The Food Entrainable Oscillator The Ghrelin and Growth Hormone Secretagogue Receptor Story

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Abstract

Food availability is a potent external cue that can uncouple endogenous rhythmicity from the master circadian oscillator. It is driven by a largely unknown entity that encompasses a network of peripheral and central systems, the food entrainable oscillator (FEO). The orexigenic gut hormone ghrelin is associated with feeding, energy metabolism and has been proposed as an entrainment signal for the FEO. This thesis set out to manipulate the FEO through scheduled feeding paradigms while studying the influence of ghrelin on FEO outputs. Energy utilization patterns in response to a scheduled meal, as examined through indirect calorimetry, exhibited entrainment to the meal timing. We further demonstrated that a restricted feeding paradigm entrained the energy utilization patterns, independently of the GHSR and circulating ghrelin. The influence of scheduled meals on the FEO was not limited to negative energy balance, freely fed CD-1 male mice exhibited food anticipatory activity (FAA) and entrained to a scheduled snack of cookie dough during their non-active phase. Intriguingly, the development and entrainment of FAA is dependent on both the type of scheduled snack and the strain of the animal model used. Circulating ghrelin levels were not necessary for the development or maintenance of the entrainment of FAA. However, central signaling of ghrelin, through its growth hormone secretagogue receptor (GHSR) may have a role in the development of the FAA in paradigms of scheduled treats. Taken together these data present evidence that ghrelin and the GHSR are not critical in mediating the systems of the FEO and its respective outputs, under various feeding paradigms.

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I dedicate this work to my Mama.
Who has sacrificed her dreams, just so I can dream without limits.
Thank You.

List of Abbreviations

α -MSH: α -melanocyte stimulating hormone, ***adlib*:** Ad libitum, **ACTH:** Adrenocorticotropin, **AGRP:** Agouti gene-related protein, **AP:** Area postrema, **ARC:** arcuate nucleus, **AVP:** Arginine vasopressin, ***Bmal1*:** Brain and Muscle-Arnt-Like 1, **BAT:** Brown adipose tissue, **BNST:** Bed nucleus of the stria terminalis, **CaBP:** calcium-binding protein calbindin-D_{28K}, **CAR:** Calretinin, **CART:** Cocaine-and amphetamine-regulated transcript, **CK1 δ :** Casein kinase 1 delta, **CKI ϵ :** Casein kinase 1 epsilon, **CCK:** cholecystokinin, ***Clock*:** Circadian Locomotor Output Cycles Kaput, **CPP:** conditioned place preference, **CRH:** Corticotrophin releasing hormone, **Cry1:** Cryptochrome 1, **Cry2 :** Cryptochrome 2, **Cyp2a5:** Cytochrome p450 2A5, **DMH:** Dorsomedial hypothalamus, **FAA:** Food anticipatory activity, **FEO:** Food entrainable oscillator, **GHSR:** Growth hormone secretagogue receptors, **GHSR-KO:**GHSR knockout, **GHSR-WT:** GHSR wildtype, **GLP1:** Glucagon-like peptide 1, **GOAT :** Ghrelin O-acyltransferase, **GRP:** Gastrin-releasing peptide, ***ghrl*-/- :** Ghrelin-deficient mice, **HF:** high fat diet, **IGL:** Intergeniculate leaflet, **IP:** Intraperitoneal injection, **icv.:** Intracerebroventricular, **LH:** lateral hypothalamus, **MCH:** melanin concentrating hormone, **NAc:** Nucleus accumbens, **NTS:** nucleus of the solitary tract, **NT:** neurotensin, **NPY:** Neuropeptide Y, **OB-R_L:** Long form leptin receptor, **OB-R_s:** Short form leptin receptor, **ORX:** Orexin, **POA:** Preoptic area, **PAS:** Period-Arnt-Single-Minded, ***Per1*:**Period1, ***Per2*:** Period 2, ***Per3*:** Period, **PBN** Parabrachial nuclei, **PR:** Progressive ratio, **POMC:** pro-opiomelanocortin, **PFC:** pre-frontal cortex **PVN:** Paraventricular nucleus of the hypothalamus, **PVN-SCG:** Paraventricular nucleus-superior cervical ganglia, **P YY₃₋₃₆:** peptide YY₃₋₃₆, **Rev-erb α :**reverse erythroblastosis virus alpha, **RGC:** Retinal ganglion cells, **RER :**Respiratory exchange ratio, **Ror α – ROR γ :** Retinoic acid-related orphan nuclear receptors, **SCN** :Suprachiasmatic nucleus, **sPVZ:** Subparaventricular zone, **SON :** Supraoptic nucleus, **TRH:** Thyrotropin releasing hormone, **VMH:** Ventromedial hypothalamus, **VIP** Vasoactive intestinal polypeptide, **VTa:** Ventral tegmental area.

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Chapter 1

1.1. Circadian Rhythms

All living organisms exhibit daily rhythms in behaviour and physiology, known as circadian rhythms, and these have evolved to synchronize physiological processes and behaviours with daily changes in light/dark cycles. In mammals, circadian rhythms coordinate processes such as feeding, energy metabolism, hormone release, body temperature, and sleep-wake cycles (reviewed in Froy, 2010).

These rhythms can be generated in the absence of environmental cues. In a seminal set of human experiments, participants placed in an underground bunker that was devoid of all external timing cues continued to display periods of activity and rest that ranged from 25 hours to 27 hours (Wirz-Justice et al., 2005). Daily environmental cues, however, reset the clock to rhythms close to 24 hours in duration via a process called entrainment (reviewed by Challet & Mendoza, 2010; Colwell, 2011). Light is the principal external cue that coordinates the body's circadian clock with its environment, known as a *zeitgeber*, German for “time-givers”. In the absence of a *zeitgeber*, organisms show a free-running rhythm that is approximately a day in length. Organisms have evolved to predict and entrain to the cycles of light-dark and modify their behaviours and physiological processes accordingly (Panda, Hogenesch & Kay, 2002).

While light may be the primary *zeitgeber* influencing circadian rhythmicity; it is not the only one. Non-photoc cues such as food availability can be a potent *zeitgeber*. Mammalian foraging behaviors are dependent on environmental cues that signify food availability (Mistlberger, 1993). The earliest recorded observations of timed food behavior were from the early 1900's from Hugo Berthold von Buttel-Reepen and August Forel describing the food-seeking behavior of bees at specific

times during the day. Buttel-Reepen observed that bees appeared in the morning when the buckwheat blossoms were open and subsequently theorized that bees had a foraging *Zietsinn* (time-sense). Swiss physician August Forel termed the behaviour as *Zeitgedächtnis* (time-memory) after observing that bees consistently appeared when he had breakfast on the patio and continued to appear even when he was indoors (Carnerio & Araujo, 2012)

The pivotal study on mammalian food seeking behavior by Richter 1922 reporting that rats housed under constant light increased their activity 2-3hrs prior to meal presentation, was the first to feature the relationship between rat activity and feeding times. Since then, a variety of different food restriction paradigms have been developed and utilized to explore this phenomenon. The main oscillator within the central nervous system largely conducts the orchestration of the circadian system (Carnerio & Araujo, 2012)

1.2. The Organization of the Biological Clock.

In mammals, the master clock that synchronizes circadian rhythms is situated within the brain, in the suprachiasmatic nuclei (SCN), bilateral hypothalamic nucleus flanking the bottom of the third ventricle, just above the optic chiasm. The SCN's role in circadian function was revealed by studies conducted in the 1970's showing that lesions of the SCN resulted in the loss of behavioural, hormonal and physiological circadian rhythms (Moore & Eichler 1972; Stephan & Zucker 1972; Raisman & Brown-Grant 1977; van den pol & Powlety 1979).

Consistent with the fact that light is the primary *zeitgeber* for circadian rhythmicity, one of the principal inputs to the SCN comes from the retina via the retinohypothalamic tract (Moore & Lenn 1972; Hendrickson et al., 1972). These retinal inputs arise primarily from retinal ganglion cells (RGC) that produce a photopigment called melanopsin. Their major target is the ventrolateral

portion of the SCN also known as the “core”. The SCN receives additional indirect photic information from the intergeniculate leaflet (IGL) a small band of cells located on top of the lateral geniculate, which is part of the geniculohypothalamic tract. SCN efferents emanate from cells in the dorsomedial portion of the SCN, a region termed the “shell” (reviewed in Antle and Silver, 2005).

The two anatomical subdivisions of the SCN, the ventral “core” and the dorsal “shell”, are also distinguished by their neurochemical content (Leak, Card & Moore, 1999; Abrahamson & Moore, 2001). Neurons in the SCN core are GABAergic and also predominantly express vasoactive intestinal polypeptide (VIP), gastrin-releasing peptide (GRP) and neurotensin (NT) (Moore, Speh & Leak, 2002). The shell also contains GABAergic neurons, however, unlike the core these cells are also arginine vasopressin (AVP) and calretinin (CAR) positive (Moore, Speh, & Leak, 2002). Trans-synaptic labeling of neuronal circuits has shown that while the shell receives dense projections from the core, there are only sparse projections from shell to core (Leak *et al.*, 1999). In addition to receiving projections from the SCN core, the shell receives inputs from the basal forebrain, the cerebral cortex, hippocampus, the cholinergic nuclei of the brain stem, and other hypothalamic nuclei including the arcuate nucleus (ARC) (Yi, van der Viet, Dai, Yin et al., 2006) paraventricular nucleus of the hypothalamus (PVN), dorsomedial hypothalamus (DMH) and the subparaventricular zone (sPVZ) (Bujis, Hou, Shinn & Renaud, 1994).

In rodents, bilateral electrolytic lesions of the SCN abolished the circadian rhythmicity of locomotor activity (Stephan & Zucker, 1972) and endocrine outputs (Moore & Eichler, 1972). Partial lesions of SCN left the locomotor component of circadian rhythmicity intact, albeit weak and variable (Eastman, Mistlberger, Rechtschaffen, 1984). Partial restoration of circadian rhythms of locomotor behavior can be achieved following implantation of fetal SCN brain grafts into the 3rd

ventricle of arrhythmic hamsters with SCN lesions (Lehman, Silver, Gladstone, Kahn et al., 1987). Transplantation of mouse fetal SCN tissue in arrhythmic mice (*Clock* mutant mice and *mCry1/mCry2* double knockout mice) restored circadian behavioural rhythmicity (Sujino, Masumoto, Yamaguchi, van der Horst, et al., 2003).

In hamsters, specific lesions of the SCN targeting calcium-binding protein calbindin-D_{28K} (CaBP)-positive cells in the core, but sparing the rest of the SCN, eliminated circadian locomotor rhythms (LeSauter & Silver, 1999). Furthermore, this group also reported that SCN grafts needed to contain CaBP cells to restore locomotor rhythms in SCN-lesioned host animals. These studies demonstrated heterogeneity and specificity of the neuronal population of the SCN.

The SCN conveys timing information to the rest of the brain and periphery mainly through its efferent projections (Figure 1.2). Extra-SCN targets in turn communicate with oscillators throughout the brain and the body. The SCN's influence as the master pacemaker is established through its output into the sPVZ that serves to amplify the SCN's signal. The ventral part of the sPVZ is directly above the SCN and is implicated in the circadian rhythms of sleep, wakefulness and locomotor activity. Whereas the dorsal region of the sPVZ, found below the PVN drives circadian rhythms of body temperature (Saper, Scammell & Lu, 2005). In addition to the sPVZ, dense SCN projections terminate in other regions including the preoptic area (POA), the bed nucleus of the stria terminalis (BNST), the DMH, the ARC and the PVN (Berk & Finkelstein, 1981; Stephan, Berkley & Moss, 1981; Watts & Swanson, 1987).

The SCN further influences rhythmicity through various parasympathetic and sympathetic connections (Bartness, Song & Demas, 2001; Buijs, la Fleur, Wortel, van Heyningen et al., 2003; Ishida, Mutoh, Ueyama, Bando et al., 2005). The SCN projects to the autonomic nervous system

through the paraventricular nucleus-superior cervical ganglia (PVN-SCG), a pathway that entrains the submandibular salivary glands (Vujovic, Davidson & Menaker, 2008). This pathway controls the release of corticotrophin releasing hormone (CRH) from the PVN, which regulates the neuroendocrine control of corticosterone secretion from the adrenal cortex (Buijs, Wortel, Van Heerikhuize, Feenstra et al. 1999). In addition to directly influencing the secretion of glucocorticoids, the sympathetic innervation of the SCN to the adrenals modulates their sensitivity to adrenocorticotrophic hormone (ACTH) (Buijs et al., 1999). Sympathetic innervation from the SCN to the liver, via the PVN, modulates the daily rhythms of plasma glucose (Kalsbeek, La Fleur, Heijningen, & Buijs, 2004). Consequently, the SCN does not simply affect hormone secretion; it also regulates the sensitivity of the target organs of those hormones in a tissue dependent manner (Guo, Brewer, Champhekar, Harris & Bittman, 2004).

1.3. Extra-SCN Clocks.

Circadian clocks are not exclusive to the SCN, rather they are present throughout central and peripheral systems. All mammalian cells contain an auto-regulatory negative feedback transcription network (Figure 1.1) that regulates gene expression throughout the organism (Lowrey & Takahashi, 2004). A circadian oscillator prepares the organism to anticipate and respond to the external environment. Guilding and Piggins (2007) identify master circadian pacemakers by four characteristics: (1) circadian pacemakers must present an independent, self-sustaining 24hr rhythm (2) these rhythms should not be influenced by changes in the external temperature (3) the pacemakers should be able to respond and entrain to changes in the external environment (4) the pacemaker should be able to communicate the external signal to tissues downstream.

Pacemakers that do not possess these attributes are categorized as either semi-autonomous oscillators or slave oscillators. Semi-autonomous oscillators are cells that generate autonomous circadian rhythms that are synchronized by the SCN. When disconnected from the SCN, these individual cells will maintain circadian rhythmicity but will fall out of phase from one another. Slave oscillators, on the other hand, generate arrhythmic patterns in isolation and are dependent on the influence of the SCN or semiautonomous pacemakers (Guilding & Piggins, 2007).

Although the SCN is undoubtedly the principal circadian oscillator in the brain, oscillators are found throughout different brain regions including hypothalamic nuclei, the amygdala, and the olfactory bulb. A comprehensive study by Abe and colleagues (2002) examined the oscillations of an important component of the clock mechanism Period1 gene (*Per1*) throughout the brain (Figure 1). The group utilized *Per1-luc* rats that express the firefly luciferase gene under the control of the mouse *Per1* promoter to visualize the rhythmicity of *Per1* in different brain regions. Out of the 27 brain regions they examined, 14 areas expressed rhythmicity. They found that although *Per1* was widely expressed in the mammalian brain, its robustness and pattern or phase of expression was dependent on its location. For example, areas such as the hippocampus expressed high levels of *Per1* but did not express any intrinsic rhythmicity, while areas such as the ARC had lower expression but were shown to have endogenous rhythmicity (Abe, Herzog, Yamazaki, Straume *et al.*, 2002). A table detailing the rhythmicity of different clock genes throughout the brain can be found in the review by Guilding and Piggins (2007). Endogenous circadian oscillators that are present in extra-SCN areas of the brain are not limited to the CNS but are also found throughout the periphery.

In vitro work demonstrated that clock gene expression was present in mammalian fibroblasts (Balsalobre, Damiola & Schibler, 1998). Furthermore, skeletal muscle, lung and liver tissue harvested

from *Per1-luc* rats also exhibit intrinsic circadian clock expression (Yamazaki, Numano, Abe, Hida et al., 2000). *In vivo* studies have shown that circadian rhythms of peripheral tissues are maintained in *mPer2^{Luciferase}* knock in mice with SCN lesions (Yoo, Yamazaki, Lowrey, Shimomura, et al., 2003). Further signifying that although the SCN entrains peripheral oscillators, peripheral tissues also express independent circadian oscillations and may be regulated by organ specific synchronizers.

Although the core genetic machinery of the circadian clock is present within all cells in the periphery, there are multiple differences between peripheral and central circadian clocks. For example, without the influence of the SCN, oscillating peripheral clocks become desynchronized from each other (Yoo, et al., 2003). When tissue specific rhythmicity was examined using engineered mice that have hepatocyte clocks that can be turned on and off, it was discovered that functional hepatocyte clocks are necessary to maintain rhythmic transcription and outputs (Kornmann, Schaad, Bujard, Takahashi & Schibler, 2007).

1.4. The Food Entrainable Oscillator

The importance of food availability as a *zeitgeber* has been demonstrated in studies where laboratory animals of different species have food availability restricted to certain times during the day. Under normal conditions, peripheral clocks are generally in synchrony with the SCN and rodents couple feeding-fasting cycles with cycles of rest-activity. However, when food availability is restricted to a limited time window during the day, the phase of gene expression in the periphery is altered. Ultimately causing the peripheral clocks to fall out of phase with the SCN (Damiola, Le Minh, Preitner & Kornmann, Fleury-Olela, & Schibler, 2000). While the SCN remains phase-locked to the light-dark cycle (Stokkan ,2001), exclusive daytime feeding in rodents reverses the phase of clock genes (*Per1*, *Per2*, *Per3* and *Cry1*) and of clock-controlled genes (*Ddp*, *Rev-erba* and *Cyp2a5*) in the

liver (Damiola, et al., 2000) (Figure 3). Phase advances in the circadian rhythm of gene expression were also observed in the liver, kidney, heart, and the pancreas, ultimately uncoupling them from the SCN's control (Damiola, et al., 2000; Hara, Wan et al. 2001). As these organs play a pivotal role in orchestrating metabolic pathways to enhance food processing, these changes optimize the processing of incoming nutrients.

Entrainment to scheduled meals is reflected in a number of behavioural outputs that are collectively referred to as food anticipatory activity (FAA); general locomotor activity, bar pressing and/or feed hopper approaches in rats living in operant boxes, as well as body temperature and corticosterone secretion are all elevated in anticipation of a scheduled meal (Mistlberger, 1994; Mistlberger, 2009). The entrainment to a scheduled meal, in particular the presentation of FAA, is exhibited in SCN-ablated animals (Stephan, 2002).

The preservation of FAA in the absence of a functioning SCN raised the possibility of an extra-SCN, food entrainable oscillator (FEO). The FEO displays circadian characteristics, in that once FAA is established (for a classic review of FAA models see Mistlberger, 1994), it persists in the absence of the light *zeitgeber*, suggesting that the FEO has the ability to generate and sustain behaviour (Mistlberger, 1994; Stephan, 2002) but within circadian limitations (Luby, Hsu, Shuster, Gallardo et al., 2012).

Restricted feeding schedules not only affect peripheral oscillators, but also alter the expression of clock related genes in a number of brain regions. For instance, both the lateral hypothalamus (LH) and ventromedial hypothalamus (VMH) show increased neuronal activity that is entrained to the time of scheduled feeding (Kurumiya & Kawamura 1991). In mice, entrainment to

feeding schedules is associated with changes in *Per1* and *Per2* expression in the hypothalamus, hippocampus, and prefrontal cortex (Wakamatsu, Pristiyazhnyuk, Kinoshita, Tanaka & Ozato, 2001).

