments of Clearene® (Surgipath Canada Inc., Winnipeg, MB, Canada) for 10, 2, and 2 minutes respectively. This was followed by serial treatments in 100%, 100%, and 70% ethanol (v/v) and subsequently three treatments in fresh distilled water for 2 minutes each.

Slide mounted stomach slices were processed for PER2 immunocytochemistry according to the following procedure. Slides were washed in phosphate buffered saline (0.1M) followed by a 15 minute quenching step using hydrogen peroxide (1%) to neutralize any endogenous peroxidase activity in the tissue. After a subsequent wash, slides were incubated for 30 minutes in a blocking solution containing bovine serum albumen (1% w/v), triton-X (0.3% v/v), and PBS. This was followed directly by 48 hour incubation, with stirring, of the primary rabbit antibody raised against PER2 proteins (1:3,000, Alpha Diagnostic Inc., San Antonio, TX) in blocking solution at 4°C. After subsequent washing, a 1 hour incubation of the secondary antibody (1:500 donkey anti-rabbit, Jackson ImmunoResearch, West Grove, PA) in blocking solution was carried out at room temperature. This was followed by 1 hour incubation, at room temperature, of an avidin-biotin complex (ABC Elite Kit; Vector laboratories, Burlingame, CA, USA) in PBS followed by a washing step. Staining was developed in 0.5% 3,3'-diaminobenzene (DAB) and 0.01% H_2O_2 in PBS until sections were fully developed. Sections were determined to be fully developed when staining appeared dark in colour and specific to the cells within the oxyntic mucosa with only a hint of non-specific background staining. This took 1 hour. Slides were then washed in PBS for five minutes and stored in PBS at 4°C and allowed to air dry.

Slides were dehydrated in serial solutions of 70%, 95% and 100% ETOH for 5 minutes each and then defatted in Clearene® (Surgipath Canada Inc., Winnipeg, MB, Canada) for 5 minutes before coverslipping with Permount (Fisher Scientific, Fairlawn, NJ) mounting medium.

2.1.4 Quantification of Immunocytochemistry

Two techniques were used to quantify immunocytochemical labeling of proteins of interest. Since cFOS is a nuclear protein that is not constitutively expressed, it was easily quantified by counting individual cells, where each stained nucleus was surrounded by an unstained cytoplasm. For PER2, it was necessary to evaluate overall expression using relative optical density (discussed in detail in section 2.1.4.2). This was due to the expression of PER2 in all experimental conditions, the presence of PER2 throughout the nucleus and the cytoplasm, and an extremely high density of cells within the oxyntic mucosa of the stomach that expressed this protein, thereby obscuring individual cells. It should be noted that a western blot analysis of the stomach may have been more appropriate to quantify the expression of PER2 in the stomach but this would require sacrificing the animals without intracardial para-formaldehyde perfusion, something which was not possible in this experiment. Furthermore, the technique employed here has been previously published (Lesauter, et al., 2009).

2.1.4.1 Stereological Estimates of Cell Density for cFOS Positive Cells

Three dimensional stereological estimates of cell density were used in order to analyze neuronal activation, using positive cFos expression, of our three experimental groups (n=5 per group). Due to various factors including: missing or ruined sections during slicing, lack of adequate baseline expression, or failure to meet an appropriate GCE (defined below) after counting, only 5 animals were analyzed for each experimental group per brain region. This analysis was carried out using the optical fractionator probe within Stereo Investigator v 9 (MBF

bioscience, Williston, VT) running on a desktop computer with Windows XP operating system (Microsoft Corp., Redmond, WA). The Olympus BX51 brightfield microscope (Olympus Canada, Markham, ON) and associated digital capture device DVC-2000C (DVC Company, Austin, TX) were also connected to this computer system via USB 2.0.

For each region of interest (ROI) (see **Table 1** for a full list of regions, their associated map coordinates, and the sampling parameters used), cell counting was performed using sampling parameters determined to be sufficient to produce a Gundersen's coefficient of error (GCE, $m=1$) less than 0.1. The GCE ($m=1$) has been determined to be the most appropriate coefficient of error estimate for all non-homogeneous biological tissues including cell populations within the brain (Gundersen & Jensen, 1987; Gundersen, Jensen, Kieu, & Nielsen, 1999) and like all coefficients of error, it is important to choose parameters that exceed the cutoff in order to ensure that the estimated cell counts do not include too much variability due to random chance. Once determined individually for each ROI, parameters were held constant across all three experimental groups. Each region of interest was outlined digitally (40x magnification) using the software provided while referring to *The Mouse Brain in Stereotaxic Coordinates 2nd Edition* (Paxinos & Franklin, 2001) for comparison. This information was then analyzed to create a volumetric estimate of each ROI measure in μm^3 . Each nucleus sampled was individually examined at very high magnification using an oil immersion lens (600x magnification) and cFos-positive cells were labeled with a digit marker. The software provided used both the planar location and depth information recorded for each marker in the analysis of cell population estimates thereby creating a three dimensional model of cells within each section from which an estimate of the total population of cells in the tissue is created. Cellular population estimates were then divided by the estimated volume in order to create an estimate of the density of cells expressing cFos such that comparisons between animals take into account the intrinsic variability between the shape and size of ROIs.

For each ROI, cell densities were analyzed using PASW statistics v 17.0 (SPSS Worldwide, Chicago, IL) to perform a one-way ANOVA and *posthoc* (LSD) test ($\alpha = 0.05$) and any group differences between experimental conditions were observed.

2.1.4.2 *Relative Optical Density Quantification of PER2 positive cells in Stomach*

A single slice of stomach (n=6 for each group) was analyzed for PER2 expression by calculating the relative optical density (ROD). The Olympus BX51 brightfield microscope (Olympus Canada, Markham, ON) and associated digital capture device DVC-2000C (DVC Company, Austin, TX) were used to observe stomach slices at 100x magnification while holding the sampling rate (25ms) and irradiance (full power with ND6 and ND25 filters engaged) constant. Images were digitized using the *image capture* function within Stereo Investigator v 9 (MBF bioscience, Williston, VT) and saved as 16bit grayscale TIFF images.

Relative optical density was calculated using the *measure* function within Image J v1.4 (National Institute of Health, USA). For each slice, the polygon selection tool was used to outline both the oxyntic mucosa and a blank area of the slide in order to measure the *mean grey values* of each. This returned a value that represents the sum of all grey values (with a possible value of 1 for black to 256 for white) for each pixel divided by the total number of pixels. In order to determine the optical density of each region, these values were then transformed to fit a linear standard curve where 100 represented the highest optical density and 0 represented the lowest $[(100-(Y/256*100))]$, where Y represents the mean grey value]. Finally, the ROD was calculated by subtracting the optical density of the background from that of the oxyntic mucosa. Values were then analyzed using PASW statistics v 17.0 (SPSS Worldwide, Chicago, IL) to perform a one-way ANOVA with *posthoc* LSD test ($\alpha = 0.05$) and any group differences between experimental conditions were observed.

2.1.5 Serum Collection and Ghrelin Assay

Approximately 0.5cc of blood was collected from the left atrium of each animal immediately prior to perfusion. It was placed in a blood collection tube deposited with anticoagulant ethyl-diamine-tetraacetic acid (EDTA) and 5 μ L of 100mM thiol protease inhibitor p-hydroxymercuribenzoate (pHMB). Tubes were stored on ice before being spun in a centrifuge at 3500rpm for 10 minutes. The blood serum supernatant was collected in 0.5ml centrifuge tubes and 25 μ L of 1M HCl was added before storage at -20 °C until analysis.

The serum concentrations of the acylated active form of ghrelin was assayed using an enzyme linked immunosorbant assay (ELISA) kit #EZRGRA-90K (Millipore, Billerica, MA). Data was collected by a spectraMAX spectrophotometer (Molecular Devices, Sunnyvale, CA) and analysed using SoftMax Pro v 5.3 (Molecular Devices, Sunnyvale, CA) which automatically created a standard curve and provided interpolated concentrations of the samples (n=6 for each group) and for our samples the intra-assay variability (IAV = $\text{meanStdev}/\text{meanConc} \times 100$) was calculated as 22.9%.

Briefly, this technique immobilizes the protein of interest onto a plate containing antibodies raised against it. Then the horse radish peroxidase enzyme (HRP) is conjugated with this complex. After washing, the concentration of enzyme-antibody conjugate is quantified by monitoring HRP enzyme activity in the presence of substrate 3,3',5,5'-tetra-methylbenzidine for exactly 15 minutes. This activity is then measure spectrophotometrically, whereby the increased absorbance of light with a wavelength of 450nm, corrected against absorbance at 590nm, is indicative of increased concentrations of the specific end-products of the enzymatic reaction. The increased absorbance at 450nm is therefore directly proportional to the concentration of active ghrelin in each sample and this allows the concentration of ghrelin to be derived by interpolation from a standard curve created using known concentrations of active ghrelin provided in the kit.

Each sample (n=6 for each group) was assayed in duplicate and the average of the two absorbance values was used to calculate serum concentrations. Any samples with absorbance values below or above the range of the standard curve were rejected without extrapolation. Concentrations were then analyzed using PASW statistics v 17.0 (SPSS Worldwide, Chicago, IL) to perform a one way ANOVA with *posthoc* LSD test ($\alpha = 0.05$) and any group differences between experimental conditions were observed.

2.2 Study 2: The Role of Ghrelin in Engaging the FEO

Several circulating hormones have been shown to rise pre-prandially and affect central mechanisms: corticosterone, insulin, and ghrelin. Some of the earliest attempts to localize the FEO involved adrenalectomy, with the idea that the adrenal gland was the source of the FEO, specifically that corticosterone rhythms were able to create an entraining signal in circadian clocks important for FAA. However, complete adrenalectomy showed no effects on FAA. While the behavioural analysis lacked sophistication, there were absolutely no differences observed in entrainment to food, or FAA expression within ADX rats (Boulos, et al., 1980; Stephan, et al., 1979a).

With the role of glucocorticoids in FAA well characterized, the secretion of ghrelin, and its potential actions in the central nervous system, is a much more recent discovery. Owing to its novelty as a centrally acting neuropeptide, its role in FAA requires investigation.