Restricted feeding schedules trigger changes in the periodic fluctuation of macronutrient availability (Woods, 2005), which in turn, might reset or/and induce clock gene expression. Food restriction is not a prerequisite for food entrainment, however, *adlib* fed animals that are also given scheduled exposure to palatable foods such as chocolate, chocolate Ensure®, or cookie dough expressed FAA entrained behavior (Mendoza, Angeles-Castellanos & Escobar, 2005, Verwey, Stewart & Amir, 2007; Angeles-Castellanos, Mendoza, & Escobar, 2007). This suggests that the FEO can be activated in the absence of a negative energy balance.

1.5. The Central Regulation of Energy Metabolism.

Homeostatic feeding is defined as feeding that provides the organism with essential macronutrients (protein, carbohydrates and fats) and micronutrients (vitamins and minerals) and is driven by the regulation of energy balance (Saper, Chou & Elmquist, 2002). In-contrast, non-homeostatic food intake, or *hedonic* food intake, is defined as food intake that occurs when an organism is in relative energy abundance and presumably satiated. Hedonic feeding is driven by a number of factors, including accessibility and palatability (Berthoud, 2006).

Energy homeostasis can be defined as the short-term and long-term regulation of cumulative energy intake and energy expenditure (Woods, Seeley, Porte, & Schwartz, 1998). In rodents, there are a number of major brain regions that are involved in conveying adiposity signals and the regulation of food intake, to maintain a homeostatic balance.

The hypothalamus contains multiple neuronal systems that are major players in food intake and body weight regulation. The hypothalamic systems that regulate both anabolic and catabolic

states are responsive to peripheral metabolic signals such as ghrelin, leptin, insulin, peptide YY₃₋₃₆ (PYY₃₋₃₆), (Batterham Cowley, Small, Herzog, et al., 2002; Abizaid & Horvath, 2008) glucagon-like peptide 1 (GLP1) (Turton, O'Shea, Gunn, Beak et al., 1997) and cholecystokinin (CCK) (Varró, Bu'lock, Williams, & Dockray 1983).

The ARC is anatomically positioned to play a critical role in the integration of signals that facilitate metabolism. Located at the base of the hypothalamus and directly above the median eminence, the ARC senses and responds to an array of circulating hormones (Stanley, Wynne, McGowan & Bloom, 2005). Schwartz *et al.* (2000) have proposed a theory in which the ARC is the primary hypothalamic area on which the peripheral hormones, such as leptin and ghrelin, act. These hormones modulate the activity of ARC neurons. The neurons of the ARC then project to other hypothalamic nuclei, such as the LH (Schwartz, Woods, Porte, Seeley & Baskin, 2000). The ARC neurons express a number of receptors, including growth hormone secretagogue receptors (GHSR) (Guan et al., 1997), leptin receptors (Elmquist, Bjobaek, Ahima, Flier & Saper, 1998) and insulin receptors (Marks, Porte, Stahl, & Baskin 1990). The GHSR is of particular interest; considering its endogenous ligand is the orexigenic gut hormone ghrelin (Kojima, Hosoda, Date, Nakazato et al. 1999), which will be further reviewed in the upcoming sections.

The ARC contains two neuronal populations that coordinate signals of nutritional status and mediate energy homeostasis. The first of these subpopulations of neurons express both pro-opiomelanocortin (POMC) and cocaine-and amphetamine-regulated transcript (CART) (Kristensen, Judge, Thim, Ribel et al., 1998). CART, a satiety factor, is identified as an anorectic peptide that is regulated by leptin (Kristensen, et al. 1998) and indirectly inhibited by ghrelin (de Lartigue, Dimaline, Varro & Dockray, 2007). POMC is cleaved into several peptides including

adrenocorticotropin (ACTH) and α -melanocyte stimulating hormone (α -MSH) a peptide that potently decreases appetite and increases energy expenditure (Millington, 2007). The α -MSH peptide exerts its effects by binding to melanocortin receptors (in particular MC3 and MC4), that in turn suppresses food intake (Wikberg, Muceniece, Mandrika, Prusis et al., 2000). Interestingly, POMC neurons are heterogeneous in responding to metabolic hormones with a subset of its cells responding to leptin and others to insulin (Schwartz, Seeley, Woods, Weigle et al., 1997; Williams, Margatho, Lee, Choi et al., 2010). Ghrelin on the other hand, indirectly inhibits POMC neurons (Cowley, Smith, Diano, Tschöp et al., 2003; Zeltser, Seeley, Tschöp, 2012).

The second subpopulation of neurons in the ARC co-express neuropeptide Y (NPY) and agouti gene-related protein (AGRP) (Hahn, Bregininger, Baskin & Schwartz, 1998), both of which have a potent orexigenic effect and decrease energy expenditure, ultimately promoting adiposity (Stanley, et al. 2005). Significant portions of NPY neurons in the ARC co-express the GHSR (Willesen, Kristenen & Romer, 1999) and both NPY and AGRP mRNA expression in the ARC is upregulated in response to exogenous ghrelin administration (Shintani, Ogawa, Ebhiara, Aizawa-Abe *et al.*, 2001; Nakazato, Murakami, Date, Kojima *et al.*, 2001). Chronic central infusion of ghrelin not only increased NPY and AGRP mRNA levels, but also increased food intake and body weight (Kamegai, Tamura, Shimizu, Ishii *et al.*, 2001). Single intracerebroventricular (i.c.v.) injections of ghrelin were enough to increase NPY mRNA expression and antagonize leptins activity via the NPY/Y1 pathway (Shintani et al., 2011). In contrast, these neurons are inhibited by both insulin and leptin (Schwartz, Sipols, Marks, Sanacora et al., 1992; Spanswick, Smith, Groppi, Logan & Ashford, 1997; Hahn, et al., 1998; Varela & Horvath, 2012). These results show that hypothalamic circuits

are dynamically regulated by peripheral signals at the level of the ARC (Hewson, Tung, Connell, Tookman, & Dickson, 2002; Pinto, Roseberry, Liu, Diano *et al.*, 2004).

Both POMC/CART and NPY/AGRP neurons project to the same downstream targets in the hypothalamus, including the PVN, the LH, the DMH, supraoptic nucleus (SON), the VMH, periventricular nucleus, the amygdala and the NAc (Millington, 2007; Morton, Meek & Schwartz, 2014). Both play a critical role in orchestrating the neurocircuitry of energy homeostasis.

The DMH is another hypothalamic area that has been associated with regulating metabolism and body weight (for review see Bellinger & Bernardis, 2002). Anatomically, the DMH is located adjacent to the third ventricle, dorsal to the VMH and caudal to the PVN. Projections of the DMH densely innervate the PVN, the LH, the preoptic nucleus, while less dense projections innervate the brainstem and telencephalon (Thompson, Canteras & Swanson, 1996). As previously mentioned, the DMH receives input from NPY/AGRP neurons that reside in the ARC, but also contains its own population of NPY neurons (Bellinger *et al.*, 2002; Lee, Kirigiti, Lindsley, Loche *et al.*, 2013).

Leptin (Elmquist, *et al.*, 1998), insulin (Wether, Hogg, Oldfield, McKinley *et al.*, 1987) and ghrelin receptors (Cowley, *et al.*, 2003) are also expressed in the DMH. Intravenous leptin injections induced an increase in Fos activity in the VMH and DMH (Elmquist, Ahima, Elias, Flier & Saper, 1998).

Selective neuronal activation of the DMH leptin receptors increased BAT thermogenesis and locomotor activity, resulting in an increase of energy expenditure and decrease in body weight. In contrast, selective deletion of DMH leptin receptors produced an increase in body weight, a reduction in locomotor activity and a decrease in energy expenditure (Rezai-Zadeh, Yu, Jiang, Laque *et al.* 2014).

Increased Fos immunoreactivity, index of cell-activation, was found in mice in the DMH in both food-restricted paradigms and after chronic IP injections of ghrelin (Blum, Patterson, Khazall, Lamont, et al., 2009; Kobelt, Wissner, Stengel, Goebel et al., 2008). Acute IP injections of ghrelin also increased neural activity in the DMH in Sprague-Dawley rats (Kobelt, et al., 2008). Interestingly, adeno-associated virus knockdown studies of GHSR1a in the DMH had no effect on body weight or food intake but under a restricted feeding paradigm, animals did show a significant decrease in FAA (Merkestein, van Gestel, van der Zwaal, Brans et al., 2014). These results further highlight that the DMH does not work independently to modulate food intake or produce FAA.

The PVN receives dense NPY/AGRP and POMC/CART projections from neurons in the ARC along with projections from orexin neurons in the LH (Elmquist et al., 1998). Neurons in the PVN produce a number of neuropeptides associated with metabolic and neuroendocrine regulation including CRH, thyrotropin releasing hormone (TRH), oxytocin and AVP. The PVN also contains receptors for ghrelin (Zigman, Jones, Lee, Saper & Elmquist, 2006) and leptin (Mercer, Hoggard, Williams, Lawrence *et al.*, 1996). Located caudally to the PVN and directly above the ARC, the VMH is considered to be the “satiety center” of the hypothalamus (Williams, Bing, Cai, Harrold et al., 2001). The VMH also expresses leptin receptors (Mercer *et al.*, 1996) and GHSR (Zigman, et al., 2004) and has direct connections with the PVN, DMH and LH.

Finally, LH controls feeding and body weight regulation and is known as the “feeding center” (Williams, et al., 2001). The LH expresses two distinct neuropeptides, melanin concentrating hormone (MCH) (Bittencourt, Presse, Arias, Peto *et al.*, 1998) and orexin (ORX) (Sakurai, Amemiya, Ishii, Matasuzaki *et al.*, 1998). ORX and MCH cells are responsive to projections from NPY/AGRP and POMC/CART neurons of the ARC (Brobeger, Lecea, Sutcliffe & Hokfelt, 1998),

pathways that are differentially modulated by circulating leptin (Elias, Aschkenasi, Lee, Kelly et al., 1999). GHSR expression is found in the LH (Guan et al., 1997; Zigman et al., 2004) and responds to fluctuations in ghrelin levels (Lawrence, Snape, Baudoin & Luckman, 2001). Interestingly, mice treated with an orexin antagonist and or mice that do not have orexin receptors do not respond to exogenous ghrelin-induced food intake under an *adlib* food paradigm (Toshinai, Date, Murakami, Shimada *et al.*, 2003) nor exogenous ghrelin- induced food intake in reward paradigms (Perello, Sakata, Birnbaum, Chuang & Zigman, 2010).

In addition to hypothalamic regions, extra-hypothalamic regions such as the ventral tegmental area (VTA) and the nucleus accumbens (NAc) are heavily implicated in the role of hedonic or reward based feeding (Alhadeff, Rupprecht & Hayes, 2011). The mesocorticolimbic reward pathway originates in the VTA, where dopaminergic neurons project onto neurons of the NAc, pre-frontal cortex (PFC), the hippocampus, the amygdala, and the hypothalamus, along with several other brain regions (Björklund & Dunnet, 2007; Meye & Adan, 2013). The VTA receives reciprocal projections from the aforementioned areas (Meye & Adan, 2013). Therefore, the VTA is of great interest in the study of the central regulation of hedonic feeding, and ghrelin has been found to impact the organization of dopaminergic neurons and increase motivation to obtain a hedonic treat when administered to this region (Abizaid, Liu, Andrews, Shanabrough et al., 2006; King, Isaacs, O'Farrell & Abizaid, 2011). The VTA also expresses insulin and leptin receptors (Figlewicz, Evans, Murphy, Hoen & Baskin 2003), suggesting that the VTA is responsive to metabolic hormones.

As expected, the maintenance of energy homeostasis requires a myriad of complex interactions between the brain and the peripheral system (Shwartz, et al., 2000).

1.6. The FEO & Central Regulation of Food Intake.

Originally, the FEO was hypothesized to function much like the SCN, in that it was hypothesized to be a single discrete area that orchestrates food intake. Following the SCN lesion studies (Stephan, 2002) a number of research groups began systematically investigating other brain regions and their potential candidacy for the FEO.

The search for the FEO has been dogged by conflicting results, and this is exemplified by studies examining the role of the DMH in FAA. Gooley and colleagues (2006) reported that a targeted full neurotoxic lesion of the DMH significantly decreased FAA rhythms and body temperature (Gooley, Schomer, & Saper, 2006). However, studies using total and partial radiofrequency lesions of the DMH (Landry, Simon, Webb & Mistleberger, 2006; Landry, Yamakawa, Webb, Mear & Mistleberger, 2007; Moriya, Aida, Kudo, Akiyama, Doi, *et al.*, 2009) did not find that the lesions significantly impacted FAA. The discrepancy between results is in no way limited to DMH findings. One study found that lesioning of the brain stem parabrachial nuclei (PBN) did not impact production of FAA (Gooley et al., 2006) while another research group found that the lesions did, in fact, attenuate FAA (Davidson, Cappendijk & Stephan, 2000). The disparities in findings between the research groups have been attributed to overall experimental design, including lesion type and the behavioral measures used to study FAA.

Researchers have continued to investigate the potential “input-output” relationship of FAA and brain regions that are involved in feeding and energy regulation. Targeted lesions and their consequences for FAA have been explored in the PVN, LH (Mistleberger & Rusak, 1988), orexin cell-specific ablation in the LH (Mistleberger Antle, Kilduff, & Jones, 2003), VMH (Mistleberger & Rechtschaffen, 1984), area postrema (AP), nucleus of the solitary tract (NTS) (Davidson, Aragona,

Houpt, & Stephan, 2001), hippocampus, amygdala and NAc (Mistlberger & Mumby, 1992).

NMDA excitotoxic lesions to the NAc core, but not the shell, produced an attenuation of FAA without disturbing the SCN-controlled nocturnal locomotor rhythm, suggesting that the effect of the lesion is specific to the FEO (Mendoza, Angeles-Castellanos, & Escobar, 2005). Davidson (2009) provides an in depth evaluation and review of the above-mentioned lesion studies.

These results shift the archetype theory that FEO resides in single brain region or is regulated by a single structure, the understanding that entrainment to food may very well be regulated by a system that recruits several central and peripheral processes.

1.7. Metabolic Factors of Entrainment

Important modulators of the FEO are hormones that influence metabolism, such as ghrelin, leptin, glucocorticoids, insulin, glycogen, and GLP-1. As mentioned previously, glucocorticoid levels exhibit a clear circadian variation across a 24 hr period and respond to changes in energy requirements through pulsatile rhythmicity (ultradian pulses, that are shorter than a day). The circadian peak of glucocorticoids occur around the time of arousal, just before dark for nocturnal species and just before light in diurnal species (Cheifetz, 1971; Van Cauter, 1990).

Produced in white adipose tissue, leptin has been well established as a potent hormonal regulator of energy balance. This 16kDa polypeptide has number of splice variants of its receptor, the long form known as OB-R_L and the short form OB-R_s (Tartaglia, 1997). Its effects on energy homeostasis are through its binding to its long-form of the receptor, which is found ubiquitously in the body and the hypothalamus (Elmqvist, et al., 1998; Coppari & Bjøbaek, 2012). Leptin's role in metabolism stems from its ability to act upon the hypothalamus, in part through the regulation of NPY and POMC neurons projecting to the LH (Elias, et al., 1999), to ultimately suppress food

intake and stimulate energy expenditure (Meier & Gressner, 2004). Circulating levels of leptin, under normal conditions, are positively correlated with the proportion of adipose tissue present in the organism. Under extreme conditions, such as obesity, leptin levels remains positively correlated with adiposity but the body becomes resistant to its effects after chronic over-exposure.

Leptin levels exhibit a clear circadian rhythm, in both gene expression and protein secretion (Froy, 2007). Plasma leptin levels rise after the onset of the dark phase and gradually drop during the light phase in nocturnal species (Sánchez, Oliver, Pico & Palou, 2004) but interestingly, are not influenced by feeding time (Kalsbeek, Fliers, Romijn, la Fleur, Wortel, Endert & Buijs, 2000; Froy, 2007). Leptin levels are low prior to the scheduled meal and rise after the meal when animals are under a restricted feeding paradigm (Martínez-Merlos, Angeles-Castellanos, Díaz-Muñoz, Aguilar-Roblero et al., 2004). SCN ablated animals have no leptin circadian rhythmicity, suggesting that the SCN is important for daily fluctuations leptin secretion (Kalsbeek et al., 2000). Furthermore, leptin insensitive *ob/ob* mice exhibited an amplified FAA that was dampened by leptin administration (Riberio, Ceccarini, Dupré, Friedman et al., 2011). Taken together this would signify that leptin may not be an essential hormonal cue for the FEO, but may play a supporting role.

1.8. Metabolic Factors of Entrainment: Ghrelin.

In contrast to leptin, ghrelin is a hormone that stimulates appetite and decreases energy expenditure to increase adiposity (Kojima, et al, 1999; Tschöp , Smiley & Heiman, 2000; Nakazato et al, 2001). Synthesized and released from the oxyntic gland X/A-like cells of the stomach, ghrelin is a 28-amino acid that is the endogenous ligand to the GHSR (Kojima *et al.* 1999). Interestingly, these oxyntic cells also express clock proteins PER1 and PER2, whose peak and trough expressions are in antiphase with ghrelin (LeSauter, Hoque, Weintraub, Pfaff & Silver, 2009). Ghrelin's rhythm

is actually abolished in clock deficient (mPER1-mPER2 mutant) mice, suggesting that clock genes are vital in ghrelin's regulation (LeSauter et al., 2009). Ghrelin receptors are found throughout the periphery and the brain; including areas that are associated with feeding and the regulation of circadian rhythms (Angeles-Castellanos et al., 2004). The acylated form of ghrelin is the result of post-translational cleaving by proteases from preproghrelin to proghrelin, proghrelin then is acetylated by ghrelin O-acyltransferase (GOAT) by the modification of its serine-3 residue (Yang, Brown, Liang, Grishin & Goldstein, 2008). Considered the “active” form of ghrelin, acyl-ghrelin (denoted simply as ghrelin in this document) is the only form known to stimulate the GHSR, stimulates the secretion of growth hormone and increases food intake behaviour (Kojima et al., 1999). The non-octanoylated form (des-acyl ghrelin) does not have the acyl group on the Ser-3 position and does not bind to the GHSR, and was previously assumed to be an inactive peptide (Hosoda, Kojima, Matsuo & Kangawa, 2000). However, des-acyl ghrelin has been found to have a biological role, independent of the GHSR (Al Massadi, Tschöp, & Tong, 2011). Des-acyl ghrelin has been shown to operate in contrast to ghrelin, in that it reduces food intake, body weight and adiposity (Asakawa, Inui, Fujimiya, Sakamaki et al., 2004). However, there are reports that show des-acyl ghrelin stimulating food intake when administered icv. but not intravenously (Toshinai, Yamaguchi, Sun, Smith *et al.*, 2006). The ratio of ghrelin to des-acyl ghrelin in various nutritional states is still not fully understood (Delhanty, Neggers & van der Lely, 2012; Al Massadi, et al., 2011).