The active, acylated form of ghrelin peaks before the active phase in *ad libitum* fed animals, and this peak can shift to occur pre-prandially, when a meal paradigm is employed. This has been demonstrated in several mammalian species including humans (Cummings et al., 2001; Drazen, Vahl, D'Alessio, Seeley, & Woods, 2006; Morgado et al., 2008; Sugino, Hasegawa, et al., 2002; Sugino, Yamaura, et al., 2002; Sutton, et al., 2008).

It has been proposed, but only indirectly demonstrated, that the active form of the stomach-derived orexigenic hormone ghrelin is under direct control of clock genes. Specifically,

peaks in plasma ghrelin concentration coincide with the nadir of both PER1 and PER2 expression in ghrelin-positive cells, suggesting that there is a clock gene found in the contra-lateral limb of the molecular clock (potentially BMAL1 or CLOCK) that increases activation of ghrelin and/or mediates its release from the stomach (Lesauter, et al., 2009). Unfortunately, there is currently no direct evidence of a clock gene interaction in the promoter region of the ghrelin gene nor is the mechanism for ghrelin release from the stomach known. Therefore, the secretion/activation of ghrelin may be constitutive, with plasma levels only coupled to the time of feeding in an hourglass manner - steadily increasing in concentration until the consumption of the next meal when a sharp drop off creates an apparent pre-prandial peak. This hypothesis has already been rejected through the observation of anticipation but not fasting producing a pre-prandial peak of ghrelin in rats (Drazen, et al., 2006). Here, the first investigation will measure anticipatory serum acylated-ghrelin levels. If the pre-prandial peak of active ghrelin is dissociated from PER2 expression in the RFAL, then it is certainly not under clock control. However if both the clock gene expression and the serum ghrelin concentrations remain correlated in our final condition, it furthers the proposal that ghrelin secretion/activation is under control of clock genes.

The second study of this thesis attempted to characterize the role of ghrelin as an important peripheral hormone necessary for the anticipation of food. Acylated ghrelin has already been implicated in FAA as ghrelin injections increase locomotor activity in rats (Jerlhag, 2008) and foraging-like behaviour in hamsters (Keen-Rhinehart & Bartness, 2005). Several hypothalamic nuclei have been proposed as the loci of its effects, we will review the data previously published by our lab (Blum, et al., 2009) and add several new pieces of data along with fresh insights into the mode of ghrelin's action within the CNS. By comparing locomotor behaviour and neuronal activation in ghrelin receptor knockout mice (GHSR_{KO}) and their wildtype counterparts, this study attempted to locate neuronal activation of select GHSR-expressing nuclei within the hypothalamus and limbic system sensitive to the pre-prandial peak of acylated ghrelin. It is

hypothesized that ghrelin secretion from the stomach represents a key component of the FEO, necessary for increased activation of central nuclei and the appropriate expression of FAA.

2.2.1 Animals

This experiment was carried out using mice with targeted mutations to the ghrelin receptor gene (GHS-R_{KO}, n=10) and their wild-type counterparts (WT, n=10). All mice were bred at the Carleton University Neuroscience Institute animal facilities and originated from breeding pairs obtained from Regeneron Pharmaceuticals in Tarrytown, New York (see Wortley et al., 2004 for more information about the generation of these mice). Before experimentation, all animals were housed in groups of 2-4 in standard mouse cages and received *ad libitum* feeding of mouse chow and water in a temperature and humidity controlled vivarium with a 12-hour Light Dark (L:D) cycle starting at 8:00 AM.

All Research was conducted under the guidelines of the Canadian Council on Animal Care and approved by Carleton University's Animal Care Committee (Protocol # P06-15).

2.2.2 Behavioural Analysis

Mice with targeted mutations to the ghrelin receptor gene (GHS-R_{KO}, n=10) and their wild-type counterparts (WT, n=10) were placed under a restricted feeding paradigm with food presented daily from ZT4-8 for 14 days. Both groups were released into *ad libitum* feeding for 3 days before undergoing a 40 hour fast. Food was removed at ZT12 of the 3rd day of *ad libitum* and replaced at ZT 4 on the 5th day, after the previous anticipatory period. This manipulation is typically employed in order to test two characteristics of a proper circadian pacemaker, namely the ability to continue oscillating at a phase previously set, hence the use of an *ad libitum* period between entrainment and fasting, and the ability to oscillate without zeitgeber input, hence the use of fasting over two cycles of entrainment.

Behaviour was recorded and analyzed as described in section 2.1.2. The data was divided into four feeding conditions for analysis 1) baseline, days one through three, 2) entrainment, days four through seventeen, 3) *ad libitum* feeding, days eighteen through twenty-one, and 4) fasting, days twenty-two through twenty-three. Repeated measures ANOVAs ($\alpha = 0.05$) were performed within each temporal category for each behavioural measure using PASW statistics v17.0 (SPSS Worldwide, Chicago, IL) to analyze the effect of experimental group, day, and any interaction between these two factors.

2.2.3 Tissue Collection and Processing

A second set of mice with targeted mutations to the ghrelin receptor gene (GHS-R_{KO}, n=6) and their wild-type counterparts (WT, n=5) were used for the immunohistological analysis of hypothalamic- and reward-related neuronal activation. All mice were placed on a restricted feeding schedule for 7 days with feeding between ZT4-8. On day 8, between ZT3-4 in anticipation of the meal, animals were injected with an overdose of sodium pentobarbital and intracardially perfused with 100ml of saline (0.9%) followed by 250ml of paraformaldehyde (4%). Brains were collected and post-fixed in 50ml of paraformaldehyde overnight and then transferred to a 30% sucrose solution until sliced on a vibrating microtome (Vibratome, Bannockburn, IL). 40 μ m slices of tissue were collected and placed into 4 serial sets. They were stored in a cryoprotectant solution at -20°C until processed for immunocytochemistry.

One set of serial brain sections was processed for cFos immunocytochemistry according to the procedure described in section 2.1.3.1. Full development took approximately 12 minutes. Sections were mounted onto glass slides and coverslipped as described in section 2.1.3.1.

2.2.4 CFos-Positive Cell Counts

Because the tissue was collected and prepared prior to the arrival of stereology equipment to our lab, it was of insufficient thickness to undergo stereological analysis. Therefore, it was

necessary to count and analyze the total number of cFOS positive cells sampled from 2 medial slices for each bilateral ROI (see **Table 2** in appendix A for a full list of brain regions examined and their stereotaxic coordinates). Digital images were collected using an Olympus BX51 brightfield microscope at 40x magnification (Olympus Canada, Markham, ON) with a DVC-2000C digital capture device (DVC Company, Austin, TX). CFos-positive cells were counted using Image J v1.4 (National Institute of Health, USA) while consulting *The Mouse Brain in Stereotaxic Coordinates 2nd Edition* (Paxinos & Franklin, 2001) for each ROI and then averaged across the two slices. For each ROI, averaged cell counts were analyzed using PASW statistics v 17.0 (SPSS Worldwide, Chicago, IL) to perform a one-way ANOVA ($\alpha = 0.05$) examining genotype differences.

Due to problems associated with the onset of torpor due to entrainment difficulties of younger, slimmer animals (body weight < 25g); several animals were removed from the restricted feeding schedule before the conclusion of the study.

3 Isolating the FEO

3.1 Results

3.1.1 Behaviour

During behavioural recording, there was no body weight difference between groups for the baseline ($F_{(2,23)} = 0.159, p = 0.854$), entrainment ($F_{(2,23)} = 1.390, p = 0.271$), or *ad libitum* conditions ($F_{(1,15)} = 0.016, p = 0.902$). A repeated measures ANOVA used to analyze food consumption showed a significant change across days during entrainment ($F_{(2,12)} = 22.694, p < 0.001$) as well as an interaction between day and group effects ($F_{(2,24)} = 4.972, p < 0.001$). Furthermore, total food consumption during entrainment was attenuated in the RF and RFAL groups as compared to AL control according to a *post hoc* Least Squares Difference (LSD) test ($p < 0.001$, and $p < 0.001$ respectively) although they did not differ significantly from each other ($p = 0.623$) according to LSD post hoc comparisons. A repeated measures ANOVA used to analyze food consumption during subsequent *ad libitum* feeding showed no change across days ($F_{(2,28)} = 0.240, p = 0.788$) although food consumption was significantly increased in the RFAL group as compared to the AL controls ($F_{(1,14)} = 34.892, p < 0.001$) in a post hoc LSD comparison (See **Figure 6** for graphical representation of these effects).

During the last seven days of entrainment there was a significant difference in both FAA and nocturnality across days ($F_{(6,120)} = 4.125, p = 0.007$ and $F_{(6,120)} = 0.240, p = 0.788$ respectively) using the Greenhouse-Geisser criteria (see **Figure 7**). Anticipatory locomotor activity was elevated in the RF and RFAL groups relative to AL controls ($p = 0.005$, and $p = 0.023$ respectively) but no difference was observed between these two groups ($p = 1.000$) in a post hoc LSD comparison. This is also obvious in the averaged activity waveforms, where there is a visible peak in locomotor activity preceding the meal in both the RF and RFAL groups but no change in nighttime activity (**Figure 8**). Nocturnality was similarly reduced in the RF and RFAL groups as compared to controls ($p < 0.001$ and $p < 0.001$ respectively) with no difference

Figure 6: Body weights and food consumption across days. **Top**, average body weights for each group at the end of each feeding treatment. **Bottom**, average daily food consumption for each group across days. Note the sharp peak in rebound feeding after a return to ad libitum. However, both the initial reduction and subsequent rebound in feeding had no effect on body weight across conditions suggesting internal metabolic compensation/protection against food restriction. Note that *ad libitum* feeding began after meal presentation on day 14.

observed between the two groups ($p = 1.000$) in a post hoc LSD comparison. During the subsequent *ad libitum* feeding period there was a significant difference in FAA but not nocturnality across days ($F_{(2,26)} = 10.229, p = 0.004$ and $F_{(2,26)} = 1.639, p = 0.218$ respectively) according to the Greenhouse-Geisser criterion. However, the expression of FAA was quickly extinguished ($F_{(1,13)} = 1.351, p = 0.266$) whereas a statistical trend remained for the nocturnality score ($F_{(1,13)} = 4.292, p = 0.059$).

Figure 7: Locomotor Activity Across Days. **Top,** Average daily cumulative values for ambulatory activity during the time of meal anticipation (ZT2-4). Note that FAA develops slowly over several days as the animals entrain to their new feeding schedule but sharply declines back to baseline values on day 15 after being returned to *ad libitum* feeding. **Bottom,** Nocturnality scores for animals during RF and subsequent AL feeding. Nocturnality is defined as the total ambulatory activity at night divided by total daily activity. During FAA, nocturnality was reduced as animals spent more time active during the day, usually centered around the time of meal anticipation, and reduced the amount of time spent active at night. Note that *ad libitum* feeding began after meal presentation on day 14 (triangle).

Figure 8: Average locomotor activity by feeding regimen. **Top,** There was an extremely pronounced increase in activity in anticipation of the meal presented at ZT 4 which quickly subsided after feeding. Activity increased throughout the 24 hour period during food entrainment and was not confined to the time of meal anticipation. **Bottom,** The anticipatory peak and the increase in night time activity was completely abolished after a return to *ad libitum* feeding, although daytime activity did appear to remain slightly higher than AL controls. Note: 1hr MA represents a smoothed curve where the moving average, the average score for all data points within the hour surrounding each time point, was plotted. Gray bars indicate the time of food availability.

3.1.2 Stereological Estimates of Cell Density for cFOS Positive Cells

Cell density estimates of cFOS positive cells revealed a distinct pattern of neuronal activation as compared to AL controls for both the RF and RFAL groups (see **Figure 9**). The following areas exhibited notable changes in the number of cFOS expressing cells between groups: AcbC ($F_{(2, 17)}=3.108, p = 0.074$)*, AcbSH ($F_{(2, 17)}=9.508, p = 0.002$), Cg1 ($F_{(2, 17)}=4.899, p = 0.023$), VTA ($F_{(2, 17)}=3.542, p = 0.058$)*, SCN ($F_{(2, 17)}=3.122, p = 0.081$)*, LH ($F_{(2, 17)}=5.408, p = 0.021$), PVN ($F_{(2, 17)}=5.127, p = 0.025$), VMH ($F_{(2, 17)}=4.680, p = 0.031$), ARC ($F_{(2, 17)}=4.227, p = 0.041$), DMH ($F_{(2, 17)}=5.372, p = 0.022$), DR ($F_{(2, 17)}=5.182, p = 0.024$), PB ($F_{(2, 17)}=33.189, p < 0.001$), NTS ($F_{(2, 17)}=7.310, p = 0.008$), LC ($F_{(2, 17)}=17.506, p < 0.001$), BNSTov ($F_{(2, 17)}=10.102, p = 0.003$), CeA ($F_{(2, 17)}=17.613, p < 0.001$), BLA ($F_{(2, 17)}=18.138, p < 0.001$), and DG ($F_{(2, 17)}=17.692, p < 0.001$).

Post hoc analysis using LSD pairwise comparisons revealed a significant increase in the number of cFOS expressing cells in the RF group, as compared to AL negative controls for the following regions: AcbC ($p = 0.048$), AcbSH ($p = 0.001$), Cg1 ($p = 0.011$), VTA ($p = 0.094$)*, LH ($p = 0.010$), VMH ($p = 0.016$), ARC ($p = 0.077$), DMH ($p = 0.022$), DR ($p = 0.008$), PB ($p = 0.001$), NTS ($p = 0.004$), LC ($p = 0.001$), BNSTov ($p = 0.002$), CeA ($p < 0.001$), BLA ($p = 0.002$), and DG ($p = 0.001$).

As hypothesized, post hoc analysis using LSD pairwise comparisons revealed continued activation within in the RFAL group, as compared to AL negative controls and RF positive controls respectively, for the following regions: ARC ($p = 0.015$ and $p = 0.380$), DMH ($p = 0.011$ and $p = 0.698$), DR ($p = 0.053$ and $p = 0.334$), PB ($p < 0.001$ and $p = 0.013$), and NTS ($p = 0.011$ and $p = 0.603$)(See **Figure 13** in Appendix A for representative images and associated brain maps).

There were also two regions that exhibited decreased activation in the RF groups as compared to AL controls, the SCN ($p = 0.046$) and the PVN ($p = 0.061$)* with a return to AL levels after return to *ad libitum* feeding ($p = 0.904$ and $p = 0.300$ respectively)(see **Figure 14** in Appendix A for representative images and associated brain maps).

Figure 9: Mean cFOS cell density (+/- SE). **A**, the appetitive network. **B**, the affective network. **C**, the metabolic and neuroendocrine network. **D**, the arousal network. Stars indicates a significant difference from AL controls ($p < 0.05$), crosses indicate significant difference from RF group ($p < 0.05$). Hollow symbols denote $p < 0.1$.

3.1.3 *Stomach Clock Gene Expression and Circulating Active Ghrelin*

Significant differences in both PER2 immunoreactivity ($F_{(2,16)}=5.408, p = 0.021$) and circulating ghrelin levels ($F_{(2,16)}=12.994, p = 0.001$)(**Figure 10**) were observed between groups when analyzed with a one-way ANOVA. Post hoc LSD pair-wise comparisons revealed the same increase in both PER2 expression within the oxyntic mucosa ($p = 0.023$) and accompanying circulating active ghrelin levels ($p = 0.001$) that have previously been reported in anticipation of a meal during RF. However, both of these increase returned to baseline levels in our RFAL group ($p = 0.904$ and $p = 0.824$ respectively).

Figure 10: Is the stomach an FEO? **A**, average ROD of PER2. **B**, Average concentrations of circulating active ghrelin. **C**, Representative images of PER2 staining in the oxyntic mucosa. Stars indicates a significant difference from AL controls ($p < 0.05$), crosses indicate significant difference from RF group ($p < 0.05$).

3.2 Discussion

3.2.1 *The Stomach Does Not Contain an Autonomous Circadian Pacemaker*

Rhythms of the stomach oscillator are normally entrained to the light-dark cycle and continue to oscillate in synchrony with the master clock even after being placed in constant darkness or undergoing food-deprivation (Lesauter, et al., 2009). The findings of this study confirm that clock protein expression within the stomach and ghrelin secretion is altered by a restricted feeding schedule. Furthermore, these are both very quickly reset to previous levels after a return to *ad libitum* feeding. While clock gene rhythms were not examined within the stomach *per se*, it is possible to extrapolate our findings based on previous observations of stomach rhythms, and circadian mechanisms as they exist elsewhere in the CNS and periphery.

If the stomach is an autonomous oscillator, then a three day period of *ad libitum* feeding may be just enough time for clock gene oscillations in the stomach to phase delay a full 8 hours. This is certainly true for rats with SCN lesions, however in intact rats an 8 hour phase delay of meal presentation can require up to 14 days for FAA to entrain to the new schedule. This suggests that the FEO is much slower to respond to shifts in meal time when signals from the SCN driving sleep-wake cycles conflict with FEO signals driving food anticipation (Stephan, 1986).

Realistically, without full rhythmic expression profiles for our animals it is very difficult to determine if this is indeed occurring, but based on the observation of a return to *ad libitum* levels for both PER2 expression and circulating ghrelin levels along with the rhythms observed by Lesauter (2009), it is likely that the stomach is resetting to the light-dark cycle much quicker than should be possible if it was an autonomous pacemaker. There is no reason to expect a sudden loss of rhythmicity or for the oscillatory mechanism within the stomach to act contrary to any other known circadian oscillator. Therefore, a mechanism is needed to explain the extremely quick resetting of stomach clock genes and ghrelin secretion observed. A gating mechanism between the SCN and FEO may be involved, one that is dependent on the same factors which negotiate

this switch in both behavioural and glucocorticoid rhythms (Stephan, 2002). This is consistent with the mechanism of the peripheral clock found in the liver (Stokkan, Yamazaki, Tei, Sakaki, & Menaker, 2001).

Clock gene expression in the liver of mice entrains to meal presentation with roughly the same time scale as FAA in naïve animals and remains entrained even after a protracted food deprivation test. This occurs in both intact and SCN ablated animals, however intact animals show a quick resetting of clock gene rhythms to the light:dark cycle after a return to *ad libitum* feeding (Hara et al., 2001). Furthermore, shifting the L:D cycle under *ad libitum* feeding also shifts liver, heart and lung clock gene expression with SCN rhythms, albeit more slowly (Yamazaki et al., 2000), but this does not occur while maintaining an RF schedule with constant meal time, at least in the liver (Hara, et al., 2001). If a similar situation is occurring in the stomach, which based on the observations of this study is true, then the stomach represents a peripheral oscillator in a hierarchical model with either the SCN or FEO, depending on feeding conditions, setting the timing of clock gene expression. This argues that the stomach does not contain an autonomous pacemaker, necessary or sufficient, for driving secretion of ghrelin and subsequently FAA at the time previously set when a food deprivation test is carried out after *ad libitum* feeding. This is consistent with our finding in the second study and will be examined further in section 4.2.1 when we discuss the role of ghrelin as a modulatory hormone necessary for the appropriate expression of FAA but not entrainment of the FEO.

While it is perhaps unfortunate, or rather less interesting, that the stomach cannot be considered an integral part of the circadian mechanism of the FEO, our observation of a return to *ad libitum* levels of circulating active ghrelin in the RFAL group can be taken as one form of evidence that our experimental paradigm was successful in removing central and peripheral cues of metabolic demand that drive FAA.

3.2.2 *Potential Confound of Entrainment Followed by Ad libitum Feeding:*

While a potential confound in this experiment may be the availability and consumption of food at the time of previous anticipation, it serves to highlight an integrated circuit which remains active in the absence of metabolic demand. Here, dissociation was observed between activation of select hypothalamic and caudal brainstem sites and certain behavioural outputs, most notably rhythms of locomotor activity. Certain nuclei in the third group (RFAL), specifically the NTS and ARC, contain different cell populations responsive to either metabolic demand, or food consumption. Therefore the apparent continued activation of these areas may not be due to internal circadian oscillations of clock genes timed to the anticipation of food but rather due to food consumed during the previous period of food anticipation. This is also suggested by the massive rebound feeding occurring in our RF then *ad libitum* fed mice. There is no way of discerning if food was indeed consumed during this period, as food was measured by hand and the presence of a researcher in the room would be an even more serious confound, acting as separate cue for food anticipation, or possibly food removal, one without a circadian component. It will require further investigation, possibly using double labeling of cFOS and the appropriate neuropeptides, NPY/AGRP versus POMC/CART in the ARC, or simply a much finer spatial analysis, to discern whether increase activation in these areas is anticipatory or in response to a recent feeding bout.