The initial role of ghrelin, the stimulation of growth hormone secretion (Seoane, Tovar, Baldelli, Arvat *et al.*, 2000), has been expanded to include additional roles in energy balance and metabolism (Tschöp, Smiley & Heiman, 2000; Theander-Carrillo, Wiedmer, Cettour-Rose,

Nogueiras, *et al.*, 2006), the promotion of wakefulness, and the release of both ACTH and cortisol (Wren, Small, Abbott, Dhillon *et al.*, 2001; Diano *et al.*, 2006).

In nocturnal animals, plasma levels of ghrelin fluctuate diurnally, peaking just before the onset of the dark cycle (Darzen, Vahl, D'Alessio, Seeley & Woods, 2006). In rodents under a restricted feeding schedule, where they only receive food for a few hours during the day, ghrelin levels rise 2 hours prior to scheduled mealtimes and decrease post-prandially (Drazen, *et al.*, 2005), suggesting that ghrelin plays a role in meal anticipation. Exogenous injections of ghrelin to non-food deprived animals, both in the periphery and icv., directly simulate appetitive behaviour, food intake (Nakazato, *et al.*, 2001; Perello *et al.*, 2010) and attenuate energy expenditure (Strassburg, Anker, Castaneda, Burget *et al.*, 2008) to promote adiposity (Tschop *et al.*, 2000; Wren, Small, Abbott, Waljit *et al.*, 2001). Ghrelin receptor knock out mice (GHSR-KO), which have preserved circulating ghrelin levels but do not respond to it, have been shown to eat less food, utilize their fat stores more readily as an energy source, and gain less weight and body fat than their wild-type counterparts (Zigman, Nakano, Coppari, Balthasar *et al.*, 2005). Furthermore, ghrelin-deficient mice (*ghrl*^{-/-}) were found to be resistant to diet induced obesity, at least when they are young. They also exhibited decreased overall adiposity and increased energy expenditure (Wortley, del Rincon, Murray, Garcia *et al.*, 2005). Therefore, a functional ghrelin signaling system lends itself to enhanced appetitive behaviours and preservation of adipose tissues.

Ghrelin's primary targets in the CNS are thought to contribute mainly to homeostatic feeding, are the NPY/AGRP neurons located in the ARC that promote feeding behaviours (Kamegai *et al.*, 2000; Mondal, Date, Yamaguchi, Toshinai *et al.*, 2005). However, the ability of ghrelin to modulate feeding behaviours is not limited to its actions on the ARC. Rats that had the ARC

pharmacologically ablated by neonatal monosodium glutamate, still exhibited FAA (Mistlberger & Antle, 1999). Ghrelin also exerts a direct effect on food intake through its action on the LH, an important area for feeding behaviours and one that promotes wakefulness during daytime food anticipation (Mieda, Williams, Sinton, Richardson et al., 2004; Ferrini, Salio, Lossi, & Merighi, 2009). Following i.p. injections of ghrelin, neuronal activation was also found in the DMH, an area that is involved not only in the regulation of food intake (Yang et al., 2008; Gooley et al., 2006) but also in circadian rhythms (Chou, Scammell, Gooley, Gaus et al., 2003).

In addition to its homeostatic role in food intake and energy expenditure, ghrelin is involved in the regulation of reward circuitry important for natural rewards, such as food (Malik, McGlone, Bedrossian & Dagher, 2008; Naleid, Grace, Cummings & Levine, 2005; Dickson, Egicioglu, Landgren, Skibicka *et al.*, 2011; Perello & Dickson, 2015). Often driven by an external cue, hedonic food intake occurs when the organism is in a positive energy balance and under no caloric or nutritional deficit (Berthoud, Lenard & Shin, 2011). The hedonic system is not entirely independent of the homeostatic system; rather they both work in conjunction with one another and contain some overlapping circuitries (Saper, et al., 2002; Lutter & Nestler, 2009). A number of palatability cues, such as smell and taste, act on these circuits to influence the rewarding property of food, reinforcing food intake behavior (Saper et al., 2002).

The reward system, specifically the mesocorticolimbic dopamine reward pathway, plays a critical role in establishing and maintaining the rewarding aspect of hedonic food intake (Kelley & Berridge, 2002). The GHSRs are found throughout the VTA, in both dopaminergic neurons and non-dopaminergic neurons (Abizaid, et al., 2006; Zigman et al., 2006). Ghrelin infusions into the VTA potentiate feeding, whereas intra-VTA infusions of a GHSR antagonist (BIM28163) block

feeding behavior initiated by peripherally administered ghrelin (Abizaid et al., 2006). Peripherally administered ghrelin stimulated dopamine turnover in the VTA in control animals but not in GHSR-KO mice. GHSR-KO mice and WT rats that had been peripherally administered a GHSR antagonist (JMV2959) exhibited decreased intake of a treat (peanut butter) in a free choice food paradigm, in which animals have access to both the treat and standard laboratory chow (Egecioglu, Jerlhag, Salomé, Skibicka et al., 2010). GHSR-KO mice that underwent a restricted feeding paradigm showed a reduction in neuronal activation (cFos IR) in the VTA and the NAc shell (Lamont, Patterson, Rodrigues, Vallejos et al., 2012). The GHSR-KO mice along with the ghrelin KO mice were observed to have fewer orexin-IR cells than their wild-type littermates, signifying that ghrelin signaling is required for a fully developed orexin system (Lamont, Patterson, Rodrigues, Vallejos et al., 2012). Ghrelin furthermore, has the ability to change the synaptic inputs of dopaminergic neurons in the VTA. Neurons of the VTA exposed to 90 min of ghrelin exhibited an increase in the number of excitatory inputs and a simultaneous marked decrease of inhibitory inputs (Abizaid, et al., 2006). These results highlight ghrelin's ability to activate the VTA dopaminergic neurons directly through action on GHSR, and indirectly by increasing the ratio of excitatory to inhibitory inputs.

Ghrelin's ability to potentiate reward based feeding is not limited to the VTA. Ghrelin administration is able to induce feeding when administered into the NAc (Naleid, et al., 2015) the lateral amygdala (Alvarez-Crespo, Skibicka, Farkas, Molnar, Egecioglu, et al., 2012) and the ventral hippocampus (Kanoski, Fortin, Ricks & Gill, 2013). Central ghrelin signaling is critical in establishing and maintaining food reward as shown in studies using conditioned place preference (CPP) tasks. Following exposure to a novel chamber, rodents are placed in a cage with two

compartments that have distinct visual cues. One of these chambers is continuously paired with a “reward” (e.g. a chocolate pellet or high fat diet pellet). Greater time spent in the reward-paired chamber, as compared to the time spent in the non-reward paired chamber, is an indicator of preference and of a learned association between the rewarding stimulus and the environment has been established. Peripheral injections of a GHSR antagonist (JMV2959) inhibited the rats’ ability to form a CPP as compared to vehicle treated animals (Egecioglu et al., 2010). GHSR-KO and their WT counterparts that were treated with a ghrelin receptor antagonist (Compound 26) did not show CPP for a high fat diet (HF) (Perello et al., 2010). Unlike the WT’s, GHSR-KO mice did not show CPP when administered with ghrelin. Interestingly, ghrelin administration to orexin-deficient mice along with WT animals treated with an orexin antagonist, also failed to induce CPP for HF (Perello et al., 2010). These data suggest that ghrelin’s actions on non-homeostatic feeding are supported by a functioning orexin system. The operant conditioning paradigm, a paradigm in which rodents are trained to produce an instrumental response to obtain a reward has been used to highlight a role for ghrelin in motivated behavior. Central and peripheral ghrelin administration has been shown to increase motivated behavior in rodents to obtain sucrose pellets (Skibicka, Hansson, Egecioglu, & Dickson, 2012) and HF (Perello et al., 2010) under progressive ratio (PR) schedules of reinforcement. In contrast, peripheral and central GHSR antagonist (JMV2959) infusion significantly decreased goal-oriented behaviors in a PR ratio operant conditioning model (Skibicka, et al., 2012).

In addition to the aforementioned observations, central ghrelin’s capacity to shift food preferences from a high-carbohydrate diet to a high-fat diet (Shimbara, Mondal, Kawagoe, Toshinai et al., 2004) along with its ability to increase consumption of sweet tasting foods (Disse, Bussier,

Veyrat-Durebex, Deblon et al., 2010) clearly signifies that ghrelin plays an important role in the acquisition and preference for fatty and sweet foods. In the limited studies currently available, energy utilization (fat vs. carbohydrate vs. protein) as examined through indirect calorimetry, show that subcutaneous injections of ghrelin increase the respiratory exchange ratio (RER: ratio of carbon dioxide utilization and oxygen consumption) (Tschop, et al., 2000). The increase in RER represents an increased ratio of carbohydrate utilization with a reduction in fat oxidation. Interestingly, *ghrl*^{-/-}, do not differ in their baseline measure of RER when compared to their WT littermates. However, once the animals are given access to a HF for 6 weeks the *ghrl*^{-/-} mice exhibited a significantly reduced RER, indicating that the animals have switched their energy utilization source from carbohydrates to fats (Wortely, Anderson, Garcia, Murray et al., 2004). However, these results are not consistent within the literature. Longo and colleagues (2008) observed an increase RER in GHSR-KO mice that were also exposed to 60% HFD *adlib* for 15 weeks (Longo, Charoenthongtrakul, Giuliana, Govek et al., 2008). Further complicating matters, Sun and colleagues (2007), analyzed the RER of both *ghrl*^{-/-} and GHSR-KO mice that were fed a 35% HF for a 10 week period and found that while RER was lower, it was not significantly different between the transgenic models and their wildtype counterparts (Sun, Butte, Garcia & Smith, 2007). The discrepancies in the literature may be attributed to a number of variables including; genetic backcrossing, the age at which the mice are exposed to the HF, the duration of HF exposure and the nutritional composition of the HF.

Restricted feeding paradigms result in increased endogenous ghrelin levels in anticipation of the scheduled meal, which correlates with FAA, as observed in the locomotor activity boxes and wheel running (Blum et al., 2009). Peripherally administered ghrelin in non-food deprived mice also increases both food intake and locomotor activity (LeSauter et al., 2009). In contrast, GHSR-KO

mice placed under the restricted feeding paradigm exhibit attenuated FAA (Blum et al., 2009; LeSauter et al., 2009) and exhibit a delay in the start of their activity bout relative to their WT counterparts (LeSauter et al., 2009). Although the WT and KO mice begin their anticipatory bouts at slightly different time points, once they start their bout they both continue running until the food is presented (LeSauter, et al., 2009).

Ghrelin's role in food anticipatory activity may not be limited to when an organism is in a negative energy balance. Rat models of hedonic food anticipation, in which *ad lib* fed rats are given a scheduled treat, have shown a positive correlation between hedonic FAA and plasma levels of ghrelin (Merkestein, Brans, Luijendijk, de Jong et al., 2012). Rats under the hedonic food anticipatory model show activation (c-Fos-IR) in the DMH, LH, PVN, PFC and the central nucleus of the amygdala (Mendoza, Angeles-Castellanos & Escobar, 2005). Exogenous central ghrelin stimulated FAA to a piece of chocolate, whereas central infusions of a GHSR antagonist (JMV2959) reduced FAA to the same treat (Merkestein, et al., 2012).

Food restricted and hedonic feeding paradigms do not simply modulate hormonal rhythms; they also synchronize circadian oscillators throughout the brain, including the basolateral nuclei of the amygdala (Lamont, Diaz, Barry-Shaw & Amir, 2005), the central amygdala (Angeles-Castellanos, Salgado-Delgado, Rodriguez, Buijs & Escobar, 2008; Angeles-Castellanos et al., 2008) the hippocampus (Waddington Lamont, Harbour, Barry-Shaw, Diaz et al., 2007), the DMH (Verwey, Khoja, Stewart & Amir, 2007), the NAc, and the PFC (Angeles-Castellanos et al., 2008).

Ghrelin, when administered to cultured SCN cells *in vitro*, has the ability to advance circadian rhythm much like other non-photoc stimuli (Yannielli, Molyneux, Harrington, & Golombek, 2007). Yannielli et al. (2007) further highlighted the ability of ghrelin to affect the clock

mechanism by demonstrating that the cycling of PER2: LUC, a component of the circadian clock, in SCN explants, phase advanced when treated with ghrelin. However, peripheral ghrelin or its analog GHRP-6, administered to *ad lib* fed mice and those in constant darkness did not influence the circadian phase of wheel-running (Yannielli, et al., 2007). However, mice administered GHRP-6 after 30 hours of food deprivation exhibited a slight phase shift, with animals increasing their locomotor activity for 1 hr after the injection. Taken together, ghrelin's influence on the SCN is more pronounced when the organism is in a negative energy state.

The key roles of ghrelin in energy homeostasis, metabolism, meal initiation, entrainment, peripheral clock regulation and its reciprocal communication with the SCN, highlight the importance of this hormone in further understanding the links between circadian rhythms and metabolism. Literature has suggested that understanding ghrelin and its interactions with the circadian clock and the multitude of metabolic pathways may help unravel the location(s) of the FEO's (LeSauter, et al., 2009; Yannielli, et al., 2007; Blum, 2010; Carneiro & Araujo, 2009). Therefore, further research is required to tease apart the role of ghrelin in modulating food entrainment under various behavioural paradigms.

This thesis will further examine the role of ghrelin and the GHSR in the entrainment of circadian rhythms when mice are under both negative and positive energy states. Given ghrelin's pivotal role in energy homeostasis, and its ability to directly and indirectly influence circadian oscillations, we hypothesize that ghrelin and the GHSR will modulate the metabolic profiles and hedonic food entrainment under a restricted feeding paradigm and a hedonic food paradigm, respectively.

Figure 1.1. The molecular components of the mammalian circadian clock, is composed of transcriptional activators, Circadian Locomotor Output Cycles Kaput (*Clock*) and Brain and Muscle-Arnt-Like 1 (*Bmal1*) that form heterodimers in the cytoplasm, through Period-Arnt-Single-Minded (PAS) domains. Dimers then enter the nucleus to bind with E-Box (5' CACGTG-3') and E-box like-sequences to mediate the transcription of Period (*Per1,2,3*) and Cryptochrome (*Cry 1, 2*) genes, activating their expression (Albercht, Sun, Eichele, & Chi Lee, 1997; Whitmore, Sassone-Corsi & Foulkes, 1998). As the PER and CRY proteins are translated and their levels accumulate in the cytoplasm, they oligomerize and translocate into the nucleus to inhibit the activity of CLOCK-BMAL1, ultimately inhibiting their own transcription thus forming a negative feedback loop (Bass & Takahashi, 2010). The PER's and CRYs are regulated by *Casein kinase 1 epsilon* (CKIε) and F-box protein FBXL3, respectively (Brown, Kowlaska, & Dallmann, 2012). CKIε and *Casein kinase 1 delta* (CK1δ) are fundamental factors that regulate core circadian protein turnover in mammals (Akashi, et al., 2001) and ultimately influence circadian period (Ko & Takahashi, 2006).

Figure 1.2. SCN Projections. The SPZ and the DMH receive the most intense outputs from the SCN. SCN fibers terminate in the ARC, the VMH and the ventral LH. The SCN selectively innervates the ventral and dorsal borders of the PVN. SCN indirectly projects to the VTA through the MPOA (Luo, & Jones, 2009).

Figure 1.3. The translocation of BMAL1:CLOCK or BMAL1:NPAS2 dimers in the nucleus additionally also binds to E-boxes of genes that code for reverse erythroblastosis virus alpha (REV-ERB α) and retinoic acid-related orphan nuclear receptors (Ror α) and (ROR γ). Ror α and ROR γ positively stimulate *BMAL1* expression through the ROR response element (RRE) while REV-ERB α inhibits *BMAL1* by recruiting histone deacetylase complexes (Albrecht & Ripperger, 2008). REV-ERB α is a pro-adipogenic transcription factor that increases significantly in response to adipocyte differentiation and its mRNA is found in fat (Chawla & Lazar, 1993), while ROR α is found in abundance in skeletal muscles and regulates lipogenesis and lipid storage in skeletal muscles (Lau, Nixon, Parton, & Muscat, 2004).

Figure 1.4. The GHSR is found, at varying densities, throughout the Central Nervous system, in regions associated with feeding and circadian regulation, also in brain regions that are involved in food anticipation. Areas such as the ARC, PVN, DMH, LH, VMH and to a certain extent in the SCN (Cowley et al., 2003; Zigman et al., 2006; Yi et al., 2006).

Chapter 2

2.1. Introduction.

In most living organisms, many physiological processes and behaviors fluctuate with the 24-hour light dark cycle and are said to show a circadian rhythm. In most mammals, circadian rhythms are dependent on a particular hypothalamic nucleus, the suprachiasmatic nucleus (Moore et al., 2002). In addition to light, circadian rhythms in behavior and physiology can also be coupled to food availability (Mistlberger, 1994). For instance, when food is only available for 2-6h in the middle of the light phase of the light/dark cycle, rats will come to show increased locomotor activity in the two hours preceding the scheduled meal, (Coleman, Harper, Clarke, & Armstrong, 1982; Rosenwasser, Pelchat and Alder, 1984). A similar pattern of behavior is seen in rats when they are fed ad libitum but given scheduled access to a palatable treat (Mendoza, Angeles-Castellanos & Escobar, 2005; Verwey, et al., 2007; Angeles-Castellanos, Salgado-Delgado, Rodriguez, Buijjs & Escobar, 2010) This increase in locomotor activity is known as food anticipatory activity (FAA) (Mistlberger, 1994).

Over repeated exposure to the scheduled meal/treat the FAA becomes entrained to food availability such that the FAA behavior persists for several days after the scheduled meal is eliminated and ad libitum food availability is restored (Mistlberger, 2011). Further evidence that locomotor activity is entrained to scheduled meal presentation comes from studies showing that even after several days of ad libitum feeding, FAA is rapidly reestablished if scheduled feeding is reinstated (Coleman, et al., 1982; Rosenwasser, et al., 1984). Together these data suggest that this type of feeding schedule is an effective entraining cue.