There are four observations in this study that counter any arguments of feeding-induced activation of brain nuclei: 1) There was no remarkable visual difference in the quantity of stomach contents (i.e. undigested food) during removal and dissection of stomachs between all three groups (personal observation), although this was not recorded or directly quantified. 2) No differences were observed in the amount of activity during the anticipatory period when given *ad libitum* access. If the animals were aroused and consuming food there should be at least some small difference in the amount of activity observed during this period due to arousal and food bin

approaches but this was not the case (**Figure 7**). 3) Within the NTS and the ARC, increases of cFOS expression observed at the time of meal anticipation in our RFAL group were not consistent with massive activation that should be occurring if the animals had recently ingested food (Angeles-Castellanos, et al., 2005). Similarly, a feeding bout occurring at the time of meal anticipation in the animals should produce a pattern of cFOS expression consistent with refeeding after fasting, not just within the DMH, ARC and NTS, but also in various other brain nuclei responsive to feeding including the PVN, VMH, and supraoptic nucleus (Angeles-Castellanos, et al., 2004; Angeles-Castellanos, et al., 2005; Poulin & Timofeeva, 2008; Singru, Sanchez, Fekete, & Lechan, 2007). 4) cFOS expression in the LH and LC, two regions extremely important for mediating arousal via orexin and noradrenaline release throughout the brain respectively, showed a return to AL levels in the RFAL group. This indicates that the internal arousal state of the animals was low, and not conducive to food anticipation or consumption. In sum, while food may have been consumed during the previously anticipatory period, this is not likely, based on the indirect observations noted above.

3.2.3 ***Appetitive System:***

As expected, an increase in cFOS within every nuclei of the dopaminergic reward system was observed in anticipation of a scheduled meal (**Figure 9**) with one exception, the prefrontal cortex. This area is known to be involved in anticipation of both aversive and appetitive stimuli, most notably the integration of associative cues such as sound, vision, and olfaction, to influence the expression of anticipatory behaviours (Groenewegen, et al., 2000; Van Eden & Buijs, 2000). This is true for the anticipation based on the presentation food-associated cues (Schiltz, Bremer, Landry, & Kelley, 2007). Associative learning of these cues alters forebrain connectivity and activation, with increases in immediate-early gene expression including *homer1*, *arc*, and *zif268* within the prefrontal cortex under a paradigm pairing food presentation with a spatial, visual, and

olfactory context. Furthermore, changes in sensitivity to context and activation of the PFC were also observed.

One possible explanation for these results is that lateralization of function within the PFC obscured significant increases due to food anticipation. Activation of the PFC by dopaminergic projections from the accumbens occurs preferentially in the right hemisphere in response to food-associated cues (Merali, McIntosh, & Anisman, 2004). However, this was likely not the case here, as cFOS expression increases within the PFC in animals anticipating a palatable food but not those under an RF schedule (Mendoza, Angeles-Castellanos, & Escobar, 2005a). Furthermore, entrainment of clock genes within the PFC does occur in RF fed mice, but the rhythms are dampened compared to those of animals entrained to a palatable meal (Angeles-Castellanos, Salgado-Delgado, Rodriguez, Buijs, & Escobar, 2008). Taken together, these studies indicate that the PFC is mildly responsive to RF but shows increased rhythmicity correlated with the appetitive value of the reward that is independent of metabolic status.

Activation of the anterior cingulate gyrus, an area traditionally considered to be critical for decision-making, error detection and integration of emotional (i.e. limbic) and cognitive (i.e. cortical) function (Allman, Hakeem, Erwin, Nimchinsky, & Hof, 2001) was also observed in anticipation of a daily meal. While there is some evidence that this area is involved in mediating the cognitive aspects of anticipation in humans (Aarts, Roelofs, & van Turenout, 2008), the results observed in this study represent a seminal demonstration that it is preferentially activated in anticipation of a timed daily meal, even when no external cues are available. It is therefore important to assess circadian clock gene expression in this area to determine if food, or some food-related cue, is able to influence the internal oscillations of this nucleus under an RF schedule.

All increases in activation observed during RF were completely abolished after three days of *ad libitum* feeding as predicted. The deactivation of this system after three days of *ad libitum* feeding provides the first evidence that this experimental paradigm was successful in abolishing the metabolic signals masking activation of nuclei responsible for the circadian component of FAA. Furthermore, none of these nuclei was implicated as a potential circadian component of the FEO which suggests a potential rapid switching of circadian rhythms, resetting rhythms to the light-dark cycle under the hierarchical control of the SCN. Of course, this last suggestion must be empirically tested by examining clock gene expression in these nuclei using the paradigm devised here, before any firm conclusion about their oscillatory mechanisms and rhythmic activity can be made.

3.2.4 Affective System:

Emotional processing of anticipatory food-related cues is important for the full expression of FAA. As predicted, a marked increase in activation occurred in all nuclei at the time of food anticipation in the RF group. This supports previous findings that RF, and not simply the access to rewarding stimuli such as sucrose or a palatable food, change cFOS expression and entrains clock gene oscillations in these areas (Verwey, et al., 2007; Waddington Lamont, et al., 2007; Waddington Lamont, et al., 2005). It has similarly been demonstrated that glucocorticoids are the zeitgeber for both the BNSTov and CeA rhythms (Segall, et al., 2006), which suggests an involvement of the HPA axis and the direct activation of the adrenal cortex by ghrelin (see section 3.2.5.2), in the entrainment of these oscillators during restricted feeding.

While all examined areas were activated as compared to AL controls, the BNSTov showed an approximately 5 fold increase in cFOS positive cell density in anticipation of a meal. This may be related to the function of the BNSTov, integrating signals from the amygdala and hippocampus, and relaying those signals to hypothalamic areas. It is possible that the activation of this nucleus occurs synergistically with interactions between its inputs and internal clock gene

oscillations to produce a strong influence on the hypothalamus at the time of meal anticipation, ultimately sculpting anticipatory responses like neuroendocrine secretion and autonomic function. The final output of this system, via the BNSTov, represents the mediation of negative and stressful effects of metabolic demand when a meal is predicted and anticipated. For further discussion of this mechanism see section 3.2.5.2.

Of significant interest is the return to baseline of all nuclei investigated in the RFAL group. Clock gene expression of these areas is typically entrained to the SCN under *ad libitum* feeding conditions (Waddington Lamont, et al., 2005), and a shift to FEO-driven rhythms occurs under RF. Since no anticipatory activation was observed after a return to *ad libitum* feeding, it is of interest to determine if clock gene expression also resets to SCN control in some of these areas. While no explicit statements about clock oscillations can be made at this time, it is unlikely that the affective limbic system contains any nuclei directly involved in driving the autonomous oscillations of the FEO. This is consistent with ablation studies revealing that these areas are not necessary for the entrainment to food or the expression of FAA (Mistlberger & Mumby, 1992).

3.2.5 Metabolic and Neuroendocrine System:

An important goal of this experimental paradigm was the removal of metabolic demand to ensure that any activation in the examined brain regions during anticipation of food in our RFAL group was uniquely due to internal circadian signals generated by the FEO. It is intuitive to think that the best place to look for the effects of these signals and their subsequent removal after a return to *ad libitum* feeding is the hypothalamus, since it is the site of homeostatic regulation of body weight and food consumption however this is not the case. Several hypothalamic nuclei are tasked with integrating signals of both metabolic demand and metabolic surplus, and these areas are preferentially activated under both conditions. Therefore, investigation of the affective and appetitive systems served to indirectly demonstrate the removal of metabolic demand under this paradigm.

The VMH alone provides insight into the state of energy balance after a return to *ad libitum* feeding. Glucose sensing neurons in the VMH (Mobbs, et al., 2001; Song & Routh, 2005) are extremely responsive to alterations in circulating glucose levels and activation of this nucleus indicates one of two occurrences: either blood sugar is unusually low, due to prolonged fasting which causes depletion of glucagon stores in the liver leading to hypoglycemia, or it is unusually high, indicating that a meal has been very recently consumed and insulin has not yet acted to reduce blood sugar levels to an appropriate homeostatic set point. In the RF group, it is believed that the former occurred, where 20 hours of fasting since the last meal presentation created hypoglycemic animals. In the RFAL group, the return of activation to AL levels indicated two things: 1) the experimental manipulation was successful at removing metabolic demand normally correlated with meal anticipation and FAA, and 2) the animals in this group were not aroused and consuming food at the time of previous anticipation, even though they had free access to food.

There is another hypothalamic nucleus that can serve to illustrate this point as well, the LH, but it will be discussed in detail in section 3.2.6, highlighting its role in arousal caused by food deprivation.

There are two particular nuclei within the hypothalamus that coordinate behaviour and neuroendocrine responses of central and peripheral nuclei. The SCN does so by providing a guiding rhythmicity to activate, or inhibit, involved nuclei according to internally-derived signals caused by entrainment of clock genes to incoming signals of daylight. The second, the PVN, does so by integrating signals of metabolic, emotional, and cognitive processes to drive secretion of various peptides that acts as central and circulating hormones. It is interesting that both of these areas were preferentially deactivated in anticipation of a meal in this study and the following two subsections attempt to deal with such perplexing observations.

3.2.5.1 Food anticipation reduces SCN activation:

A reduction in cFOS activation within the SCN was observed in our RF group and this is in agreement with similar observations in rats during FAA (Carolina Escobar, Martínez-Merlos, Angeles-Castellanos, Miñana, & Buijs, 2007).