In addition to locomotor activity, the timing of food availability can also entrain physiological processes such as, body temperature, hormone secretion and metabolism (Honma, Honma & Hiroshige, 1983; Damiola, et al., 2000; Díaz-Muñoz, Vázquez-Martínez, Aguilar-Roblero & Escobar, 2000). For example, the circadian pattern of hormone secretion is associated with feeding and energy balance, such as leptin, insulin and ghrelin, can all be entrained by a scheduled meal (Escobar, Díaz-Munóz, Encinas & Aguilar-Roblero, 1998; Davidson & Stephan, 1998; Blum et al., 2009; Riberio, et al., 2011). Restricted feeding schedules also influence metabolic rate hence potentially allowing organisms to optimally utilize energy resources (Satoh, Kawai, Kudo, Kawashima et al., 2005; Hattori, Vollmers, Zarrinpar, DiTacchio et al., 2012). For instance, nocturnal rodents with *ad libitum* access to food exhibit a circadian rhythm of respiratory exchange ratio (RER) such that, coincident with food intake, RER peaks during the dark phase of the light-dark cycle indicating utilization of carbohydrates. During the light-phase of the cycle when little food is ingested RER declines signifying a switch to lipid oxidation (Satoh et al., 2005; Hattori et al, 2012). When food is available only during the light phase of the cycle as with the restricted feeding paradigm, however, RER peaks during the light phase (Hattori et al., 2012; Satoh et al., 2005).

Food entrainment, is not dependent on an intact SCN, but instead is the product of multiple systems including the organism's homeostatic state, reward, arousal and external and internal cues (Antle & Silver, 2009; Chavan, Albrecht & Okabe, 2017). The exact mechanisms that control food and metabolic entrainment remain largely unknown, but given that the SCN is not crucial for this process (Stephan, 1984; Stephan, 2002; Guilding & Piggins, 2007), and several other brain regions have also been eliminated as candidates for an anatomically discrete food entrainable oscillator (FEO; Mistleberger, 1994; Chavan, Albrecht & Okabe, 2017), many accept the notion that the FEO is a

distributed system that encompasses central and peripheral mechanisms (Stephan, 2002; Mendoza, 2006; Mohawk, Green & Takahashi, 2012; Mistlberger & Antle, 2011; Mistlberger, 2011; Dibner, Schibler & Albrecht, 2010; Chavan, Albrecht & Okabe, 2017).

One hypothesis is that peripheral signals associated with feeding, digestion, and energy balance, and that are regulated by nutrients, target a number of brain regions to stimulate the activity of the FEO network, resulting in behavioral and metabolic changes that facilitate the ingestion and utilization of nutrients from the expected meal. While the signals that convey this information remain elusive, it has been proposed that the signals: (1) are present prior to a scheduled meal; (2) stimulate food intake and alter metabolism; (3) should shift the phase of food-entrained rhythms; (4) are correlated with FAA; (5) the manipulation of the signals, or of their receptors, should enhance or attenuate FAA (Mistlberger, 1990; LeSauter, et al., 2009; Escobar, Cailotto, Angeles-Castellanos, Delgado & Buijs, 2009; Mistlberger, 2011; Patton & Mistlberger, 2013).

One signal that meets these criteria is the hormone ghrelin. Ghrelin is a stomach derived orexigenic hormone that acts in the pituitary to stimulate growth hormone release (Wren, et al., 2000). In humans, plasma ghrelin levels increase preprandially (Cummings, et al., 2000) and in ad libitum fed rodents, peak just before the onset of the dark phase of the light/dark cycle (Sánchez, Oliver, Picó & Palou, 2004). Additionally, peripheral and central administration of ghrelin stimulates food intake in freely fed animals (Wren, et al., 2001; Tschop, et al., 2000).

Scheduled meal presentation alters the timing of ghrelin secretion so that it peaks just prior to the scheduled meal (Cummings, Frayo, Marmonier, Aubert & Chapelot, 2004; Lesauter et al, 2009; Blum et al., 2009) and hence peak circulating ghrelin concentrations correlate well with FAA in rodents (Tschöp, et al., 2000; Drazen, et al., 2006; Bodosi, et al., 2004). It is probably the rapid

increase in ghrelin that occurs prior to the timed meal, that best correlates with FAA. Indeed, when rats are given access to two meals, ghrelin levels rise prior to the first meal and remained elevated until the second meal whereas locomotor activity increases prior to the first meal and then declines (Patton, Katsuyama, Pavlovski, Michalik et al. 2014).

Ghrelin has also been implicated in increasing the consumption of palatable food intake in sated animals (Egecioglu, et al., 2010; King, et al., 2016). Consistent with this, Merkestein and colleagues reported that plasma ghrelin correlated with increased FAA in free-fed rats that are entrained to palatable snack (Merkestein, et al., 2012). Evidence for a potential causal role for ghrelin in FAA is that peripheral administration of ghrelin increases FAA in sated rodents (LeSauter et al., 2009).

The actions of ghrelin are mediated through its binding to a G-protein coupled receptor, the growth-hormone secretagogue receptor (GHSR1a), which is found throughout the peripheral and central nervous system. (Zigman, et al., 2006). Animals with targeted mutations of the GHSR (GHSR-KO) that renders them insensitive to the actions of ghrelin, show attenuated FAA (Abizaid, et al., 2006; Blum et al., 2009; LeSauter et al., 2009; Davis, Choi, Clegg & Benoit, 2010; Lamont et al., 2014), suggesting that functioning GHSR signaling is required to establish a robust FAA.

In addition to its established role in increasing food intake ghrelin alters metabolism by promoting the utilization of carbohydrates as a source of fuel, while sparing the use of fat (Tschöp, et al., 2000). For instance, mice given peripheral or central ghrelin injections and then placed in metabolic chambers, show an increase in carbohydrate utilization as reflected in a higher RER, (Tschöp et al., 2000). Mice with mutations of the ghrelin gene (*ghrelin*^{-/-} mice) or GHSR^{-/-} mice show higher utilization of fat as an energy source than their wild type littermates (Wortley, et al.,

2004), and the latter also show attenuated locomotor activity particularly during the dark cycle (Zigman, et al., 2005).

Given the role of ghrelin in regulating feeding and energy balance together with its ability to act at a wide number of targets within the brain it is a good candidate for coupling food availability to a wide array of physiological (e.g. metabolic rate) and behavioral processes (FAA). While ghrelin influences FAA in restricted feeding schedules (Blum et al, 2009, Le Sauter et al, 2009), it is not known whether ghrelin or GHSR activity are important for the metabolic entrainment that is observed in mice and rats subjected to restricted feeding paradigms. In the current set of studies we investigated whether ghrelin receptor activity makes an important contribution to the development and maintenance of metabolic entrainment of scheduled meals, under a restricted feeding paradigm and a HF treat paradigm.

2.2. Materials and Methods

2.2.1. Animals.

All methods described below were approved by the Carleton University Animal Care Committee, and followed the guidelines of the Canadian Council for Animal Care. Adult CD-1 male mice (Charles River St. Constant QC), weighing 30-35g at the start of the experiments, were subjects in Experiments 1 and 2. Mice were single housed on arrival and given a week to acclimate to standard laboratory conditions. Male adult mice with targeted mutations to the ghrelin receptor gene (GHSR-KO) and their wildtype (WT) littermates obtained from a breeding colony at Carleton University were used in experiment 3. The original breeding pairs for this colony originated from Regeneron Pharmaceuticals in Tarrytown, NY, USA. The metabolic profile and phenotype of these mice was described by Wortley et al (2005), and by Pfluger, Kirchner, Gunnel, Schrott *et al.*, 2007. All mice were housed in a temperature and humidity-controlled room with 12:12hr light-dark cycle, with lights on at 8:00AM (*zeitgeber* time (ZT) 0)). Standard laboratory chow (SCH) (3.3 kcal/g, 70% carbohydrates) was provided ad libitum during the baseline period and when indicated throughout the experiments.

2.2.2 Indirect Calorimetry and Locomotor Activity.

Mice were placed in the metabolic chambers (TSE Phenomaster; TSE Systems, Inc., Chesterfield MO, USA) that measured energy utilization and locomotor activity. Indirect calorimetry measures including oxygen consumption (VO_2), carbon dioxide production (VCO_2), respiratory exchange ratio (RER), and locomotor activity were obtained in 30 min bins. The measurement of RER is used to estimate the energy utilization based on the number of oxygen molecules required for the oxidation of glucose versus fatty acids (Lusk, 1923; Weir, 1949). The system calculates RER as

$VCO_2[\text{ml/h/kg}]/VO_2[\text{ml/h/kg}]$, where VO_2 is $\text{FlowML}[\text{ml/h}] \cdot (V1[\%^2] + V2[\%^2]) / (N2Ref[\%] \cdot \text{bodyweight}[\text{kg}] \cdot 100[\%])$ and VCO_2 is $\text{FlowML}[\text{ml/h}] \cdot dCO_2[\%] / (N2Ref[\%] / (\text{bodyweight}[\text{kg}] \cdot 100[\%]))$. This calculation is based on metabolic estimated provided by Weir (1949). Breaks in infrared light beams, emitted from both x and y-axes, were used to measure total locomotor activity.

2.2.3 Blood Sample Analyses.

Immediately following decapitation, trunk blood glucose levels were measured using glucose strips and a Contour glucose meter (Bayer Corp., Pittsburgh, Pennsylvania). The remaining trunk blood was collected in ethylenediaminetetraacetic acid (EDTA) coated tubes and kept on ice. All blood samples were centrifuged at 3000-x *g* for 15 minutes (at 4°C) to obtain a plasma layer that was aliquoted into three separate 1.5ml microcentrifuge tubes. To protect the acylated ghrelin molecule from degrading, one 50 µl plasma aliquot was treated with 2.7 µl of 0.1M HCl and 5 µl of 100 mM of p-hydroxymercuribenzoic acid (PHMB). Acylated ghrelin levels were measured in duplicate using a commercially available ELISA kit (Millipore, St.Charles, MO, USA) with an intra-assay variability of 9.5%. Plasma corticosterone levels were measured in duplicates using a commercially available RIA kit intra-variability of 10% (CN Biomedicals, Inc., Aliso Viego, California).

2.2.4 Circadian Analyses.

Measurements of acrophase and amplitude of RER and locomotor activity were made using Acro software (Acro v3.5; Refinetti, 2017). The acrophase represents the time at which the peak of the rhythm occurs whereas the amplitude is the difference between the peak and the mean of the sine wave (Refinetti, Corné Lissen & Halberg, 2007). These measurements standardize the rhythmicity

of RER and locomotor activity and allow us to compare and correlate the metabolic and locomotor measurements.

2.2.5 Experiment One-

Does exposure to RF and to a scheduled HF treat change the circadian rhythm of RER?

In rodents, evidence of entrainment of FAA, hormone concentrations, and other physiological measures become apparent within 4-5 days after the onset of the restricted feeding schedule, or after repeated presentation of a palatable diet (Blum et al., 2009; Merkestein, Brans, Luijendijk, de Jong et al., 2012). In this study, we investigated the RER patterns of animals that were fed adlib chow, underwent a RF paradigm and freely fed mice that had access to a scheduled HF treat.

2.2.6 Procedure.

Body weight and food intake were measured daily throughout the study. Following a one week baseline period, mice were assigned to one of three weight matched groups: ad libitum fed (AL; n=8), restricted fed (RF; n=8) and high fat exposed (HF; n=8). Animals in the RF group were fasted overnight and given limited access to standard laboratory chow for four hours each day (10:00- 2:00 pm), while mice in the HF group, had access to a high fat (HF) treat (60% fat by weight, Harlan Laboratories, Madison, WI) for two hours per day (10-12:00pm) as well as ad libitum access to chow. Both the restricted feeding and HF paradigm were adapted from work our lab has previously published Blum et al., 2009 and King et al., 2016, respectively. Mice assigned to the AL group continued to be treated as they did during the baseline. Mice were placed into the metabolic chambers at the end of the baseline period, and again at the end of the experimental phase. Due to

limited number of metabolic boxes, animals from each group were placed into the metabolic boxes in a balanced and staggered fashion. All the animals and the groups underwent the same total number of experimental days and were all sacrificed on the same day. While in the metabolic chambers, RER and locomotor measurements were collected from each mouse every 30 minutes for 48 hours. However, only the recordings from the last 24 hours were used, to account for potential acclimation variability. At the end of the experimental phase, mice were sacrificed by rapid decapitation and trunk blood was collected for analysis of glucose, corticosterone and acylated ghrelin levels.

2.2.7 Experiment Two-

Determining the onset and duration of metabolic entrainment to restricted feeding schedules.

We conducted experiment 1 to determine whether RF or scheduled HF feeding would alter patterns of RER in the same way that they do patterns of locomotor activity. In experiment 2 we used a modified version of the paradigm to investigate whether these altered metabolic patterns were actually entrained to the scheduled meal by measuring their persistence when food was once again presented *ad libitum* as well as how rapidly they were re-established when RF itself was reinstated.

2.2.8 Procedure.

As in experiment 1, following a baseline period of one week, mice were assigned to either: *ad libitum* fed (AL, n=5) or restricted feeding schedule (RF, n=5) groups such that groups were matched for food intake and bodyweight. Mice in the AL group were given *ad libitum* access to chow throughout the experiment. Animals in the RF group received free access to chow from (ZT4-ZT8) for a 7-day period after which they were exposed to a recovery phase for 26h during which food was again available *ad libitum*. Animals were food restricted for 24 hours before being placed once again

in 36 hours recovery. Finally mice were food restricted once more and metabolic rate parameters were measured. The timeline of the food restriction was designed in order to illustrate food entrainment in a time-restricted manner.

2.2.9. Experiment Three-

Examining the contribution of GHSR signaling to metabolic entrainment by restricted feeding.

A number of studies support the notion that GHSR signaling is important for the expression of FAA in mice and rats subjected to restricted feeding schedules (Blum et al, 2011; LeSauter et al., 2011). Here we tested the hypothesis that GHSR signaling is also important for metabolic entrainment. Thus, the response of male ghrelin-receptor knockout (GHSR-KO) (n=10) mice to the paradigm described in Experiment 2 was compared with that of their wild-type littermates (GHSR-WT) (n =9). Mice weighed between 25-35 g at the beginning of the experiment and groups were weight- and aged-matched prior to commencing the experiment.

2.2.10. Statistical Analyses.

Data were analyzed using a ANOVA and post-hoc analyses were performed using Least Significant Difference (LSD) test with significant values set at $P < 0.05$. Where applicable data were analyzed using independent t –tests. Daily measures were analyzed using Repeated Measures Analysis where appropriate and Bonferroni post-hocs. Correcting for violations of Sphericity, the Greenhouse-Geisser correction was used when the epsilon (ϵ) values were $\epsilon < 0.75$ however, for $\epsilon > 0.75$ the values from the Huynh-Feldt correction were used (Verma, 2016). Statistical analysis was performed with SPSS Statistics version 20 (IBM©, 2011). The alpha level was set at $p < 0.05$ for all statistical

comparisons. Graphs were constructed using Prism 5 (Graphpad Software, INC., 2008) and all data are shown as mean $\pm$ SEM.

2.3. Results.

2.3.1. Experiment One-

Restricted feeding paradigms influences circadian rhythmicity of energy metabolism and increases home cage activity an hour prior to the scheduled meal.

As anticipated there were no differences in RER levels or rhythmicity between the groups during the Baseline condition (Figure 2.1, Panel A). During the experimental phase however there were clear differences between the groups such that although mice in the RF group showed lower RER than those in the other two groups just after lights on (8:00), they showed a rapid increase in RER in the period just prior to and during food presentation and this higher RER persisted until close to lights off (see Figure 2.1, Panel B). This pattern of results gave rise to a significant main effect of time ($F(3.170, 66.56) = 4.169, p < 0.05$) and a significant group x time interaction ($F(6.339, 66.56) = 9.213, p < 0.05$) but no significant main effect of group ($p > 0.05$). Post hoc tests showed that the RF group had significantly lower RER than the AL group and the HF group ($p < 0.05$) at 8:00 (Figure 2.1, Panel B). The RER of the RF group did not differ from AL and HF groups at 9:30 and 10 (>0.05), but was greater than that of these groups at 11:00 o'clock and remained so until 19:30 ($p < 0.05$) when it dropped to levels lower than the AL and HF groups. Comparison of the acrophase and amplitude of the RER cycle among the groups shows that RF shifted the peak of the RER cycle to the light phase and also increased its amplitude relative to the other two groups which did not differ significantly on either measure (Table 2.1). There were no differences between the

groups ($p>0.05$) with regards to either acrophase or amplitude of the RER or locomotor activity, during the baseline measures (Table 2.1).

Total activity did not differ among groups at baseline ($p>0.05$) (Figure 2.2, Panel A). During the experimental phase there was a main effect of time ($F(5.273, 105.458) = 3.493$), but no main effect of group ($F(2,20) = 1.624$, $p>0.05$) or time x group interaction ($F(10.546, 105.458) = 1.643$, $p>0.05$). Because we, and others had previously shown that the RF paradigm reliably produces an increase in locomotor behavior in the 2h prior to the scheduled meal (Blum et al., 2009; Waddington-Lamont et al., 2014) we had hypothesized that this would also occur in the current experiment. Based on this a priori expectation we did planned comparisons that confirmed that the RF group exhibited more total activity than the AL and HF groups ($p<0.05$) one hour prior to the scheduled meal (9:00-10:00) (Figure 2.2, Panel B). As with RER, analysis of the acrophase and amplitude of the locomotor activity rhythm shows that the peak of the rhythm was significantly shifted toward food presentation in the RF group. There was also a trend towards a significantly higher amplitude in locomotor rhythm, in the RF group.

Manipulating feeding schedules changes caloric intake and body weight as well as plasma glucose and glucocorticoid levels.

Mice in the RF group lost weight as soon as they were exposed to the restricted feeding schedule (Figure 2.3B). Analyses of body weight data yielded significant main effects of days ($F(32, 672) = 4.520$, $p<0.05$), group ($F(2,21) = 25.251$, $p<0.05$) and a significant days x group interaction ($F(64, 672) = 24.489$, $p<0.05$). Pairwise comparisons showed that mice in the RF group weighed significantly less than both the AL and HF groups ($p<0.05$) from experimental day 2 (Ex2) onwards.

Analyses of the food intake data showed significant main effects of days, group and a days x group interaction ($F(32,576) = 4.117$, $p < 0.05$; $F(2,18) = 28.451$, $p < 0.05$; and $F(64, 576) = 2.559$, $p < 0.05$, respectively). Pairwise comparisons showed that mice in the HF group ate more calories than mice in the AL and RF groups, and AL mice ate more calories than mice in the RF group ($p < 0.05$).

As Figure 2.4 shows plasma glucose levels were lower in the RF mice than in the AL and HF groups ($F(2,21) = 4.671$, $p < 0.05$; pairwise post hoc comparisons show that the RF had lower glucose levels than both the AL and HF groups $p < 0.05$. The HF group did not differ from the AL group with regards to blood glucose levels $p > 0.05$. A similar pattern was seen for plasma corticosterone concentrations ($F(2,21) = 21.655$, $p < 0.05$; pairwise post hoc comparisons indicated that the RF displayed lower levels of cort when compared to either the AL or HF $p < 0.05$. The HF group did not differ from the AL ($p > 0.05$). In contrast, acylated ghrelin levels were significantly higher in the RF group than in AL and HF groups ($F(2,12) = 10.250$, $p < 0.05$). Levels in the AL and HF groups did not differ significantly ($p > 0.05$) (Figure 2.4)

2.3.2. Experiment Two-

RER entrains to RF

Similar to the pattern observed in experiment 1, the average RER of the RF group on experimental days 1-day 6 of exposure to the restricted feeding schedule was significantly lower than that of the AL group following lights on (8:00-11:00) but rose sharply at 11:30-12:00. From the onset of the scheduled meal (12:00) until just prior to lights off (19:30) the RER levels of the RF group remained higher than those of the AL group (Figure 2.5). RER in the RF group then decreased to levels lower than the AL group. Analysis of these data resulted in main effects of time ($F(47, 376) = 53.098, p < 0.05$) and a significant time x group interaction ($F(47, 376) = 57.268, p < 0.05$). Overall RER was lower in the RF than AL group giving rise to a significant effect of group ($F(1, 8) = 46.905, p < 0.05$). As in Experiment 1, RF significantly altered the rhythms of both RER and activity such that the peak of both was shifted to the light phase of the cycle. However, in this study the amplitude of the RER rhythm was decreased by exposure to RF and that of the locomotor rhythm was unaffected (Table 2.2).

A day-by-day analysis of RER rhythmicity indicated that the AL and RF groups were significantly different from one another for the entire duration of the experimental period (Figure 2.6). This difference translated in to the phase advance in the acrophase and the respective differences in the amplitude of the RER levels (Table, 2.3).

To further investigate the relationship between RER and activity we investigated whether or not the levels were correlated. There was no correlation between RER and activity levels in either the AL group ($r = -0.291, p > 0.05$) or the RF group ($r = -0.190, p > 0.05$).

Food anticipatory activity, that is total activity during the 2-hours prior to the scheduled meal, was measured throughout the experiment and is shown in Figure 6, Mice in the RF group showed higher levels of activity than AL mice as early as experimental day 3, and this was still seen on the first day of recovery. Notably, after the decline on the second day of recovery there was a rebound in locomotor behavior in the re-exposure phase. This pattern of behavior resulted in a significant main effect for days and a significant day x group interaction ($F(11,77) = 3.707, p < 0.05$; $F(11,77) = 6.001, p < 0.05$). There was also a trend towards significance for the main effect of group ($F(1,7) = 4.699, p = .067$) Pairwise comparisons showed that the RF group exhibited significantly higher FAA than the AL on EX3, EX4, EX6 and EX7 as well as RC1 and Rex (Figure 2.7).

Restricted feeding schedules influence metabolic end-points.

As in Experiment 1, plasma acylated ghrelin levels were significantly higher in mice in the RF group compared to mice in the control AL group ($t(8) = -4.044, p = .004$). In this experiment, however, there were no significant differences ($t(8) = .029, p > 0.05$) in plasma glucose levels between the control and the RF mice (See Figure 2.8, panels A & B).

As expected, animals in the RF group ingested significantly fewer calories than those in the AL group during the experimental period and consequently lost weight. Daily caloric intake analysis yielded a significant main effect of days ($F(12,96) = 81.713, p < 0.05$), a main effect of group ($F(1,8) = 12.941, p < 0.05$) and a significant days x group interaction ($F(12,96) = 80.198, p < 0.05$). Pairwise comparisons further illustrated the RF ate less than AL during the experimental period but more during the recovery period (Figure 2.8, Panel C). Analyses of daily measurements of body weight generated a significant main effect of days ($F(12,96) = 101.865, p < 0.05$), and a trend towards

significance in group ($F(1,8) = 5.060, p = .055$). There was also a days $\times$ group interaction $F(12, 96) = 97.427, p < 0.05$ with pairwise comparisons indicating RF group weighing less than the AL throughout the experimental period but showing a rebound in weight during the recovery and re-exposure phases (Figure 2.8, Panel D).

2.3.3. Experiment Three-

The restricted feeding paradigm drives GHSR-KO animals to immediately utilize their fat stores, whereas the WT's gradually make the switch from carbohydrate utilization to fat utilization.

In Experiment 2, we found that a 7 day exposure to the restricted feeding paradigm was sufficient to entrain both locomotor activity and RER. To investigate the role of ghrelin in the entrainment of these endpoints, GHSR-KO animals and their WT counterparts underwent the same paradigm described in Experiment 2.

Overall RER levels averaged over the experimental period (experimental day 2 to experimental day 6) showed a main effect of time ($F(47, 799) = 328.85, p < 0.05$) and an interaction of time and genotype that approached significance ($F(47, 799) = 1.347, p = .063$) but no main effect of genotype ($F(1, 17) = 2.981, p < 0.05$). Pairwise comparisons of time highlighted that the GHSR-KO animals had significantly lower RER levels during portions of the dark phase ($p < 0.05$) (Figure 2.9).

During the experimental phase (as the aforementioned timeline), the acrophase ($t(17) = .593, p > 0.05$) and the amplitude ($t(17) = -1.104, p > 0.05$) of the RER did not differ between the genotypes. Likewise, the acrophase ($t(16) = -.798, p > 0.05$) and amplitude ($t(16) = -1.464, p > 0.05$) of the total activity during the experimental period did not differ between the genotypes on average (Table 2.4).

To examine whether the GHSR activation is important for the coupling of RER to the scheduled meal, we compared the daily RER pattern during the experimental, recovery and re-exposure phase of the study between GHSR-KO and their WT littermates (see Figure 2.10). On the last day of baseline GHSR-KO mice had significantly lower RER than WT mice only during the beginning of the light/dark cycle (significant effects of time ($F(47, 799)=19.033$, $p<0.05$) and time by genotype interaction ($F(47,799)=3.644$, $p<0.05$ post hoc) but no main effect of genotype .

On experimental day 1 there were clear differences in the response to food restriction in the WT and GHSR mice. While all mice showed a marked decrease in RER throughout the day and early in the dark period (significant main effect of time ($F(47, 799)= 28.308$, $p<0.05$), GHSR KO mice showed a higher RER than WT mice from 13:00-14:30 and 16:30-23:30. This resulted in significant effects of genotype ($F(1,17)= 11.751$, $p<0.05$), and a significant genotype x time interaction ($F(47, 799)=1.450$, $p<0.05$) (see Figure 2.10 Panel B).

On experimental days 2-5 (Figure 2.10, Panels C, D,E, and F), daily RER recordings were similar for both groups until the onset of dark phase, at which time GHSR-KO mice showed a significantly lower RER than WT mice. This persisted until about an hour before the next scheduled meal, when both KO and WT mice exhibited a rise in RER that lasted until approximately an hour after the meal. After this, two groups again diverged, with KO mice remaining at a higher RER compared to WT mice. Analyses of these data resulted in significant effects of group, time and a significant group x time interaction ($p. < 0.05$). The differences in RER between GHSR-KO and WT mice persisted until the fourth day of the restricted feeding schedule, at which time the RER of the GHSR -KO group was higher than that of WT only for the period between lights-on and meal

presentation. The circadian pattern of RER did not differ between the genotypes for the remaining days (Panels G, H) of the restricted feeding schedule ($p > 0.05$).

Notably, on the day the mice were given access to ad lib food (RC 1), the circadian pattern of RER of mice in both groups became less pronounced but no group differences were observed. More importantly, when access to food was removed again to test for entrainment, the circadian pattern of RER was restored to mimic that seen at the end of the restricted feeding schedule, with no differences between GHRSR-KO and WT mice ($p > 0.05$; See Figure 2.10).

Overall, there were no differences in overall 24-hour locomotor activity between GHRSR-KO and WT mice during the baseline ($p > 0.05$). FAA was measured on the last re-exposure day and, as shown in Figure 2.11 GHRSR-KO mice had reduced FAA compared to WT mice

On experimental day two, when the groups had been fasted the night before, WT mice showed an RER acrophase that was significantly later than that shown by GHRSR KO mice ($t(17) = 2.533$, $p < 0.05$). However, GHRSR KO and WT mice expressed the same RER amplitude ($t(17) = .314$, $p > 0.05$) and locomotor activity acrophase ($t(16) = .489$, $p > .05$). Interestingly, the KO's expressed a higher amplitude in their locomotor activity which approached significance ($t(16) = -1.986$, $p = .076$). Following experimental day 2, there were no differences between the genotypes with regards to acrophase or amplitude in neither their RER and locomotor measures (Table, 2.5).

The restricted feeding paradigm did not elicit significant differences metabolic measures between the genotypes.

Interestingly, the genotypes did not differ in body weight throughout the experiment ($F(1,16) = .413, p > 0.05$). Both WT and GHSR KO mice weighed less during the experimental period (main effect of time-point ($F(2,32) = 16.040, p < 0.05$) (Figure 2.13, A). Similarly, caloric intake analysis indicated no differences between the genotypes ($F(1,17) = .293, p > 0.05$). But yielded a significant main effect of time ($F(2,34) = 89.003, p < 0.05$), both genotypes ate significantly fewer calories during the experimental phase and significantly more calories during the recovery phase when compared to their baseline caloric consumption ($p < 0.05$) (Figure 2.13, B). Plasma glucose levels did not differ ($t(17) = 1.518, p > 0.05$) between the WT and GHSR-KO mice (Figure 2.13, C)

2.4. Discussion

In these experiments, we studied whether metabolism and nutrient utilization is coupled to the FEO in mice exposed to a restricted feeding schedule, or in mice given access to a palatable snack. We then examined whether the ghrelin receptor was important in mediating the entrainment of metabolic rhythms to scheduled meals. We found that nutrient utilization as reflected in RER has a circadian rhythm that is tightly coupled to the time of access to food. Furthermore, our results suggest that, the GHSR is not critical in the ability of scheduled meals to entrain rhythms of RER and locomotor activity.

Consistent with previous reports (Blum et al., 2009), the restricted feeding (RF) group demonstrated elevated plasma ghrelin levels and attenuated levels of blood glucose in anticipation of the meal that correlated with increases in food anticipatory activity. Interestingly neither the time of peak RER of the mice that were fed high fat (HF) diet nor the amplitude of its rhythm differed significantly from that of the AL group. These data are inconsistent with previous literature showing that mice given exclusive access to a HF diet during nighttime feeding exhibited attenuated levels of RER (Satoh, et al., 2006) and that chronic *ad libitum* HF feeding decreases RER (Wortley, et al., 2005; Sun, et al., 2007; Longo, et al., 2008) as well as nighttime locomotor activity (Zigman, et al., 2005). It is possible that the relatively short period of daily HF diet availability in the current study, 2 hour access versus the 6 hour access in the literature, contributed to these discrepancies.

The present results are consistent with those studies in which mice were given access to food for nine hours (Satoh et al., 2006) or twelve hours, (Bray, Ratcliffe, Grenett, Brewer *et al.*, 2013) that showed that RER levels were entrained to meal presentation, with the scheduled meal animals exhibiting higher RER levels. To our knowledge, however, the current study is the first examination

of RER levels in animals under a restricted feeding paradigm in which food is presented for four hours in the middle of the light phase for an extended period.

In experiment one, we found that the RF mice exhibited a robust change in RER rhythmicity. Experiment 2 set out to study the progression of that change and whether the RF paradigm entrained RER rhythmicity. Therefore, we examined the rhythmicity of RER during restricted feeding, recovery and the re-exposure phases. The reversal of the RER rhythm induced by RF in Experiment 1 was also seen in Experiment 2. Interestingly, food anticipatory activity was not correlated with RER levels, suggesting that RER levels are not directly influenced by changes in locomotor activity. Previous studies have shown RER levels are linearly related to the locomotor activity of mice, only when mice are running at high speed (Speakman, 2013).

Studies from our lab and others have shown that mice with targeted deletions of the ghrelin receptor gene (GHSR-KOs) exhibited attenuated food anticipatory activity to a scheduled meal (Blum et al., 2009; Lamont et al., 2014; LeSauter et al., 2009) that corresponded with decreased levels of cFOS activation in a number of hypothalamic regions (Blum et al. 2009). Furthermore, GHSR-KO's, and ghrelin-knockout (GHRL) mice also display attenuated feeding responses under a food-restriction paradigm (Abizaid, et al., 2006). However, under baseline conditions, both GHSR-KO & GHRL-null animals show similar RER levels to their WT littermates (Ma, Lin, Qin, Lu et al., 2011). Studies investigating the role of ghrelin in RER have been limited to those in which the mice have been fed a high fat diet (Tschop, et al., 2000; Zigman, et al., 2005; Sun, et al., 2007; Longo, et al., 2008). Baseline measures of RER between GHSR-KO and their WT littermates have consistently yielded no differences (Sun et al., 2007; Longo et al., 2008; Patterson, Khazall, MacKay, Anisman, Abizaid, 2013).

These findings led us to investigate the role of the GHSR in metabolic entrainment to a restricted feeding paradigm. We found that there were no differences at baseline between the genotypes in RER levels averaged over 24 hours, but that GHSR-KO mice exhibited higher RER levels at certain time points following lights on.

At the initiation of the food-restricted paradigm the differences between the genotypes was exaggerated. Time specific differences continued throughout experimental days with the GHSR-KO's now exhibiting significantly lower RER levels. One day two of experimentation, the acrophase of the WT's was significantly later than that of the KO's. However, following day 2 the acrophase and amplitude of both the RER and locomotor rhythmicity did not differ between the genotypes.

By Experimental days 3, 4 and 5, the differences of RER rhythmicity between the groups were limited to the WT express higher RER than the KO's from lights on till 11 am, in addition to a few random time points throughout the respective days. Both genotypes displayed entrained behaviour as evident by their RER rhythms during the days of recovery and re-exposure.

We hypothesized that the WT animals would exhibit elevated RER levels that in turn would indicate the promotion of carbohydrate utilization and that this elevation would be maintained throughout exposure to the food restriction paradigm. Contrary to that expectation, our results showed that GHSR-KO's exhibited blunted RER levels in comparison to the WT's at the initiation of the RF feeding, which would suggest the utilization of predominately fat stores. That difference gradually decreased as the experiment progressed. What is interesting in these data is the WT animals gradually began to utilize more of their fat stores as the experiment progressed.

Contrary to previous studies (Blum et al., 2009) we found that GHSR-KO had increased overall activity on specific days of the experimental paradigm. Baseline measures of increased activity

from the GHSR-KO models have been previously reported (Pfluger, Krichner, Gunnel, Schrott et al., 2008). Waddington-Lamont et al., (2014) reported that GHSR-KO displayed higher levels of activity in a RF paradigm under constant light.

Our lab has repeatedly shown that the GHSR-KO animals gradually show equivalent locomotor behavioural entrainment to the WT's (Blum et al. 2009; Waddington Lamont et al., 2014). We could not accurately measure FAA, in our third study, due to the disruptions that occurred as a result of timing of body weight measurements. However, once animals were left undisturbed during their final day of fasting, we observed that the KO animals exhibited significantly less FAA than their WT counterparts. Suggesting perhaps that the KO's may respond to external stressors, such as handling, during the experimental period more readily than the WT's.

It should be noted that the acrophase of the RER and the locomotor activity was not correlated in either of the genotypes. Further suggesting that RER rhythms were not influenced by locomotor activity.

Genetically modified animals lend a certain level of complexity in identifying energy utilization differences and effectively interpreting those results (Tschop, Speakman, Arch, Auwerx *et al.*, 2012). When exposed to a metabolic challenge in which a meal is scheduled during a non-active phase, the GHSR-KO animals may rely on a number of compensatory mechanisms to ensure that they obtain adequate nutrition and effective energy utilization.

Together these data further provide evidence that ghrelin and the GHSR play a role in energy utilization. Given the nature of the restricted feeding paradigm, this nutritional challenge is incredibly potent in resulting in increased levels of terminal ghrelin and consequently FAA. To this end the RER rhythmicity of RF animals was significantly phase advanced to match that food

presentation. These FEO outputs were not apparent in freely fed animal models that were given a scheduled HF treat, suggesting that the type FEO outputs may be dependent on the energy status of the animal. Therefore, grouping together FEO outputs under a negative energy balance and those presented under a positive energy balance may be misleading. To fully appreciate the prospective role of ghrelin in mediating the metabolic profiles, without the potential limitations of compensatory mechanism, future studies should be conducted to characterize the roles of central versus peripheral ghrelin signaling.

2.5. Graphs & Tables

Baseline	RER		Activity	
	Acrophase	Amplitude	Acrophase	Amplitude
AL	5.75 ± 2.34	0.149 ± .05	3.98 ± 0.918	1478.5 ± 334.5
RF	3.73 ± .84	0.105 ± .01	3.87 ± 0.771	1362.4 ± 168.3
HF	4.01 ± 1.53	0.118 ± .009	5.41 ± 2.512	1411 ± 93.2
Experimental				
AL	3.71 ± .83	0.100 ± .012	4.67 ± .44	1273.68 ± 135.3
RF	15.98 ± .37*	0.192 ± .015*	9.73 ± 1.08*	3463.75 ± 1183.9 ^ψ
HF	4.13 ± .76	0.103 ± .008	6.38 ± 1.07	1702.3 ± 194.1

Table 2.1: The influence of RF & HF diets on the acrophase and amplitude of RER and locomotor activity across the baseline and experimental periods.* p<0.05 RF v.s AL and HFp <0.09 RF v.s AL and HF.

A.

B.

Figure 2.1. The average RER for all groups across the light-dark cycle during baseline (Panel A) and experimental (Panel B) conditions. In Panel B the gray shading represents the time at which the RF animals were exposed to standard laboratory chow (10:00-14:00) while the superimposed outline (10:00-12:00) denotes the time frame in which the HF group received their high fat pellets. Significance ($P < 0.05$) between the RF group in comparison to the AL group and the HF group is denoted with (*), the significance between the RF group and the AL group is denoted with (δ) and the significance between the RF group and the HF group is denoted (ψ). Horizontal black bars represent the dark-phase (8pm-8am) of the light-dark cycle.

A.

B.

Figure 2.2. The average of the total activity during the baseline measurements of the AL, HF and RF groups (Panel A). In Panel B the gray shading represents the time at which the RF animals were exposed to chow (10:00-14:00) while the superimposed outline (10:00-12:00) denotes the time frame in which the HF group received their high fat pellets. Significance ($P<0.05$) between the RF group in comparison to the AL group and the HF group is denoted with (*). Horizontal black bars represent the dark-phase (8pm-8am) of the light-dark cycle.

Figure 2.3. The caloric intake fluctuated across the duration of the experiment (Panel A). Significance ($P<0.05$) between the RF group in comparison to the AL group and the HF group is denoted with (*), the significance between the RF group and the AL group is denoted with (δ), the significance between the RF group and the HF group is denoted (ψ) and finally the significance between the AL and HF group (α). Panel B depicts the daily body weight change across the experimental period between the AL, RF and HF groups.

Figure 2.4. Plasma glucose (A), Plasma corticosterone levels (B), Plasma acylated-ghrelin (C) concentrations in *ad libitum*-fed mice and in mice under a restricted feeding schedule or given daily 2 hour access to a high fat diet. The RF and HF group were sacrificed prior to their respective meals, the AL group were also staggered during those time-points. The RF group displayed lower blood glucose and corticosterone levels then both the AL and HF groups. Increased levels of acylated ghrelin were apparent in the RF group in comparison to both the AL and HF group (* $P < 0.05$).