In terms of both evolutionary adaptation, as well as economy of function, the necessity for SCN-uncoupling during FAA is not surprising. In many single-celled organisms and plants, a single circadian pacemaker appears sufficient for orchestrating all cellular processes and producing coordination and metabolic efficiency (Wijnen & Young, 2006). In higher-order eukaryotes, such as the fungus *Neurospora crassa* (Bell-Pedersen, Crosthwaite, Lakin-Thomas, Merrow, & Okland, 2001), insects like *Drosophila melanogaster*, birds, and mammals, multiple distributed clocks like the LEO and FEO become necessary (Bell-Pedersen et al., 2005). Each must oscillate independently, and any mechanisms affecting a switch from the primary clock to a secondary one must occur not at the level of the pacemakers themselves, but rather at the level of pacemaker input and/or output.

As previously discussed, the GHT is a secondary pathway providing light intensity information to the ventrolateral SCN, and it is responsible for integrating non-photic signals entering the lateral geniculate. Food is a potent non-photic cue to this nucleus and there are several studies implicating the IGL as a site sensitive to metabolic status (for review see Challet, 2010). Neuronal activation in the IGL, as measured by cFOS expression, increases preprandially under a RF schedule (Challet, Jacob, Vuillez, Pevet, & Malan, 1997). Conversely, the behavioural phase advances typically observed in metabolically-challenged rats are abolished with bilateral lesions of the IGL (Challet, Pevet, & Malan, 1996). The sensitivity of the IGL to metabolic status is believed to be conferred by afferent projections originating from glucose sensing neurons of the VMH (Vrang, Mrosovsky, & Mikkelsen, 2003).

The majority of neurons projecting from the IGL terminate directly within the SCN. These terminals express neuropeptide Y (NPY), co-localized with GABA (Harrington, Nance, & Rusak, 1987; Moore & Card, 1994) and have been directly implicated in non-photic phase shifting of behaviour. Intra-SCN infusion of antisera raised against NPY attenuates phase advances normally associated with non-photic zeitgebers, *i.e.* access to a novel running wheel (Biello, Janik, & Mrosovsky, 1994). Furthermore, NPY release into the SCN has been shown to produce non-photic phase shifts during the day, and the application of glutamate (as a mimic of photic input) blocks this non-photic phase shifts *in vitro* (Biello, Golombek, & Harrington, 1997). Finally, application of GRP blocks these non-photic phase shifts *in vivo* (Kallinger & Mintz, 2007). This further supports light-dependent modulation of SCN rhythms by non-photic signals.

There is a second NPY-dependent mechanism through which the SCN is modulated by food restriction, and this occurs through direct projections from the ventromedial arcuate nucleus (vmARC). The vmARC may serve as a much more localized site, where both short- and long-term metabolic status are able to influence the daytime activity of the SCN. The intravenous application of a ghrelin mimetic induces cFOS expression in NPY-positive cells of the vmARC and subsequently reduces early daytime cFOS in the SCN cells to which they project (Yi et al., 2006). These same vmARC cells are not only activated directly by ghrelin, but they also receive projections from other brain regions that influence food-directed behaviour, such as the nucleus accumbens, which is responsible for the increased salience of food (Zheng, et al., 2003), the DMH (Yi, et al., 2006), the PB (Ricardo & Koh, 1978) and the NTS (Sim & Joseph, 1991). The last two brain regions mentioned receive visceral information about nutrient status *via* vagal afferents and both are activated during RF and remain activated in the RFAL group. The reduction of cFOS immunoreactivity during the day is of particular interest, as it represents a direct demonstration that SCN daytime activity is temporally reduced by the presence of a humoral factor signaling food deprivation, namely ghrelin, which is under food-entrained

circadian control and disappears shortly after feeding (Drazen, et al., 2006; Lesauter, et al., 2009).

The purpose of these non-photoc inputs to the SCN, including those induced by a negative metabolic state, may be to alter the strength of SCN output signals. *In vivo* microdialysis has demonstrated that extracellular AVP in the SCN is decreased but remains rhythmic and un-shifted in animals on a RF schedule (Kalsbeek, van Heerikhuize, Wortel, & Buijs, 1998). Andrade et al. (2004), implementing stereological analysis of immunoreactive cells, observed that RF decreases the expression of both VIP and AVP in the core and shell of the SCN respectively. This further strengthens the proposal that the effect of RF is not due to any change in the timing of the molecular clocks found within the SCN but rather is the result of the suppression of output of the LEO. Since this is not due to changes in the rhythmicity of light-driven clock genes in the SCN itself (Hara, et al., 2001; Wakamatsu, et al., 2001), it is likely that a signal from the FEO, through some as yet unknown neural and/or hormonal mechanism, is exerting control on the synthesis or degradation of these peptides in the SCN.

It has been proposed that AVP is a potent synchronizer of the firing of the SCN shell, leading to mass organization of neuronal firing and strengthening of circadian rhythms of the endogenous pacemaker (Ingram, Snowball, & Mihai, 1996; Mihai, Coculescu, Wakerley, & Ingram, 1994), and the number of AVP-immunoreactive cells negatively correlates with overt rhythmicity of locomotor activity (Gerkema, Van der Zee, & Feitsma, 1994). This explains the finding that normal rats under daytime RF remain entrained to the L:D cycle, even while entrained simultaneously to food availability. This demonstrates a distinct splitting of biological rhythms driven by the SCN and the FEO. Replicating the findings of Kalsbeek et al. (1998), a study investigating the effects of RF on the DMH also showed a distinct blunting of SCN-derived AVP secretion in anticipation of the meal while the phase of AVP release remained entrained to the light/dark cycle (Gooley, et al., 2006). In rats that do not produce AVP in the hypothalamus, such as the Brattleboro rat, it was found that daytime RF caused a complete shift of the circadian

system producing completely diurnal animals (Murphy, Wideman, & Nadzam, 1996). It follows that transient AVP depression during the daytime in the SCN, even without a shift in its temporal phase of expression, is important for allowing an uncoupling of the SCN. This uncoupling is key for the expression of rhythms driven by the putative FEO, allowing time-coded information about food availability to influence specific hypothalamic populations that would normally be suppressed by the outputs of the SCN during the light phase. This argument is further strengthened by the finding that Brattleboro rats are extremely adept at adapting to phase advances of restricted food presentation but not light entrainment whereas Long-Evans rats have much more difficulty adjusting to changes in the schedule of food presentation, especially when it is presented during the light period when SCN function and AVP expression peaks (Murphy, Wideman, & Nadzam, 1998).

SCN-derived AVP also plays a critical role in driving rhythmic activation of the HPA axis and subsequent glucocorticoid rhythms, and it has been shown to have a potent inhibitory effect on basal plasma corticosterone in rats (Kalsbeek, et al., 1992). This is not due direct interactions with corticotrophin releasing hormone- (CRH) positive neurons within the PVN but rather as a result of an integrative secondary circuit found within the DMH. AVP rhythms within the DMH are responsible for low corticosterone levels in the early subjective day (Kalsbeek, van Heerikhuize, Wortel, & Buijs, 1996), when AVP release is at its peak, as well as the preprandial/pre-waking surge of corticosterone observed just before the onset of the active phase, when AVP is at its nadir (Kalsbeek, van der Vliet, & Buijs, 1996). It has been recently proposed that the role of the DMH in rats as a GABAergic inhibitory intermediary (Hermes, Coderre, et al., 1996; Hermes, Ruijter, Klop, Buijs, & Renaud, 2000) between SCN-derived AVP and the HPA axis provides an explanation for the reversal of HPA but not SCN activity rhythms in nocturnal as compared to diurnal species (Kalsbeek, Fliers, Hofman, Swaab, & Buijs, 2010). When considering the blunting of AVP secretion in metabolically challenged animals, this would appear to provide the necessary mechanisms for producing the preprandial daytime peak in

corticosterone rhythms observed in rats anticipating a meal under a RF schedule (Mistlberger, 1994). Furthermore, it presents an interesting role for the DMH as an integrator of SCN-derived AVP rhythms with internal clock gene oscillations as well as several other signals generated by the metabolic state of the animal.

Determining the direct cause of these changes in neuropeptides expression, the reduction in activation of the SCN during food anticipation, and their subsequent effects may be the most important aspect for understanding how SCN signals are interrupted in a temporally-restricted manner to allow FAA outside of the typical period of arousal without a change in the rhythmicity of the pacemaker within the SCN.

3.2.5.2 *Food anticipation reduces PVN activation:*

It has been demonstrated that the preprandial rise in corticosterone observed in anticipation of the meal is both CRH and ACTH independent (Belda, et al., 2005; Pesonen, et al., 1992; Poulin & Timofeeva, 2008). While cFOS activation of the PVN was not expected, the observation of a decrease in activation as compared to controls and entrained then *ad libitum* fed animals was surprising. A similar reduction has only been reported once, and even then, it was in rats fasted for 64 hours, not in rats entrained to a daily meal (Sanchez et al., 2008). Mice are much more vulnerable to fasting, potentially making the 20 hour fast occurring daily in our mice comparable to that of this previous study. However, a direct comparison of the time-course of metabolic demand created by fasting and its temporal effects in central activation within mice versus rats has never been assessed.

There are several possible mechanisms for the reduction in cFOS observed within the PVN. The first is the direct inhibition of PVN neurons by other central nuclei, one of which is the BNSTov, which was highly activated in the RF group. The PVN is heavily innervated by the BNSTov (Dong, Petrovich, Watts, & Swanson, 2001) via GABAergic projections (Cullinan, Helmreich, & Watson, 1996; Cullinan, Ziegler, & Herman, 2008). Ablation of the posterior

BNST has been shown to increase cFOS expression and CRH release in the PVN, indicating that the BNSTov plays a significant role in the suppression of these two mechanisms within the neuroendocrine system (D. C. Choi et al., 2006). It is proposed by Cullinan et al. (1996) that this represents a braking mechanism through which afferents of the forebrain limbic system can modulate the stress response, and this agrees with the results achieved in this experiment. While acute food deprivation has been shown to increase the stress response, as animals have no indication of the time or availability of a future meal, repeated exposure to a predictable meal schedule should ideally decrease the stress response due to affective and cognitive anticipation of the predicted availability of food.

The PVN is also directly innervated by both glutamatergic and GABAergic projections from the DMH which are limited to mutually exclusive cells populations (Herman, Tasker, Ziegler, & Cullinan, 2002; Ziegler, Cullinan, & Herman, 2002). Interestingly, even though both excitatory and inhibitory cells are found in the DMH, lesions of the DMH lead to increased HPA axis responses to stressors (Bealer, 1986). Conversely, pharmacological stimulation of DMH neurons produces inhibitory post-synaptic potentials in neuroendocrine cells of the PVN (Boudaba, Szabo, & Tasker, 1996). This is consistent with a strictly inhibitory role of the DMH on the PVN but implies an ability of the DMH outputs to switch from inhibitory to excitatory depending on the intrinsic rhythms of the animal, nocturnal or diurnal, and this has been recently proposed (Kalsbeek, et al., 2010). Without further analysis of the animals in this study, it is impossible to determine precisely which cell populations are activate in the DMH of the RF and RFAL mice. Although the observed suppression of the PVN in the RF condition implicates the GABAergic phenotype, the subsequent dissociation in our RFAL group may or may not depend on the state of the clock gene oscillations within the DMH in the experimental paradigm used here. Nevertheless, the DMH still represents a potential central nucleus involved in sending suppressing GABAergic signals to the PVN during food anticipation.

There is a second mechanism through which activation of the PVN may be reduced. Typically, both direct and indirect signaling from the SCN, via AVP and inhibition of GABAergic DMH projections respectively, increases activation of CRH and AVP positive cells in the PVN. However this mechanism would appear to be transiently disengaged under RFS conditions. As previously discussed, signals from the SCN are temporally suppressed by several mechanisms involved in food anticipation and this may also account for some of the reduced activation of PVN cells at the time of food anticipation.

There is a final mechanism that may be responsible. Inhibitory feedback by circulating glucocorticoids interacts with negative glucocorticoid receptors in the PVN (Kageyama & Suda, 2009). Ghrelin, however, peaks in anticipation of a meal, and both ghrelin and GHSR are expressed within the human adrenal cortex (Ueberberg et al., 2008). Activation of GHSR has been shown to increase the release of glucocorticoids from cultured adrenal cells (Rucinski, Ziolkowska, Tyczewska, & Malendowicz, 2009) and is also implicated in increased ACTH release from the pituitary (Stevanovic, Milosevic, Starcevic, & Severs, 2007). Negative glucocorticoid receptors within the PVN have been shown to decrease both cFOS and CRH expression as a feedback mechanism for regulating the stress response (Jacobson, Sharp, & Dallman, 1990; Kageyama & Suda, 2009). It is possible that in the animals in this study, the increased corticosterone levels observed in anticipation of a meal, due to increases of ACTH by the pituitary, and direct release of corticosterone from the adrenal cortex by GHSR activation, was suppressing PVN activation and resultant CRH expression and release.

Further complicating the observed reduction is the excitation of the PVN directly by catecholamines from the NTS (Plotsky, 1987; Plotsky, Cunningham, & Widmaier, 1989) and 5-HT from the raphe nucleus (Pan & Gilbert, 1992; Van de Kar et al., 2001), both of which were highly activated in the RF condition in direct opposition to the suppression of the PVN.

Activation of the PVN during food anticipation represents direct competition between various central and peripheral signals, all of which act upon the PVN to control the innate reactivity of the HPA axis and modulate the stress response at the time of meal anticipation.

3.2.6 Arousal System:

Since neither large nor orexin-specific ablations of the lateral hypothalamus affect the ability of mice and rats to entrain to a daily meal or to exhibit FAA, activation of the LH was expected only in RF entrained animals. This was confirmed by our analysis of cFOS expression within this nucleus where a sharp increase of activation was observed in the RF group but returned to AL control levels after a return to *ad libitum* feeding. Food anticipatory activity and food entrainment persist after ablations of the lateral hypothalamus, and after specific lesions of the orexin system (Mistlberger, et al., 2003; Mistlberger & Rusak, 1988). Furthermore, the lateral hypothalamus has been implicated as an integral output of the FEO responsible for modulating wakefulness in anticipation of a meal without a role in the entrainment process *per se* (Kaur, et al., 2008; Mieda, et al., 2004). Therefore, activation of this nucleus was expected only in the RF group. This was confirmed by the analysis of cFOS expression within this nucleus, where a sharp increase in activation was observed in the RF group but returned to control levels following *ad libitum* feeding..

The LH is also of particular interest as evidence that this experimental paradigm did not lead to food consumption at the time of previous anticipation. Both increased glucose concentrations and circulating leptin strongly inhibit activity of orexin neurons in the LH (Yamanaka, Beuckmann, et al., 2003). It was expected that cFOS expression in the LH would be suppressed to levels below those of the AL group, whose food consumption was restricted to the dark phase, if food was consumed at or near the time of previous anticipation. Since no such suppression of activation was observed in the RFAL group this adds more weight to the arguments put forth in section 3.2.2.

The locus coeruleus is an additional nucleus integral to the arousal pathways that mediate sleep and wakefulness. Its noradrenergic forebrain connectivity, typically under SCN control by way of an SCN-DMH-LH circuit, is capable of increasing arousal when this nucleus is activated. Since interactions at the SCN-DMH interface become so crucial for the expression of FAA, the LC, an output of this interaction, is a region of interest for the appropriate expression of FAA (Aston-Jones, Chen, Zhu, & Oshinsky, 2001). The orphan nuclear receptor *Ear2* is expressed uniquely in this brainstem nucleus, and genetic ablation of *Ear2* results in a loss of approximately 70% of all neurons within the LC. Furthermore, *Ear2*-deficient mice exhibit reduced FAA as compared to wildtype controls (Warnecke, Oster, Revelli, Alvarez-Bolado, & Eichele, 2005). Given this information, it was expected that the LC should be activated in anticipation of a meal. While this region is implicated in the maintenance of forebrain circadian oscillators, via noradrenergic signaling to the frontal cortex (Aston-Jones, et al., 2001), it does not appear to be necessary for entrainment of central clock genes to food presentation. The lack of continued activation in the RFAL group suggests that the LC, and hence noradrenergic signaling, is not an integral part of the circadian component of the FEO system.

3.2.7 Activation of FEO Correlates in the Brainstem and Hypothalamus:

Observations of increased activation in the brainstem in anticipation of a meal, as well the continued activation of the NTS after a return to *ad libitum* feeding conflicts with the findings of another group (Angeles-Castellanos, et al., 2005). In this study, the authors maintain that there was no anticipatory cFOS expression observed in the NTS, PB or the area postrema (AP) as compared to *ad libitum* fed controls, and activation was only observed after the feeding bout. While it was true that there was increased activation (almost four-fold) observed after feeding in all three of these brain stem nuclei related to visceral signals of satiety, a closer inspection of their results 1 hour prior to the expected presentation of food showed apparent increases in activation of the medial NTS during food entrainment and the medial PB during fasting after entrainment.

These time points and nuclei correspond directly with the RF group and the RFAL group of this study. These changes appeared statistically significant, although this was not tested in their study, but were much smaller in magnitude to the drastically increased activation observed after feeding. Furthermore, this study used a single 40 μ m brain slice for cell counting in each region of interest. By comparison, this study performed a stereological investigation of each region creating an estimate of the activation in its entirety and was therefore much more likely to observe small but significant changes both in anticipation of the meal (RF) and at the time of previous anticipation (RFAL).

Interestingly, there is evidence that local delivery of ghrelin to the caudal brainstem, when injected into the fourth ventricle, increases cFOS expression within the NTS but not the ARC or PVN, and this marked increase in activation occurs in the non-catecholaminergic cell population (Faulconbridge, Grill, Kaplan, & Daniels, 2008). Since forebrain delivery of ghrelin has been shown to activate the ARC, PVN, and several other hypothalamic structures as well as the NTS of the brainstem (Lawrence, Snape, Baudoin, & Luckman, 2002), it would suggest that the NE-independent activation observed after direct application is unidirectional. Whereby, output signals from the ghrelin receptive cells of the NTS only travel towards the periphery and not up to the forebrain. Perhaps this represents a feedback mechanism exerting control on ghrelin secretion.

In this study, several areas remained activated at the time of previous anticipation in the RFAL group. This includes the ARC, DMH, PB, NTS, and DR. This suggests that some, if not all, of these nuclei are involved in a distributed circuit responsible for maintaining circadian oscillations timed to the consumption of a daily meal. Many of these nuclei have been individually implicated in the circadian entrainment to food (Angeles-Castellanos, et al., 2005; Davidson, et al., 2000; Gooley, et al., 2006; Mieda, et al., 2006; Minana-Solis, et al., 2009; Verwey, et al., 2007; Verwey, Lam, & Amir, 2009). However, this is the first demonstration that

activation was increased across all regions at the same time, within the same intact animals, in anticipation of a meal.

Using previous neuroanatomical investigations, it is possible to map out a direct circuit from the NTS to the PB of the hindbrain, then to the ARC and DMH of the forebrain and finally back to the DR of the midbrain with many reciprocal and cross connections between these nuclei in both rats and mice (Azmitia & Segal, 1978; Bouret, et al., 2004; Dietrich & Horvath, 2009; Kita & Oomura, 1982; Ricardo & Koh, 1978; Thompson & Swanson, 1998). If each of these nuclei contains a molecular clock entrained to some particular aspect of food anticipation or consumption, sensitive to shared but potentially different zeitgebers, this would explain why ablation of a single nucleus within this pathway does little to prevent entrainment to food, even if it is capable of altering the FAA response. It also suggests that entrainment would be spared even upon removal of a single zeitgeber, for instance ghrelin, such that rhythmicity is maintained even when one or more nuclei are not receiving the appropriate entraining signals. This is not a new conceptual model of the FEO and has been proposed several times, gaining acceptance as more observations of the character and loci of FEO function are investigated (Carneiro & Araujo, 2009; C. Escobar, et al., 2009; Verwey & Amir, 2009). However, strengthening of this theory requires the demonstration of clock gene oscillations within each of the nuclei highlighted after a return to *ad libitum* feeding using the paradigm described in this experiment.