Baseline	RER		Activity	
	Acrophase	Amplitude	Acrophase	Amplitude
AL	10.73 ± 3.65	.122 ± .057	6.07 ± 3.24	2336.6 ± 142.5
RF	9.99 ± 3.94	.166 ± .012	7.75 ± 3.05	2292.86 ± 145.8
Experimental				
AL	23.04 ± .44 *	0.265 ± .006*	3.25 ± 1.05*	1967.05 ± 266.52
RF	17.26 ± .11*	0.225 ± .004*	18.43 ± 1.09*	2404.48 ± 370.84

Table 2.2: The influence of RF on the acrophase and amplitude of RER and locomotor activity across the baseline and experimental periods. *p<0.05 AL v.s RF.

		RER		Activity	
		Acrophase	Amplitude	Acrophase	Amplitude
Exp1	AL	23.3 ± .078*	.527 ± .236	20.8 ± .42	2473.6 ± 152.9
	RF	13.27 ± .999*	.565 ± .004	17.25 ± 1.88	2268 ± 145.5
Exp2	AL	23.16 ± .353*	.146 ± .004*	17.93 ± .723	1706.7 ± 763.3
	RF	15.76 ± .353*	.183 ± .082*	21.05 ± .65	2173.12 ± 236.1
Exp3	AL	23.48 ± .087*	.159 ± .003*	17.38 ± 3.14	1643.1 ± 170.1
	RF	16.67 ± .239*	.207 ± .092*	20.29 ± 0.81	2320.25 ± 279.1
Exp4	AL	23.38 ± .161*	.166 ± .005*	18.89 ± 2.03	2075 ± 197.6
	RF	17.34 ± .287*	.198 ± .088*	19.46 ± .45	2018 ± 494.6
Exp5	AL	22.43 ± .308*	.17 ± .01	19.57 ± 1.34*	2332.75 ± 289.9
	RF	17.14 ± .333*	.194 ± .087	11.56 ± 1.49*	1935.7 ± 234.4
Exp6	AL	22.02 ± .463*	.553 ± .008	18.76 ± 1.5	2743.62 ± 414.8
	RF	18.72 ± .272*	.582 ± .26	8.25 ± 3.42	1814.3 ± 243.3

Table 2.3: The influence of RF on the acrophase and amplitude of daily measurements of RER and locomotor activity across the entire period of the experiment. *p<0.05 AL v.s RF *continued...*

		RER		Activity	
		Acrophase	Amplitude	Acrophase	Amplitude
Exp7	AL	22.25 ± .297*	.155 ± .003*	18.61 ± 1.46*	3734.87 ± 467.05
	RF	17.17 ± .234*	.207 ± .092*	12.75 ± .57*	2675.8 ± 308.9
Exp8	AL	23.48 ± .185*	.162 ± .003*	18.01 ± 1.33*	3026.5 ± 587.9
	RF	22.51 ± .183*	.597 ± .267*	8.11 ± 1.33*	2661.6 ± 441.9
Rec1	AL	24.31 ± .168*	.141 ± .008	19.08 ± 1.62	3164.1 ± 538.1
	RF	18.78 ± .755*	.088 ± .039	12.64 ± 2.6	2450.1 ± 238.1
Rex1	AL	23.06 ± .225*	.506 ± .003*	23.06 ± .225*	2451.13 ± 656.8
	RF	15.36 ± .057*	.604 ± .27*	15.36 ± .057*	2295 ± 232.5
Rex2	AL	23.48 ± .22	.165 ± .006	19.91 ± 1.17*	1776.75 ± 243.1
	RF	19.3 ± 3.37	.238 ± .106	13.28 ± 1.5*	2031.5 ± 186.1
Rec2	AL	23.66 ± .106*	.583 ± .003*	21.54 ± 1.26	2375.12 ± 270.7
	RF	16.28 ± 2.48*	.683 ± .305*	15.47 ± 3.44	3029.6 ± 487.4

Continued.. **Table 2.3:** The influence of RF on the acrophase and amplitude of daily measurements of RER and locomotor activity across the entire period of the experiment. *p<0.05 AL v.s RF

A.

Figure 2.5. The RER levels averaged across the experimental days to visualize a 24-hour span, indicates a clear difference in circadian rhythmicity of the RER between the AL group and the RF group. Period of food presentation is denoted with a vertical shaded whereas the dark horizontal bar represents night-phase RER levels. Food accessibility influenced RER circadian rhythmicity; the RF group displayed lower levels of RER prior to their scheduled meal and during the dark phase.

Figure 2.6 The RER rhythmicity of RF and AL throughout the entire duration of the experiment, representing Baseline, Experimental Day1, 2, 3, 4, & 5. The red line represents the time in which food was removed from the RF animals while the grey overlay is the time the food was presented. The white and black bar corresponds with the light-dark phases, respectively. *P<0.05 RF vs. AL *Continued..*

G.

H.

I.

J.

K.

L.

Continued... **Figure 2.6** The RER rhythmicity of RF and AL throughout the entire duration of the experiment, representing Experimental Day 6, 7, 8, Recovery Day 1 & Re-exposure Day 1 & 2. The red line represents the time in which food was removed from the RF animals, the grey bar represents the time food was left in the cage for the RF animals, whereas, the grey overlay is the time the food was presented. The white and black bar corresponds with the light-dark phases, respectively. * $P < 0.05$ RF vs. AL

Continued.

Figure 2.6 The RER rhythmicity of RF and AL throughout the entire duration of the experiment, representing Recovery Day 2 & Re-exposure Day 3. The red line represents the time in which food was removed from the RF animals for their final overnight fast. The white and black bar corresponds with the light-dark phases, respectively. *P<0.05 RF vs. AL

Figure 2.7. Indicates that average FAA behaviour displayed ($*P<0.05$), which demonstrates that the RF group entrained to the scheduled restricted feeding paradigm. The last day of RC (recovery) due to cage changes.

Figure 2.8. Effects of restricted feeding paradigm on metabolic end points of acylated ghrelin levels (A), blood glucose levels (B), caloric intake (C) and body weight (E). Baseline period is denoted as B1-B4; experimental period as EX1-EX7; recovery (RF group returned to baseline conditions) as RC; re-exposure (RF group re-exposed to RF paradigm) as Rex. Shaded areas visually represent the time point in which the RF group underwent the RF paradigm. * $P < 0.05$ relative to the AL control.

Baseline	RER		Activity	
	Acrophase	Amplitude	Acrophase	Amplitude
WT	3.68 ± 2.23	.164 ± .015	6.50 ± 3.19	1332.06 ± 111.96
KO	5.28 ± 2.19	.143 ± .017	3.83 ± 2.40	1518.07 ± 151.92
Experimental				
WT	17.17 ± .14	0.189 ± .06	19.08 ± 0.64	1266.19 ± 422.06
KO	17.05± .13	0.203 ± .007	19.85± .715	1531.74 ± 510.58

Table 2.4: The influence of the GHSR on mediating the effects of a RF paradigm on the acrophase and amplitude of RER and locomotor activity across the average experimental period.

		RER		Activity	
		Acrophase	Amplitude	Acrophase	Amplitude
Exp1	WT	13.14 ± .73	.207 ± .04	20.06 ± .89	1145.94 ± 64.45*
	KO	12.61 ± 1.42	.157 ± .018	21.4 ± .84	1973.72 ± 158.13*
Exp2	WT	18 ± .718*	.187 ± .011	19.5 ± 0.59	1210 ± 111.16
	KO	16.08± .233*	.182 ± .012	20.95± .89	1513.33 ± 115
Exp3	WT	15.65 ± 1.7	.149 ± .02	18.95 ± .73	1381.1 ± 136.6
	KO	16.89 ± .17	.188 ± .01	19.97 ± .81	1406.83 ± 152.2
Exp4	WT	17.47 ± .52	.152 ± .03	19.48± .94	1095.28 ± 110.56
	KO	16.72 ± .13	.194 ± .004	18.75 ± 1.42	1347.72 ± 155.72
Exp5	WT	18.32 ± .52	.589 ± .008	16.02 ± 1.94	1184.167 ± 162.66
	KO	18.73 ± .30	.586 ± .006	17.16 ± 1.65	1674.33 ± 190.31

Table 2.5: The influence of the GHSR on mediating the effects of a RF paradigm on acrophase and amplitude of RER and locomotor activity across the experimental period. *p <0.05 WT v.s KO.

Figure 2.9. Average experimental RER levels, averaged of Ex1 – Ex6, over a 24-hour period (A) $*P < 0.05$. The shaded overlay displays the time-point in which the scheduled meal was presented, dark bar indicates dark-phase activity.

Figure 2.10 The RER rhythmicity of WT and KO throughout the Baseline, Experimental Day1, 2, 3, 4, & 5. The red line represents the time in which food was removed from both genotypes while the grey overlay is the time the food was presented. The white and black bar corresponds with the light-dark phases, respectively. *P<0.05 WT vs. KO *Continue...*

ntinued... **Figure 2.10** Representation of Experimental Day 6, 7, 8, Recovery Day 1 & Re-exposure Day 1 & 2, in RER rhythmicity. The red line represents the time in which food was removed from both genotypes, the grey bar represents the time food was left in the cage for the RF animals, whereas, the grey overlay is the time the food was presented. The white and black bar corresponds with the light-dark phases, respectively. * $P < 0.05$ WT vs. KO *Continued...*

Continued ... **Figure 2.10** The RER rhythmicity of WT and KO during Recovery Day 2, Re-exposure Day 3 and the overnight fast. The red line represents the time in which food was removed from the both groups. The white and black bar corresponds with the light-dark phases, respectively. * $P < 0.05$ WT vs. KO.

Figure 2.11 Total during the experimental phase, the shaded overlay displays the time-point in which the scheduled meal was presented, dark bar indicates dark-phase activity.

Figure 2.12 The undisturbed FAA, measured two-hours prior to the scheduled meal, * $p < 0.05$ vs. WT.

Figure 2.13 Body weight (A) and caloric intake (B) was measured throughout the experiment and partitioned into Baseline, Experimental and Recovery phases. During the experimental phase both genotypes weighed significantly less then they did during baseline and recovery (significance denoted by δ). Consequently, during the experimental period both genotypes ingested less calories then what they ingested during baseline (significance denoted by δ). During the recovery both genotypes ingested significantly more calories then the had during baseline and experimental (significance denoted by α). Plasma blood glucose did not differ, between the genotypes at the end of the experiment (C).

Chapter 3

3.1. Introduction.

When animals are only given access to food at a discrete and regular time periods within a day, circadian locomotor activity patterns change. Following that 3-4 days of scheduled meal presentation, a marked increase in activity is observed in anticipation of the scheduled meal. This increase in locomotor activity is known as food anticipatory activity (FAA) and it is generally manifested 2-3hrs prior to mealtime (Mistlberger, 1993). When an animal is entrained to an environmental cue, FAA will reliably occur at the same time during the exposure to the cue and is maintained for a couple of days when the cue is removed (Mistlberger, 2011). In addition to locomotor activity, body temperature, plasma corticosterone levels (Boulos & Terman, 1980) and plasma ghrelin levels (Darzen, et al., 2006) are also increased in anticipation of and entrained to, the scheduled meal.

Food entrainment, unlike other environmental signals, appears not to depend on the hypothalamic suprachiasmatic nucleus (SCN), which, in mammals, is critical for synchronizing circadian rhythms of physiological function and behavior with relevant environmental stimuli (see Mistlberger, 2011). Within peripheral oscillators, scheduled food availability influences the timing of genes associated with circadian rhythm expression, realigning their expression to the specific time of the scheduled meal without affecting patterns of clock gene expression within the SCN itself (Waddington-Lamont, et al., 2007; Verwey & Amir, 2009). Changes in patterns of circadian gene expression, do not appear to be essential for food entrainment since mice with global deletion of some clock genes demonstrated “normal” FAA under a restricted feeding paradigm (Storch & Weitz 2009)

Lesion studies have not been able to identify a specific brain area that satisfies all the criteria for a food entrainable oscillator (reviewed by Davidson, 2006). The failure to identify a single neural substrate for FAA has led to the suggestion that the timing mechanism controlling the food entrainable oscillator (FEO), is a system that is comprised of a network of brain and peripheral oscillators that integrate hormonal signals, along with time cues to produce rhythms that match the timing of the meal (Davidson, 2006; Blum, Waddington Lamont, Rodrigues & Abizaid, 2012; Martinez-Merlos, et al., 2004; Davidson & Stephan, 1999; Bodosi, et al., 2004).

One of the signals thought to provide critical input to the FEO is the orexigenic gut-hormone ghrelin. Ghrelin, a 28 amino-acid peptide produced primarily by oxyntic cells of the stomach lining, increases food intake and alters metabolism to increase adiposity (Tschop, et al., 2000; Nakazato, et al., 2001). Plasma ghrelin levels rise prior to a scheduled meal (Darzen et al, 2006; Blum et al, 2009; Cummings et al, 2003), and peripheral administration of ghrelin increases daytime FAA and food intake in adlib fed mice (LeSauter et al., 2009). In contrast, mice with targeted mutations of the growth hormone secretagogue receptor gene (GHSR-KO mice) show attenuated FAA under a food-restricted paradigm in which the scheduled meal is presented in the middle of the light cycle (Blum, et al., 2009; Le Sauter et al, 2009; Davis et al, 2010; Waddington-Lamont et al, 2011).

In the restricted feeding paradigm used in most studies of FAA, animals are in a negative energy state when the meal is provided. FAA can also be reliably observed, however, in rats that are fed ad libitum and given a scheduled highly palatable snack during their non-active phase (Mistlberger & Rusk, 1988; Mendoza et al., 2005; Angeles-Castellanos, et al., 2008; Verwey, Khoja et al., 2007). The fact that ghrelin administration induces increased intake of palatable foods in sated

animals suggests that this hormone is a good candidate for the signal that drives FAA in the scheduled treat context and this possibility is addressed in the current experiments.

Mice, like rats, entrain to a restricted feeding schedule, but unlike rats they do not readily show entrainment to a high fat snack (Hsu, C.T., Patton, D.F., Mistelberger, R.E., Steele, A., 2010). Unpublished results from our own recent studies suggest that mice given scheduled access to high fat between ZT4 and ZT6 (12:00-14:00) show neither FAA nor display alterations to their metabolic profiles as measured via respiratory exchange ratios. Thus, here we first investigated whether presenting a scheduled treat of cookie dough, which combines high fat with high sugar, would induce FAA activity in mice and lead to behavioural entrainment. Once having established this paradigm we then investigated whether circulating ghrelin levels and or ghrelin sensitivity would increase in anticipation of daily exposure to a palatable treat and would become entrained to that treat. Finally we used mice with a targeted deletion of the ghrelin receptor (GHSR KO mice) to determine whether global deletion of ghrelin receptor activation would influence entrainment to a scheduled treat.

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3.2. Materials and Methods

3.2.1. Animals.

Adult CD-1 male mice (Charles River St. Constant QC), weighing 20-25g at arrival) were subjects in Experiments 1,2, and 3. Mice were single housed on arrival and given a week to acclimate to standard laboratory conditions. Male adult mice with targeted mutations to the ghrelin receptor gene (GHSR-KO) and their wildtype (WT) littermates were subjects in Experiment 4. These mice were obtained from a breeding colony at Carleton University and backcrossed to a C57bl/J6 strain. The original breeding pairs for this colony originated from Regeneron Pharmaceuticals in Tarrytown, NY, USA and were a kind gift from Dr. Mark Sleeman and Dr. Tams Horvath. All mice were housed in a temperature and humidity-controlled room with 12:12hr light-dark cycle, with light onset at 8:00AM (*zeitgeber* time (ZT) 0). All methods described below were approved by the Carleton University Animal Care Committee, and followed the guidelines of the Canadian Council for Animal Care.

3.2.2. Scheduled Treat Procedure.

Across all the experiments daily measurements were taken of food intake, body weight and locomotor activity. All mice had *ad libitum* access to standard laboratory chow (18% caloric content from fat; 3.3 Kcal/g Harlan, Mississauga, ON, Canada) and tap water. After one week of baseline, mice in the experimental condition were given two hour access to commercial cookie dough (Pillsbury Chocolate Chip™, Fat 0.22 g, Carbs .63 g , Protein .031g ; 4.45 kCal/g) from ZT 6- ZT8 . The length of the experimental period varied between the experiments but all animals were sacrificed 2 hours prior to the scheduled snack.

3.2.3. Behavioural Monitoring.

Following the acclimation period, animals were single-housed and their home cages placed within locomotor activity frames (Omnitech Electronics, Inc.) for the entire duration of the experiment. Activity levels from each mouse were measured in 10 min bins for the duration of the experiment. All mice, regardless of experimental condition were housed in the same room throughout the experiment.

3.2.4. Blood Sample Analyses.

Blood glucose levels were measured from trunk blood immediately following decapitation, using glucose strips read on a Contour glucose meter (Bayer Corp., Pittsburgh, Pennsylvania). Trunk blood was then transferred to EDTA-coated tubes kept on ice, and centrifuged at 3000-x *g* for 15 minutes (at 4°C). The plasma layer was collected with a micropipette, and aliquoted in three separate 1.5ml microcentrifuge tubes. One 50µl plasma aliquot was treated with 2.7µl of 0.1M HCl and 5 µl of 100 mM of esterase inhibitor p-hydroxymercuribenzoic acid (PHMB) to protect plasma acylated ghrelin from degrading. Samples were then stored at -80°C until hormone analyses were conducted. Plasma acylated ghrelin and leptin levels were measured using commercially available ELISA kits (EMD Millipore, St.Charles, Missouri, Cat. # EZRGRA-90K intra-variability of 11.8% and Cat. #EZRL-83K intra-variability of 35%, respectively). An RIA assay (CN Biomedicals, Inc., Aliso Viego, California) with 10% intra-assay variability was used to measure plasma corticosterone levels.

3.2.5. Experiment One -

Does scheduled cookie dough presentation elicit FAA and is this associated with an increase in acyl-ghrelin levels?

After one week of baseline, mice were randomly assigned to control (n=10) and experimental conditions (n=10). Mice in the control group were given ad libitum access to standard chow. Those in the experimental group received ad libitum access to chow plus two hour access to cookie dough from ZT 6- ZT8 daily for 17-days. After 17 days of scheduled cookie dough exposure, mice underwent a recovery phase in which they were placed in control conditions and were not disturbed, no measurements of body weight nor food intake were taken during this time. Following the 6 days of recovery, animals were rapidly decapitated between ZT4 & ZT5. Carcasses were frozen at -80°C and later analyzed for body composition using an EchoMRI-1100 (System ID EF-020) at the Nutrition Research Division of Health Canada.