4 The Role of Ghrelin in Engaging the FEO

4.1 Results

4.1.1 Behaviour

During behavioural recording, there were no body weight difference observed between groups for the baseline ($F_{(1,18)} = 1.580, p = 0.225$), entrainment ($F_{(1,18)} = 1.991, p = 0.176$), *ad libitum* ($F_{(1,18)} = 0.625, p = 0.441$) or fasting conditions ($F_{(1,18)} = 0.594, p = 0.452$) (**Figure 11A**). Furthermore, total food consumption was identical between groups during baseline ($F_{(1,18)} = 0.013, p = 0.911$), entrainment ($F_{(1,18)} = 0.771, p = 0.682$), and *ad libitum* conditions ($F_{(1,18)} = 0.061, p = 0.817$) (**Figure 11B**).

Using repeated measures ANOVAs for analysis (see **Figure 11F**), there was no difference in FAA observed across day ($F_{(2,30)} = 2.747, p = 0.101$), using the Greenhouse-Geisser criteria, or between groups ($F_{(1,15)} = 2.667, p = 0.123$) for the baseline condition. During the last seven days of entrainment, there was a significant difference in FAA across days ($F_{(6,90)} = 3.478, p = 0.019$), using the Greenhouse-Geisser criteria, with a reduction in FAA of the GHSRKO group ($F_{(1,15)} = 9.779, p = 0.007$) as compared to wildtype controls. This reduction continued into *ad libitum* feeding ($F_{(1,15)} = 6.551, p = 0.022$) but disappeared in our fasting condition ($F_{(1,15)} = 2.280, p = 0.152$). A post hoc t-test performed for the last day of fasting revealed a trend towards group differences ($p = 0.08$)*. In order to test whether these observed differences were due to increased FAA or simply an increase in total daily activity, a ratio between FAA and total activity was calculated. Comparisons of the daily average for each groups showed a trend towards a difference during the entrainment condition ($t = -2.066, p = 0.057$)* but not during fasting (**Figure 11D**).

Figure 11: Behavioural analysis of GHSRKO mice. The attenuation of food anticipatory activity seen in GHSR $-/-$ as compared to WT mice applies only to the restricted feeding period and is not observed after a 42 hour fast, post-entrainment **A**, Average body weight for each behavioural condition, no genotype differences were observed **B**, Total daily food consumption over experimental days, no genotype differences were observed. **C**, Average ambulatory activity for FAA (ZT 2-4) during each behavioural condition. There is a significant effect of genotype during entrainment ($t = -3.127$, $p = 0.007$) and ad lib feeding ($t = -2.560$, $p = 0.022$) but not baseline ($t = -1.633$, $p = 0.123$) or fast ($t = -1.510$, $p = 0.152$). **D**, Average ratio of food anticipatory activity over total daily activity ($\times 100$) for the entrainment and fasting conditions. Interestingly only the entrainment period showed a trend towards genotype differences ($t = -2.066$, $p = 0.057$), **E**, Average waveforms of ambulatory activity over the last seven days of entrainment. **F**, daily food anticipatory activity (ZT 2-4) over the entire experiment. * represents all significant differences between conditions. ** represents all significant genotype differences. # represents a statistical trend. Shaded area represents food restriction, RF with presentation at ZT 4, and F with total food deprivation.

4.1.2 *cFOS-positive Cell Counts*

One way ANOVAs revealed significant attenuation of cFOS expression in anticipation of a timed meal within three of the hypothalamic nuclei investigated (**Figure 12**) including: the PVN ($F_{(1,10)} = 6.505, p = 0.031$), DMH ($F_{(1,10)} = 11.243, p = 0.008$), and LH ($F_{(1,10)} = 5.159, p = 0.049$). Differences were also observed in the VTA ($F_{(1,10)} = 12.844, p = 0.012$) and a trend was noted in the AcbSH ($F_{(1,10)} = 4.590, p = 0.065$) of the reward system. However there were no changes detected in the SCN ($F_{(1,10)} = 0.579, p = 0.466$), ARC ($F_{(1,10)} = 2.762, p = 0.135$), AcbC ($F_{(1,10)} = 1.895, p = 0.206$) or Cpu ($F_{(1,10)} = 0.350, p = 0.570$). See **Figure 15** for representative images and associated brain maps of key hypothalamic regions.

Figure 12: Cell counts of cFOS positive cells in GHSR KO and WT mice. Top, hypothalamic nuclei involved in food entrainment. **Bottom,** appetitive nuclei involved in food reward. Stars represent significant group differences ($p < 0.05$). Note that the AcbSH exhibits a trend towards significance ($p = 0.065$)

4.2 Discussion

In this study, the role of ghrelin in the expression of behaviour appropriate for the anticipation of a timed meal was examined. As previously discussed and demonstrated in the first study, ghrelin levels were increased at the time of meal anticipation under a restricted feeding schedule but quickly subsided after a return to *ad libitum* feeding.

The findings of this second study suggest that activation of GHSR in central, and possibly peripheral, nuclei is necessary for the full expression of FAA and the activation of several limbic and hypothalamic nuclei involved in the appetitive and homeostatic mechanisms driving food consumption and food seeking behaviour.

4.2.1 Behavioural Analysis Reveals Ghrelin-dependent FAA

Behavioural analysis of entrainment to a fixed meal and the expression of FAA in GHSR KO mice revealed marked differences in the level of locomotor activity during food anticipation (**Figure 11**). Furthermore, these differences were not due to abnormalities in basal locomotor activity, as both baseline and nocturnal activity during entrainment were comparable in GHSRKO and WT mice. Once placed back on an *ad libitum* diet, FAA quickly subsided to levels just slightly above baseline, but differences were still apparent between phenotypes. This indicates differing levels of daytime arousal as FAA is extinguished and a switch between food-entrained and light-entrained rhythms occurs, with GHSR KO mice showing a quicker resetting of nocturnality. Application of a 36 hour fast over two cycles of daily clock oscillations revealed that differences in FAA observed during entrainment tend to be conserved after the animal re-establishes the appropriate expression of FAA typically observed in this paradigm.

While it would appear from this study that GHSR KO mice take longer to exhibit FAA than their wildtype counterparts, this is not the case. Analysis of a previous experiment using running wheels instead of locomotor activity boxes, which reduces noise during the inactive phase and is a much better measure of the motivational aspects of locomotor activity, revealed

that both phenotypes show significant FAA at or around day three of entrainment (Blum, et al., 2009).

Together, this information suggests that while activation of GHSR is important for the full expression of FAA, it is not critical to the entrainment processes of the FEO, and therefore is not a probable zeitgeber for food entrainment. Taking the findings from the first study and the discussion in section 3.2.7, it becomes apparent that this study cannot completely rule out the possibility of ghrelin being a zeitgeber since the distributed and varied nature of the FEO may allow for entrainment to occur even if one particular zeitgeber or brain region is removed. FEO entrainment is thereby protected by a distributed and interconnected system of clocks receiving various signals derived from food anticipation and consumption. This simultaneously removes the ability to concretely discuss the possibility of ghrelin as a zeitgeber but also highlights its role in the expression of FAA. For a single genetic ablation to have such a marked effect on the expression of FAA, it must be critical to the process. Only the genetic ablation of the orexin and melanocortin systems have been successful in reducing FAA, with behavioural expression following patterns similar to that of our own study (Akiyama, et al., 2004; Mieda, et al., 2004; Sutton et al., 2010; Sutton, et al., 2008).

4.2.2 Differential cFOS activation in hypothalamic and reward pathways

Ghrelin receptors are expressed in various central nuclei including the DR, VTA, hippocampus, ARC, PVN, and LH (Guan et al., 1997). Ghrelin binding has been confirmed in these hypothalamic nuclei, where it is observed to occur on presynaptic terminal boutons (Cowley et al., 2003), indicating a potential role for ghrelin in the modulation of neurotransmitter release or reuptake.

Differential expression of cFOS activation occurred in several of the nuclei examined including the PVN, DMH, LH, and VTA and with an apparent trend observed in the AcbSH. In all cases, GHSRKO mice exhibited a reduction of activation in these areas. This is consistent with

cFOS expression observed in these nuclei after peripheral injections of ghrelin or synthetic ghrelin analogues (Blum, et al., 2009; Kobelt, et al., 2008; Lawrence, et al., 2002). Furthermore, these studies along with others (Carlini et al., 2004) implicate activation of both the NTS and the DR in a ghrelin-dependent manner.

The behavioural results observed here implicate at least one of these nuclei in the reduced generation of anticipatory activity. In fact, it is more likely that they were all activated independently and in parallel to enhance food anticipation through various output mechanisms. Of particular interest is the reduction of activation observed in the DMH as it is implicated as a functional locus for the FEO (recall the discussion of the DMH as a putative FEO in section 1.4.5 and revisited in section 3.2.5). However, the failure to localize GHSR to this region implies that ghrelin is having an indirect effect via some other afferent nuclei, most likely melanocortin signaling from the ARC (Cone, 2006; Kobelt, et al., 2008; Riediger et al., 2003) which is sensitive to GHSR activation as observed in this study. This suggests that, at least for the food-entrained clockwork found in the DMH, ghrelin is not a zeitgeber. Furthermore, recent evidence shows that melanocortin 3 receptor (MC3r) knockout mice also display an attenuated FAA responses and alterations in clock protein expression in the cortex (Sutton, et al., 2008). MC3r expression is also necessary for metabolic adaptations to RF, and MC3r knockout mice showed severe deficits in liver clock entrainment to a scheduled meal (Sutton, et al., 2010). This once again implicates the ARC, the DMH, and the PVN (which also expresses MC3r) as important for the appropriate expression of FAA and various metabolic functions necessary for adaptation to a scheduled meal. The present study now suggests a direct involvement of ghrelin signaling in these critical processes. This signaling may be occurring directly at the ARC, but it is also likely to be occurring via ascending noradrenergic pathways as peripheral ghrelin stimulates cells in the PB and NTS (Angeles-Castellanos, et al., 2005; Davidson, et al., 2000).

Here, a decrease in PVN cFOS activation in the GHSR knockout mice as compared to the wildtype animals was observed. This was expected as there are previous reports of increased cFOS activation in the PVN after peripheral ghrelin administration (Blum, et al., 2009; Kobelt, et al., 2008), and the elevated levels of preprandial ghrelin in entrained mice (RF) should produce the same pattern of activation.

If the proposed feedback mechanism (discussed in section 3.2.5.2) was dependent upon release of glucocorticoids from the adrenal cortex due to GHSR activation by ghrelin, then an observed increase in PVN cFOS in our GHSR knockouts should be expected, due to reduced ghrelin-dependent glucocorticoid release. However, this was not the case. One possible explanation is the differential development and subsequent tuning of the HPA axis in GHSRKO mice. Ghrelin has been shown to produce anxiolytic and anti-depressant like properties, protecting animals from chronic stress, and this protective effect is abolished in GHSR KO mice (Lutter et al., 2008). Furthermore, GHSR KO mice do not show the typical metabolic responses to unpredictable stressors and display vulnerability to stress-induced pathologies (Patterson, Ducharme, Anisman, & Abizaid, 2010). From these studies, and the one presented here, it is clear that ghrelin has a role in the activation of the PVN. What is unclear is exactly where this effect is occurring, whether it is due to direct activation of GHSR receptors within the PVN, the pituitary, the adrenal cortex, or even from other forebrain structures responsible for controlling appropriate responses to predictable stressors such as those involved in food anticipation.

Here, the findings of decreased activation within the LH were significant. Orexin expression within this nucleus has been directly implicated in the arousal necessary for the appropriate expression of FAA (Akiyama, et al., 2004; Harris, Wimmer, & Aston-Jones, 2005; Kaur, et al., 2008; Mieda, et al., 2004; Mistlberger, et al., 2003). This study presents the first evidence that this is modulated by activation of GHSR and whether this occurs directly or indirectly remains to be investigated.

With the hypothalamic effects of GHSR knock out detailed above, a discussion concerning the role of ghrelin in the dopaminergic reward system in anticipation of a meal is necessary (**Figure 12**). Not surprisingly activation of the VTA was reduced in our GHSR KO mice, as previous reports indicate that ghrelin signaling in this nucleus is important for mediating dopamine release to the nucleus accumbens and feeding behaviours (Abizaid, et al., 2006; Naleid, et al., 2005). Of particular note in this study was the preferential decrease in activation of the shell but not the core of the accumbens due to a lack of GHSR signalling. This is most likely due to differences in the inputs, outputs, and function of these two spatially proximal but functionally different nuclei. The shell interacts with limbic system processing of appetitive aspects of food from reciprocal connections with the VTA, amygdala, and LH, as well as direct input from noradrenergic and serotonergic brainstem nuclei (Berthoud, 2007). It was not surprising therefore to observe ghrelin-dependent activation of this nucleus as many, if not all, of its inputs are sensitive to activation by ghrelin. The core on the other hand, receives cortical input and projects to the motor system in the SNC and the caudate putamen (Heimer, et al., 1991). It represents a central output of anticipatory locomotor activity and the finely tuned generation of appropriate patterns of food-seeking locomotor behaviour. The lack of differential activation in both the NAcB and the putamen indicate that ghrelin is not affecting the locomotor outputs of the FEO. Therefore, the reduced FAA in GHSR KO mice observed here is likely due to reduced appetitive drives and/or suppressed arousal in these animals.

5 Conclusions

This study highlights multiple brain regions, spanning a large rostral to dorsal area, including the DMH, ARC, DR, PB, and NTS, each activated in an anticipatory manner even after a return to *ad libitum* feeding. Each of these brain regions has previously been shown to exhibit alterations in clock gene expression under RFS and further examination of these areas is warranted, using the experimental paradigm employed by this study, to ensure that clock gene oscillations remain entrained to the presentation of a meal even after the removal of food restriction and a return to *ad libitum* feeding. Rhythmicity of certain nuclei projecting to these areas should also be investigated in order to determine if cFOS activation is due to internal or incoming signals to these nuclei.

This study furthers the search for an integrated neural system tasked with the circadian entrainment to daily meals (the FEO) and presents an experimental paradigm which has been shown to reduce all ancillary central activation due to non-circadian components of food anticipation and FAA.

Furthermore, a role for ghrelin has been established in the appropriate expression of FAA through the activation of several hypothalamic and limbic nuclei, including the PVN, DMH, LH, NAcSh, and VTA, in response to metabolic challenge and/or as anticipatory output of the FEO, while simultaneously eliminating it as the entraining signal of the central FEO.

6 References

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7 Appendix A: Supplemental Tables and Figures

Table 1: Regions of interest for stereological analysis of cFOS positive cell densities in CD1 mice

Region of Interest	Abbr.	Grid Size	<i>Stereotaxic Coordinates</i>
Arcuate Nucleus	ARC	100µm x 100 µm	1.22mm – 2.46mm posterior
Basolateral Amygdala	BLA	100µm x 200 µm	1.06mm – 1.94mm posterior
Oval Nucleus of the Bed Nucleus of the Stria Terminalis	BNSTov	100µm x 100 µm	0.38mm – 0.14mm anterior*
Central Amygdala	CeA	100µm x 200 µm	1.2mm – 1.70mm posterior
Cingulate Gyrus	Cg1	100µm x 200 µm	2.34mm – 0.22mm anterior
Dentate Gyrus	DG	100µm x 100 µm	1.34mm – 2.46mm posterior
Dorsomedial Hypothalamus	DMH	100µm x 200 µm	1.46mm – 2.18mm posterior
Dorsal Raphé	DR	100µm x 200 µm	4.04mm – 4.96mm posterior
Locus Coeruleus	LC	100µm x 100 µm	5.34mm – 5.80mm posterior
Lateral Hypothalamus	LH	100µm x 200 µm	0.34mm – 2.80mm posterior
Nucleus Accumbens Core	NAcbC	100µm x 200 µm	1.94mm – 0.74mm anterior
Nucleus Accumbens Shell	NAcbSH	100µm x 200 µm	1.94mm – 0.74mm anterior
Nucleus of the Tractus Solaris	NTS	100µm x 100 µm	6.42mm – 7.92mm posterior
Parabrachial Nucleus	PB	100µm x 100 µm	5.02mm – 5.68mm posterior
Prefrontal Cortex	PFC	100µm x 200 µm	3.08mm – 1.54mm anterior
Paraventricular Hypothalamic Nucleus	PVN	100µm x 100 µm	0.58mm – 1.22mm posterior
Suprachiasmatic Nucleus	SCN	100µm x 100 µm	0.22mm – 0.82mm posterior
Ventromedial Hypothalamus	VMH	100µm x 200 µm	1.06mm – 2.06mm posterior
Ventral Tegmental Area	VTA	100µm x 200 µm	2.92mm – 3.88mm posterior

All coordinates displayed relative to bregma according to *The Mouse Brain in Stereotaxic Coordinates* (Paxinos & Franklin, 2001) except for * taken from *Basic organization of projections from the oval and fusiform nuclei of the bed nuclei of the stria terminalis in adult rat brain* (Dong, et al., 2001). The counting frame was kept at 100µm² for all regions and the grid size was altered based on initial parameter estimates.

Table 2: Regions of Interest for cFOS positive cell counts of GHSR KO and WT mice

Region Of Interest	Abbr.	<i>Stereotaxic Coordinates</i>
Arcuate Nucleus	ARC	1.22mm – 2.46mm posterior
Caudate Putamen	CPu	1.94mm – 0.34mm anterior
Dorsomedial Hypothalamus	DMH	1.46mm – 2.18mm posterior
Lateral Hypothalamus	LH	0.34mm – 2.80mm posterior
Nucleus Accumbens Core	NAcbC	1.94mm – 0.74mm anterior
Nucleus Accumbens Shell	NAcbSH	1.94mm – 0.74mm anterior
Paraventricular Hypothalamic Nucleus	PVN	0.58mm – 1.22mm posterior
Suprachiasmatic Nucleus	SCN	0.22mm – 0.82mm posterior
Ventral Tegmental Area	VTA	2.92mm – 3.88mm posterior

All coordinates displayed relative to bregma according to *The Mouse Brain in Stereotaxic Coordinates* (Paxinos & Franklin, 2001)

Figure 13: Representative images showing cFOS expression in key nuclei involved in the circadian component of FAA. Note the increased cFOS expression in the RF group as compared to AL controls and that this increase is maintained even after FAA is abolished.

Figure 14: Representative images showing suppression of cFOS expression in the PVN and SCN. Notice that cFOS appears preferentially suppressed in the core nucleus of the SCN but not the shell, whereas the PVN is suppressed in both the parvocellular and magnocellular nuclei.

Figure 15: Representative images showing decreased cFOS expression in key hypothalamic nuclei of GHSR KO mice in anticipation of a meal.

8 Appendix B: Ethics Protocol Approval

Carleton University
Animal Care Committee

Ethics Clearance Form

This is to certify that the Carleton University Animal Care Committee has examined the application for ethical clearance. The committee found the research project to meet appropriate ethical standards as outlined in the *CCAC Guide to the Care and Use of Experimental Animals* and policies set up by the *Tri-Council, Ethical Review of Research Involving Animals*.

New clearance

X Renewal of original clearance

Original date of clearance: October 26, 2006

Researcher: Alfonso Abizaid

Department: Neuroscience (Psychology)

Title of Project: Modulation of Food Reward by Ghrelin (P06-15)

Date of Clearance: October 22, 2009

Next Renewal Date: n/a

Expiry Date: October 31, 2010

Funding Link: NSERC

All researchers are governed by the following conditions:

Annual Review Form: You are required to submit an Annual Review Form to the Animal Care Committee, at least one month prior to the expiry date. Failure to submit the required document will result in the immediate suspension of the project by Research Services. Funded projects will have accounts suspended until the report is submitted and approved.

Amendments: Any changes to the project must be submitted to the Animal Care Committee on an Amendment Form. All changes must be approved prior to the continuance of the research.

Suspension or termination of approval: Failure to conduct the research in accordance with the principles of the *CCAC* and the *Carleton University Animal Care Committee* may result in the suspension or termination of the research project.

A handwritten signature in black ink, appearing to read 'Matthew Holahan', with a long, sweeping horizontal line extending to the right.

Chair of the Carleton University Animal Care Committee,
Dr. Matthew Holahan