3.2.6. Experiment Two –

Do satiated mice entrain to the palatable snack and is this associated with a specific pattern of activation within hypothalamic nuclei?

Mice were randomly assigned to either the control group (n=10) or experimental group (n=10). After one week of baseline, experimental animals were given two hours access to the palatable snack at ZT6 to ZT8 for 21 days followed by a 6-day recovery period in which the treat was not presented and finally a 6-day re-exposure period during which scheduled treat presentation resumed. This procedural timeline was adapted from our restricted feeding paradigm as published in Blum et al., 2009.

On the last day of the study, mice were deeply anaesthetized with sodium pentobarbital and transcardially perfused with 0.9% sterile saline, followed by 4% paraformaldehyde, during ZT4-ZT6. Brains were dissected, post fixed overnight and cryoprotected with a 30% sucrose solution for 48 hours before being sectioned on a cryostat (Thermofisher, Shandon). One out of every four 60 μ sections were serially collected making four sets of sections per mouse. One of these sets was used to compare brain activation in anticipation of the palatable treat using cFos immunohistochemistry. Following phosphate buffer (PBS) washes (5mins x 5 washes), sections were incubated with 0.3% H₂O₂ in 0.1M PBS for 30 mins at room temperature. Sections were washed (5mins x 3 times) and then incubated on shaker for 60 mins at room temperature with a preblocking solution (0.1M PBS + Triton X + Albium Bovine fraction (BSA)). Sections were then incubated for 48 hours using a 1:10,000 cFOS (Oncogene Science, Boston, MA, USA) primary diluted in a solution of PBS + Triton X + BSA. Following PBS washes (5mins x 3 washes), sections were incubated in a 1:250 biotinylated anti-rabbit IgG made in donkey (Jackson ImmunoResearch Laboratories, Inc., Westgrove, PA, USA) diluted in a solution of PBS + Triton X + BSA. Sections were then washed in PBS (5mins x 3 washes) and incubated in an AB complex – avidin-biotin-peroxidase complex (Vectastain Elite ABC Kit; Vector Laboratories, Inc., Burlington, ON, Canada) for 1 hour. After a final PBS wash (5mins x 3 washes), sections were incubated for 5 minutes in 0.05% 3,3'-diaminobenzidine (DAB) diluted in PB and for an additional 10mins in a DAB + 1% H₂O₂. Sections were rinsed, mounted onto gel-coated slides, and dehydrated using a series of alcohol and Clearene (Surgipath, Lecia, Microsystems Inc., Concord, ON, Canada). Slides were coverslipped using PermOUNT (Fisher Scientific, Toronto, ON, Canada). Sections were examined using an Axioplan Universal microscope (Carl Zeiss Light Microscopy, Gottingen, Germany) and the attached

OptixCam SummitK2 was used to capture digital images (Microscope, Roanoke, VA, USA). Bilateral counts were taken from each animal for the SCN (Bregma -0.34 to -0.82), the ARC (Bregma -1.46 to -1.82), the VMH (Bregma -1.46 to -1.82), the DMH (Bregma -1.46 to -1.82), the LH (Bregma -1.06 to -1.58), and the PVN (Bregma -0.94 to -1.22). Bregma points were determined using Paxinos & Franklin's 2nd edition of The Mouse Brain. Cells were manually counted using open-source Image J (<https://imagej.nih.gov/ij/>). Image was adjusted to the threshold of 135-145 and the pixel was set to 10=140 with circularity set to 0.5-1. Four sections per animal were taken of the ARC, DMH, VMH, LH, SCN and three sections were taken per animal from the PVN. The average number of Fos +ve cells per section for each area of interest was calculated for each mouse and these data were used for analysis. Counter was blind to the groups in which the animals belong too.

3.2.7. Experiment Three-

Do levels of ghrelin, its receptors or expression of other metabolic peptides change across the period of entrainment?

Mice (n=60) were assigned to control (n=6/ per timepoint) or experimental conditions (n=6/ per timepoint) and killed at ZT5 on five different days across the entrainment period: Baseline Day 7 (B7) Experimental day E2, E4, E9, or E14. These time points were selected on the basis of demonstration of FAA development in experiment 1 and 2.

Brains were collected immediately after decapitation, flash frozen in liquid nitrogen and stored at -80°C until processing for RT-qPCR. At that time, brains were thawed, and sliced to collect 1mm coronal sections using a brain-slicing matrix (Zivic Instruments) to micro dissect different brain regions with a procedure that was adapted from the classic Palkowitz, 1972. Using the

ventral view of the brain, we extracted bilateral micro punches of the SCN , ARC-VMH complex, DMH, LH, dorsal hippocampus and VTA. Once collected, punches from each region were stored in microfuge tubes with 500ml of Trizol at -80°C. For RNA extraction, brain punches were homogenized in Trizol and precipitated with 13µl of linear acrylamide then spun. The quality and concentration of the RNA was determined by the absorbance at 280nm and 260nm with a Thermo Scientific Nanodrop 100 spectrophotometer (Thermo Scientific, Rockland, Illinois). To synthesize cDNA, a commercially available kit was used, iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Mississauga, ON, Canada). Samples were incubated in a PTC-200 Thermal Cycler (MJ Research, Watertown, Massachusetts) using the reaction protocol provided by the manufacturer. Samples were stored at -20°C. RT-qPCR was used to determine fold changes using the $2^{-\Delta\Delta C_t}$ method (Schmittgen & Livak, 2008) using β -actin as a control transcript. Setting up the PCR plate, 5µl of each cDNA sample was added, in duplicates, along with 2µl of the GHS-R/NPY/AGRP primer solution, 3µl of Milli-Q water, and 10µl of SYBR Green Super Mix with fluorescein (Bio-Rad Laboratories, Mississauga, ON, Canada). The plate was run on a CFX Connect Real-Time System (Bio-Rad Laboratories, Mississauga, ON, Canada) for 30 sec at 95°C followed by 40 cycles of: 10 sec at 95°C, 40 sec at 60°C, then 65°C for 5 sec and 96°C for 50 sec.

Primer	Forward (5'-3')	Reverse (5'-3')
GHSR	CTCAGGGACCAGAACCACAAAC	ACAAAGGACACCAGGTTGCAG
AgRP	CGGAGGTGCTAGATCCACAGA	AGGACTCGTGCAGCCTTACAC
NPY	TACCCCTCCAAGCCGGACAA	TTTCATTTCCTCATCACCACATG
β-actin	GAACCCTAAGGCCAACCGTG	GGTACGACCAGAGGCATACAG

3.2.8. Experiment 4: What is the role of ghrelin receptor activation in the entrainment to palatable snacks in satiated mice models of FAA?

Following a nine-day baseline, GHSRKO and WT mice were given two- hour access to a palatable snack at ZT 6- ZT 8 for fifteen days followed by a seven-day recovery period, in which feeding conditions were returned to baseline. Mice were then re-exposed to the experimental conditions for two-days.

3.2.9. Statistical Analysis.

Where appropriate, data are expressed in graphs as mean $\pm$ SEM. Data are analyzed using a ANOVA and post-hoc analyses were conveyed using Least Significant Difference (LSD) test with significant values set at $P < 0.05$. Daily measures were analyzed with repeated Measures Analysis where appropriate, with Bonferroni post-hocs. For violations of Sphericity, the Greenhouse-Geisser correction was used when the epsilon (ϵ) values were $\epsilon < 0.75$ however, for $\epsilon > 0.75$ the values from the Huynh-Feldt correction were used (Verma, 2016). Where applicable data were analyzed using independent t –tests with Levene’s corrections when necessary. Significance criterion was set at $P < 0.05$. Statistical analysis was performed with SPSS Statistics version 20 (IBM©, 2011). Data were visualized using Prism 5 (Graphpad Software, INC., 2008).

3.3. Results.

3.3.1 Experiment One -

Light-phase scheduled cookie dough presentation induced FAA in satiated mice but did not result in increased plasma levels of acylated-ghrelin.

Mice in the cookie dough (CD) group exhibited greater locomotor activity in the 2hrs prior to the scheduled snack than control mice across the first 16 days of cookie dough exposure and this behaviour persisted across the recovery period (Figure 3.1, Panel A). Analyses of these data yielded statistically significant main effects for group ($F(1,17)=6.441, p<0.05$), day ($F(25,4425)=5.215, p<0.05$) as well as a day x group interaction ($F(25, 425)=3.239, p<0.05$). Post hoc pairwise comparisons showed that mice in the CD condition moved more than those in the control condition on Days E3, E6-E8, E11-E13, E16, R2 & R5 ($p<0.05$), with time points E1, E14, E15, R3, R4, R6 being marginally significant ($p>0.05$ but $p<0.1$). Repeated measures analysis of the average time-points (baseline, experimental and recovery) yielded significant main effects of time-points ($F(1.155,19.641)=6.884, p<0.05$) and group ($F(1,17)=5.770, p<0.05$) in addition to a time-point x group interaction $F(1.155,19.641)=4.778, p<0.05$). Pairwise comparisons disclosed that the CD group displayed an increase in FAA when compared to the control group during the experimental period and the recovery period (Figure 3.1, Panel B).

Figure 3.2 presented that neither plasma acyl ghrelin (Panel A), leptin (Panel B), corticosterone (Panel C) nor glucose (Panel D) concentrations differed significantly between groups

($t(16) = .054$, $p > 0.05$; $t(16) = -.189$, $p > 0.05$; $t(16) = -.097$, $p > 0.05$; $t(17) = 1.772$, $p > 0.05$ respectively.

Caloric intake did not differ between the CD and control groups ($p > 0.05$) (Figure 3, Panel C) but there was a trend towards a higher average body weight for CD group ($t(17) = 1.957$, $p = .067$) (Figure 3.3, Panel A). Similarly percent body fat did not differ between groups ($t(17) = 1.100$, $p > 0.05$), interestingly; there was a trend towards a significantly greater fat body mass in CD than in the control group ($t(17) = -2.033$, $p = .058$) (Figure 3.3, Panel B).

3.3.2. Experiment Two –

Animals developed entrainment of locomotor activity to scheduled cookie dough presentation accompanied by activation of a number of hypothalamic areas

In this experiment we first aimed to replicate the finding that mice show FAA to scheduled presentation of cookie dough found in Experiment 1 and to elaborate on this by investigating whether or not satiated mice could develop behavioural entrainment to a scheduled treat. To investigate this a re-exposure phase was introduced after the recovery phase of the experiment, in which animals were presented with the CD again. FAA was monitored during the experimental, recovery and re-exposure phase. As in experiment 1, mice in the experimental condition presented with FAA, 2 hours prior to cookie dough presentation. Analysis of the daily FAA behaviour resulted in significant main effect of days $F(22,594) = 1.631$, $p < 0.05$, group $F(1,18) = 11.610$, $p < 0.05$) and a days x group interaction $F(33,594) = 2.081$, $p < 0.05$). Pairwise comparisons indicated that the experimental group displayed a higher level of FAA when compared to the controls during E7-E11,

E13, E16-R2, R4 and REX4-REX6. On days E6 and E14 the experimental FAA approached significance ($p>0.05$ but $p<0.1$) (Figure 3.4, Panel A). Average FAA across the experimental time points (Baseline, Experimental, Recovery and Re-exposure) indicated no main effect of time points ($p>0.05$) but a main effect of group ($F(1,18)=9.061$, $p<0.05$) and a time point x group interaction effect ($F(1.936, 34.855)=5.059$, $p<0.05$). Pairwise comparisons showcased that the experimental group displayed significantly higher FAA behaviour during the Experimental, Recovery and re-exposure time points when compared to the control group (Figure 3.4, Panel B).

Experimental animals demonstrated increased C-Fos activation in the DMH, VMH and ARC.

To investigate which hypothalamic regions were activated in anticipation of a scheduled treat, the brains from mice entrained to CD and those of controls were processed for c-Fos immunohistochemistry. Representative sections are shown in Figure 3.6. There were greater numbers of c-Fos immunoreactive cells in the DMH, VMH and ARC of the experimental mice than in the controls (DMH $t(11.157)=2.816$, $p=.017$; VMH $t(9.847)=2.272$, $p=.047$; ARC $t(14)=3.620$, $p=.003$) and a trend towards higher levels in the PVN ($t(14)=2.038$, $p=.061$). Counts of cFos positive cells in the SCN and LH did not differ between groups (Figure 3.5).

Entrainment did not associate with elevated levels of acylated ghrelin, but was associated with higher plasma leptin levels.

Similar to the results of experiment 1, acyl ghrelin levels $t(14)=-.396$, $p>0.05$ (Figure 3.7, Panel A) and blood glucose levels at the time of decapitation did not differ between groups $t(17)=1.009$, $p>0.05$ (Figure 7, Panel B). However, in this experiment terminal leptin levels were

significantly higher in the experimental group than the controls $t(15) = 2.199$, $p = .044$ (Figure 3.7, Panel C).

Body weight gain relative to baseline did not differ between the groups over any of the phases of the experiment ($p > 0.05$). Similarly, although analysis of the average daily calories consumed across baseline, experimental, recovery and re-exposure, indicated a significant main effect of phase of the experiment time points $F(1.940, 32.976) = 6.726$, $p < 0.05$, but no main effect of group ($p > 0.05$). (Figure 3.7, panels D & E). There was a significant phase $\times$ group interaction ($F(1.940, 32.976) = 11.558$, $p < 0.05$). Pairwise comparisons showed that the experimental animals ingested significantly fewer calories than controls during the recovery phase when cookie dough was not available ($p < 0.05$).

3.3.3. Experiment Three-

Increased GHSR expression in the ARC in anticipation of a scheduled treat.

The results of experiments 1 and 2 demonstrated that mice showed both food anticipatory activity and entrained to daily scheduled exposure of a treat in the form of cookie dough. Data obtained at the termination of these experiments did not show any increases in circulating ghrelin levels associated with FAA. In this experiment we investigated whether ghrelin levels and or sensitivity to ghrelin, as reflected in changes in expression of the GHSR were associated with the development and entrainment of FAA by measuring these endpoints on different days across the experiment.

Plasma acyl ghrelin levels increased across time and were higher on E9 and E14 for both control and experimental groups (significant main effect for time ($F(4,47) = 6.294$, $p < 0.05$) there

was no the main effect for group nor the group x time interaction effect were statistically significant (Figure 3.8, A)

Expression levels of GHSR, in the ARC, DMH, LH, SCN, VTA, Hipp (Figure 3.10) were compared between experimental and control mice. There were no group differences found in DMH, LH, VTA, Hipp or SCN (Figure 10, Panels B, D, F, E and A, correspondingly). In the LH there was a main effect of time point ($F(4,47)=6.294$, $p<0.05$), pairwise comparisons indicate that levels were elevated during E4 & E5. There was an effect of time in the SCN $F(1,41)=6.167$, $p=.001$ with significantly higher GHSR expression on E4 than any other day of sacrifice. Interestingly there were higher levels of GHSR in the ARC of the experimental mice than in controls $F(1,43)=6.901$, $p=.012$) and this was modified by a trend towards an interaction between cohort and group $F(3,43)=2.410$, $p=.080$ reflecting the higher levels of GHSR expression on E4 and E9 in the experimental group (Figure 10, Panel C). This spurred us to further examine the GHSR pathways within the ARC, specifically looking at changes in NPY and AGRP expression. There were no main effects ($p>0.05$) or significant interactions between the groups and cohorts in either the NPY mRNA levels $F(3,42)=.415$, $p>0.05$ or AGRP mRNA levels $F(3,45)=.807$, $p>0.05$ (Figure 11, Panels A & B, respectively).

Caloric intake did not differ between groups or across time ($p>0.05$) and although all groups gained weight ($F(4,51)=7.366$, $p>0.05$) they did so at a similar rate (Figure, 3.9).

3.3.4. Experiment Four -

GHSR-KO animals and their WT counterparts expressed FAA in response to a scheduled treat, but did not entrain to presentation of cookie dough.

Daily FAA measurements showed significant main effect of time across the baseline, experimental, recovery and re-exposure time points ($F(5.247, 62.961) = 5.109, p < 0.05$), but no main effect of genotype ($F(1, 12) = .406, p > 0.05$) nor a genotype x time interaction, ($F(5.247, 62.961) = .858, p < 0.05$) (Figure 3.12, A) suggesting that both groups showed FAA. To that effect, the analysis of FAA across baseline, experimental, recovery and re-exposure revealed a main effect of time ($F(3, 36) = 14.228, p < 0.05$) but no main effect of genotype ($F(1, 12) = .540, p > 0.05$) nor a time point x genotype interaction ($F(3, 36) = 1.079, p > 0.05$). Pairwise comparisons of time points showed both genotypes expressed robust FAA behaviours during the experimental phase. But unlike the patterns of behavior seen in Experiments 1 and 2, FAA was immediately extinguished once the treat was removed (Figure 3.12, B). Hence, indicated that there was no evidence of entrainment displayed by either group.

GHSR KO mice had significantly lower glucose plasma levels ($t(12) = 2.176, p = .050$) than WT controls but leptin levels did not differ between the genotypes ($t(11) = -.074, p > 0.05$).

Weight gain did not differ between groups during the experimental ($t(12) = 1.341, p > 0.05$) and recovery periods ($t(12) = 1.169, p > 0.05$) but the WT group did show a greater weight gain in the re-exposure phase although this difference was only marginally significant ($t(12) = 2.084, p = .059$).

There were no effects of either genotype or phase of the experiment on total caloric intake time ($F(1.024, 6.146) = .640, p > 0.05$) or genotype ($F(1,6) = .024, p > 0.05$) or their interaction ($F(1.024, 6.146) = .690, p > 0.05$) on total caloric intake (Figure, 3.14).

3.4. Discussion

The purpose of this set of experiments was to determine whether mice would entrain to a palatable snack as robustly and consistently as rat models, and the contribution of ghrelin and its receptor to palatable snack entrainment as reflected in food anticipatory behaviour (FAA). Our results show that mice established FAA in anticipation of a palatable snack and that this increase in FAA is associated with increased hypothalamic activation as measured by Fos expression. We found that although our scheduled treat paradigm reliably produced FAA in different strains of mice, that was not the case for behavioural entrainment, which was shown only by certain strains.

The finding that satiated mice will show FAA to a timed palatable meal suggests that in the mouse that being in a negative energy balance is not necessary for scheduled food presentation to become a *zeitgeber*. While there are a number of studies showing that rats anticipate a variety of palatable snacks (Table 1), experiments using mice have not consistently nor reliably in observing

robust FAA in anticipation of palatable foods (Table 2). This discordance in results might be associated with differences in activity recording and the type of treat presented. In our study, we used locomotor activity frames that measure the number of beam breaks caused by a moving animal within its home cage. Bake et al (2014), used a similar method of recording locomotor activity under a reverse light-dark cycle and demonstrated FAA and entrainment to a palatable snack (high fat diet pellets). Other methods that have been used to measure FAA includes wheel running (Mistlberger & Rusak, 1988; van der Vinne, Akkerman, Lanting, Reide et al., 2015), food hopper approaches (Hsu et al., 2010) and video tracking (Gallardo, Gunapala, King, & Steele, 2012).

The form of the scheduled treat is clearly also an important factor in discrepancies in results between studies. Commercial chocolate pieces and chocolate ensure presentation reliably produced FAA in rat models of palatable food anticipation (Table 3.1) but Hsu et al. (2010), found that these treats did not induce FAA in mice. Rather high-fat diet was the only diet that elicited FAA behaviours as measured by food hopper approaches. On the basis of these results the authors concluded that, in mice, FAA is diet and behaviour specific Hsu et al. (2010).

In experiment 1 we found that food anticipatory activity developed during the experimental phase and was maintained during the recovery phase in which mice were placed back in baseline conditions. The scheduled treat did not invoke a strong shift in caloric intake or body weight and contrary to our hypothesis FAA did not correlate with increased levels of ghrelin.

Paradoxically, animals in Experiment 2 that were exposed to a scheduled treat expressed elevated levels of leptin but not acyl ghrelin levels. Leptin role in anticipatory feeding behaviours under a scheduled treat paradigm is undecided (Bake, Baron, Duncan, Morgan & Mercer, 2017). Leptin is known to decrease food intake and increase energy expenditure (Friedman, 2009). Under a

RF paradigm physiological doses of leptin in *ob/ob* mice inhibited the development of FAA (Ribeiro, et al. 2011). Whereas other studies have reported that leptin KO mice established FAA like behaviours (Gunapala, Gallardo, Hsu & Steele, 2011).

Overall, there was an increase in Fos expression throughout the hypothalamus of mice that were given daily timed access to the cookie dough and this increase was statistically significant in the ARC, VMH and DMH. All three of these areas play an important role in energy metabolism (Morton, Meek & Schwartz). The ARC contains the anabolic set of neuropeptides, AGRP and Neuropeptide-Y (NPY). Studies show activation of AGRP neurons influences feeding behaviour (Krashes, Koda, Ye, Rogan et al., 2011), and that these neurons modulate the negative affective state that is associated with hunger (Betley, Xu, Cao, Gong et al, 2015). Whether a similar negative affective state is produced by daily timed presentation of a snack is not known, but this idea, along with our Fos data, would predict that repeated presentation of a palatable snack induces feeding through the stimulation of AGRP neurons.

The mechanism through which ghrelin enhances FAA in animals on restricted feeding schedules is unclear. It is likely that, in this context, higher ghrelin titers influence the activity of a number of peripheral organs and ascending vagal information as well as hypothalamic and extrahypothalamic brain regions to enhance FAA and food entrainment (Blum et al, 2009; Blum et al, 2011; Blum et al, 2012).

Ghrelin's role in promoting food-related behaviours in response to a scheduled treat may be through its stimulatory actions on the ARC. Ghrelin activates that GHSRs that are co-expressed on NPY/AGRP –expressing neurons in the ARC (Mondal, et al., 2004) to induce food intake and bodyweight (Kamegai, Tamura, Shimizu, Ishil et al., 2001).

Under a restricted feeding paradigm, we know that GHSR signaling in the DMH and VMH influence the development and expression of FAA (Merkestien et al., 2014). The VMH is classically known for its role in satiety (Hetherington & Ranson, 1940; King, 2006) but additionally as an area that is activated during the anticipation of a meal (Ribeiro, Sawa, Carren-LeSauter, LeSauter et al., 2007). The DMH has also shown cFos activation in anticipation of scheduled meal (Angeles-Castellanos, Aquilar-Robiero, Escobar, 2006) but its specific role in food anticipatory activity is controversial (Landry, et al., 2005; Gooley, et al., 2006; Landry, Yamakawa, Webb, Mear & Mistlberger, 2007; Moriya, et al., 2009). Interestingly, rat models of non-homeostatic entrainment to a scheduled treat have not reported cFos activation in the hypothalamic regions (Mendoza, et al., 2005).

Because ghrelin activates NPY/AGRP neurons (Chen, Chen, Zhou, Pardhan et al., 2017), one would expect that ghrelin concentrations to rise in anticipation of the palatable snack, and/or that ghrelin sensitivity in the brain increases with the number of days in which an animal is exposed to the scheduled cookie dough delivery. In experiment 3, we did observe a gradual increase in acyl ghrelin concentrations, with concentrations after 9 and 15 days of cookie dough exposure, for both control and experimental groups. This comes in contrast to Merkestein et al., 2010, who reported that FAA was correlated with increases in plasma ghrelin in rat models of palatable food anticipation. These data suggest that in mouse models of palatable food anticipation, plasma ghrelin concentrations may not be important for the ontogeny of FAA in anticipation to cookie dough.

In contrast to the data obtained on ghrelin concentrations, we observed changes in the expression of GHSR mRNA within the hypothalamus across the entrainment period. Indeed, between days 4 and day 9 of cookie dough presentation, we observed an increase in GHSR mRNA

expression in the SCN and ARC, an effect not observed in other hypothalamic or extrahypothalamic regions. These data support the notion that the ontogeny of FAA in response to daily scheduled palatable treats involves an increased sensitivity to ghrelin in the SCN and ARC, regions that are important for the entrainment of metabolic signals to the circadian system (Blum et al., 2009). It remains unknown whether ghrelin sensitivity in these areas, is required for the maintenance of FAA driven by cookie dough.

To further investigate the consequences of increased expression of the GHSR in the ARC, we examined mRNA levels of NPY and AgRP across all the time points of experiment 4. We hypothesized that the increase of GHSR expression in the ARC would relate to increases in expression in NPY or/and AgRP levels. However, we did not find any statistically significant changes in the expression of either peptide. This was surprising considering that the GHSR has been reported to mediate ghrelin's effects on the NPY/AgRP neurons (Abizaid et al., 2008), however its also been reported that ghrelin excites the anorexic pro-opiomelanocortin (POMC) neurons (Chen, et al. 2017). In addition to being co-expressed with NPY/AgRP neurons, the GHSR is also colocalized with melanocortin-3 (MC3R) receptors (Rediger, Peichowski, Yi, Tarnow et al, 2011) and G protein-coupled receptor (GPCR) 83 in the ARC (Müller, Müller, Habegger, Meyer, et al., 2013). Interestingly, POMC neurons in the ARC have been implicated in influencing MC3R neurons in the ventral tegmental area (VTA) that ultimately regulates palatable food intake (Pandit, Omrani, Luljendijk, Vrind et al., 2016).

In experiment 4 we used GHSR-KO animals and their wild type counterparts to further investigate the role of the GHSR in development of the FAA. While both the WT's and KOs established FAA, neither group entrained to the paradigm. Their behaviour levels dropped down to

baseline almost immediately after the removal of the scheduled treat. Suggesting perhaps that FAA and entrainment to a scheduled treat may be differentially exhibited in various strains. Interestingly, Gallardo et al., 2012 reported strain differences in the development of FAA between experiments utilizing C57/BL6 and an inbred 129S1 strain. Although both strains exhibited FAA under a negative energy balance, the 129S1 strain failed to develop FAA to a scheduled treat under a positive energy balance. There are currently no studies that directly investigate the potential metabolic differences between the aforementioned strains. However, neuroanatomical differences have been shown between C57/BL6, 129S1 and CD-1 mice (Chen, Kovacevic, Lobaugh, Sled et al., 2006) highlighting the need to further consider strain differences under metabolic challenges.

The question that remains unanswered is related to what maintains FAA to palatable foods if not plasma ghrelin secretion and/or sensitivity to ghrelin. One potentially could argue that ghrelin facilitates cognitive and reward processes associated with the encoding of environmental stimuli associated with the presentation of the oncoming palatable snack. This would include the time at which the lights go on in the experimental room, and interoceptive signals that are reset by the consumption of the snack the day before. Once those memories are formed, ghrelin may not be required for FAA as the cues may then elicit memories associated with the meal and these may be the ones driving the behavior. In other words, ghrelin may facilitate the formation of association between temporal and visceral cues and the daily presentation of the snack, but once these associations are made, the behavior is maintained by the cues themselves. This possibility requires further inquiry focusing on ghrelin's actions on the reward pathway, the hippocampus and the amygdala, areas that are involved in cue-potentiated feeding and memory.

3.5 Graphs & Tables

A.

B.

Figure 3.1. Monitored on a day-by-day basis, the FAA development and maintenance (Panel A) comparing the control (CON) and the cookie dough (CD) group. Average FAA grouped by baseline, experimental and recovery periods (Panel B), * $P < 0.05$ vs Control. Behavioural data from days E2, E10 and R1 were removed from the analysis due to disruptions caused by cage changes.

Figure 3.2. Illustrates the metabolic endpoints of the cookie dough (CD) and control (CON) group throughout the experimental period. Plasma measurements of Acylated Ghrelin (Panel A), Leptin (Panel B) Corticosterone (Panel C) and Blood glucose (Panel D) levels.

Figure 3.3. The CD weighed in slightly heavier than the CON ($p=0.067$) (Panel A). Body composition measurements comparing lean body mass and body fat (Panel B). Total calories ingested from standard laboratory chow at baseline in compared to the experimental period (Panel C). The CD group ingested approximately 32% of their daily calories from cookie dough (Panel D).

Figure 3.4. Panel A. depicts the daily patterns of FAA across the time course of the experiment. Panel B., shows the average FAA activity in the CON and CD animals across the set timepoints of baseline (Base), experimental (Exp), recovery (Rec) and re-exposure (ReEx). Days E2, E8, E15, R1 and RE2 were moved due to disruptions associated with cage changes.

Figure 3.5. Depicts histological results of cFOS activation of hypothalamic regions comparing control and CD animals, $*P < 0.05$ vs CON.

Figure 3.6. Panel A is the visual representation of the range of bregma levels (Bregma -1.46 to -1.82) used to quantify the ARC, VMH and DMH). These figures were taken from *The Mouse Brain in Stereotaxic Coordinates*, Second Edition (Paxinos & Franklin, 2001). Panel B, C are visual representations of the ARC, from a control and experimental animal, respectively. *Continued ..*

*Continued ...***Figure 3.6** Representation of the control DMH (Panel D.) and experimental DMH (Panel E.). Panel F and G represent the VMH in control and experimental animals.

Figure 3.7. Describes end-point metabolic profiles of Blood glucose (A), Acylated ghrelin (B) and Leptin (C). Daily measurements of body weight (D) and caloric intake (E) are represented as averages across the different time points of the experiment. Although no differences were found between the groups in terms of caloric intake, the CD group ingested 38% and 34.4% of their calories from cookie dough during the experimental (Exp-CD) and re-exposure phase (Rex-CD), respectively. * $P > 0.05$ vs CON.

Figure 3.8. Blood glucose did not differ between the CD groups or cohorts (A). Acyl-ghrelin levels did not differ between groups but did differ between cohorts. Time point E9 and E 14 presented with significantly higher levels of acyl-ghrelin then those animals in B7, E2 and E4 $\delta P < 0.05$.

Figure 3.9. Total calories ingested between the groups did not diff across the time points (A). There was a gradual increase in body weight as the experiment progressed. There was a steady, increase in percent of calories ingested from cookie dough. The E2 group ingested 11.5% of their total caloric intake from cookie dough for the two-day period. The E4 group on average ingested 13.1% of their total calories from cookie dough. The E9 group ingested 20% of their total calories from cookie dough, whereas the E14 group ingested, on average, 31.1% of their calories from cookie dough (Panel C).

Figure 3.10. In anticipation of the scheduled treat, the GHSR fold changes were investigated in the SCN (A), DMH (B), ARC (C), LH (D), HIPP (E) and the VTA (F) across all the time points. Both control and experimental groups at the SCN E4 was significantly higher then all other time points $*P<0.05$. In the ARC, the CD groups were consistently higher across the time points $^{\delta}P<0.05$.

Figure 3.11. In the ARC, fold changes of NPY expression (A) and AgRP expression (B) were examined at all time points.

Figure 3.12. Daily measurements of FAA across the duration of the experiment (A), grey overlay indicates experimental period. The average FAA behaviour across the duration of experimental procedures, $^{\delta}P < 0.05$. Elevated levels of FAA were only expressed during the experimental phase of the experiment. The lack of FAA expression during recovery suggests that the GHSR-KOs and their WT counterparts did not entrain to the scheduled treat (B).

Figure 3.13. Metabolic measures of Blood glucose levels (A) and Leptin levels (B), *P < 0.05 vs. WT.

Figure 3.14. Average body weight changes from baseline across the experimental time points (A). Average of total caloric intake (B) during baseline, experimental, recovery and re-exposure phases of the experiment. Panel C illustrates the percentage of calories ingested from cookie dough. During the experimental phase the WT group (n=3) ingested 21% of their calories from cookie dough while the KO (n=5) ingested 13.4%. During the re-exposure phase, the WT group ingested 13% of their calories from the cookie dough while the KO group ingested 3.6%.

Authors	Title	Paradigm
Mistlberger & Rusak, 1987	Palatable daily meals entrain Anticipatory activity rhythms in free-feeding rats: dependence on meal size and nutrient content	9 parts purina chow+ 1 part Hershey chocolate syrup + 1 part icing sugar
Mendoza,J., Angeles-Castellanos, M., & Escobar C. (2005)	Entrainment by palatable meal induces food-anticipatory activity and c-Fos expression in reward-related areas of the brain	Chocolate Bar (10%P, 51% C, 34%F)
Verwey, M., Khoja, Z., Stewart, J., & Amir, S., (2007)	Differential Regulation of the expression of Period 2 protein in the limbic forebrain & Dorsomedial hypothalamus by daily limited access to palatable food in food deprived and free fed rats	Chocolate Ensure
Angeles-Castellanos, M., Salgado-Delgado, R., Rodriguez, K., Buijs, R.M., Escobar,C. (2008)	Expectancy for food or expectancy for chocolate reveals timing systems for metabolism and reward	Chocolate Bar (10%P, 51% C, 34%F)
Dailey, Stingl, Moran	Dissociation between preprandial gut peptide release and food anticipatory activity	Hershey chocolate (50%F, 5% P, 45% C)
Merkestien, Brans, Luijendijk, de Jong, Egecioglu, Dickson, Adan, 2012	Ghrelin Mediates the Anticipation to a Palatable Meal in Rats	Chocolate (35.4%F, 6.1%P, 54.7%C) Chocolate Milk
Blanacas, Gonzalez, Garcia, Rodriguez & Escobar 2014	Progressive anticipation in behaviour and brain activation of rats exposed to scheduled daily palatable food	Chocolate (10.3% P, 54.2% C, 35.5% F)

Table 3.1 A selection of studies that presented FAA in freely fed rat models.

Authors	Title	Strain	Diet	Paradigm
Hsu, C.T., Patton, D.F., Mistelberger, R.E., Steele, A. (2010)	Palatable meal anticipation in Mice	<ul style="list-style-type: none"> Male C57BL/6J (ages: 10-12 weeks, 6 weeks) Female C57BL/6J 	<ul style="list-style-type: none"> CR + FC FC (1hr)+ FC (2hr)+ Chocolate Slimfast + Hershey Chocolate Chips 	<ul style="list-style-type: none"> Food hopper approaches Video recording (home cage activity) Running wheel
Gallardo C.M., Gunapala K.M., King, O.D., & Steele A.D. (2012)	Daily scheduled high fat meals moderately entrain behavioral anticipatory activity, body temperature, and hypothalamic c Fos activation	<ul style="list-style-type: none"> Male C57BL/6J Male 129S1 	<ul style="list-style-type: none"> Peanut Butter Havarti Cheese 	<ul style="list-style-type: none"> Video camera tracking ZT 10
Bake, Murphy, Morgan & Mercer 2014	Large, binge-type meals of high fat diet change feeding behaviour and entrain food anticipatory activity in mice	<ul style="list-style-type: none"> Male C57BL/6J 	<ul style="list-style-type: none"> HFD (20%P, 20%C, 60%F) 	<ul style="list-style-type: none"> Binge Model- 5 Phases Reverse light cycle
V. van der Vinne, J. Akkerman, G.D. Lanting, S.J. Reide, R.A. Hut 2015	Food reward without a timing component does not alter the time of activity under positive energy balance	<ul style="list-style-type: none"> Male CBA/CaJ (6-20 weeks) 	<ul style="list-style-type: none"> Chocolate pellets 	<ul style="list-style-type: none"> Wheel running – reward ratio Infrared detector over the food hopper-

Table 3.2 Studies that have investigated the FAA in mouse models. CR= Caloric restriction
FC= Fruit Crunchies.

Chapter 4

General Discussion

In this thesis we examined the hypothesis that, like locomotor activity rhythms, metabolic rate entrains to restricted feeding schedules. In addition we examined if metabolic rate as well as locomotor activity rhythms entrained to daily presentation of a palatable snack. Finally we studied the role of ghrelin receptor signaling in mediating these processes. Results from these experiments show that metabolic rate entrains to a restricted feeding schedule (but not to daily presentation of a snack). In addition, we found that snacks can produce FAA and entrainment. Finally, while GHSR signaling may contribute to these processes, it is not critical for either of the processes.

Studies in Chapter 2 confirmed previous work showing that rhythms of RER, like rhythms of locomotor activity, entrain to restricted feeding schedules. These results highlight the ability of organisms to adapt and use the appropriate fuel under a nutritional challenge. The ability of metabolic rate to adapt to these changes is critical as it may allow the organisms to prepare for an oncoming caloric load, and for the use of the appropriate type of calories as they become available. Thus, while it might be critical to utilize long-term energy stores after prolonged periods (i.e. 6 hours or more) of fasting, it is also critical that animals are able to switch to the use of carbohydrates as they become available. Of note is the fact that RER begins to rise prior to the presentation of the meal indicating that animals begin burning carbohydrates before they actually get their timed meal. The mechanisms underlying this are not yet determined but they may involve the previously reported preprandial insulin release in animals under a restricted feeding schedule (Drazen, et al., 2006). This cephalic insulin rise seen in schedule fed animals may rapidly increase the use of glucose and ultimately raise RER before any food has been consumed.

In contrast, there were no alterations of RER rhythmicity in mice fed a HF snack. One reason for that could be that, because this paradigm does not result in metabolic imbalance, and because mice generally utilize a higher ratio of fat as a source of fuel during the day, daily limited presentation of a high fat treat has little impact on RER rhythms that are already set in place. A caveat to this is that, as seen in Chapter 3, locomotor activity rhythms do entrain to palatable meals and hence, may cause increases in energy expenditure at this time. In Chapter 2, we did not observe FAA in response to the HF diet, so this may not be the case in animals in this experiment, but it might be the case in experiments in Chapter 3, although RER was not measured in those experiments. Finally, while no differences in FAA were observed in HF fed mice in Chapter 2, it is possible that entrainment occurred but not observed with the measures used. Others have shown FAA in response to scheduled access to a HF treat using different methods to track FAA or exposing the test subjects to the HF for prolonged periods of time (ex. 6 hours access for 5 weeks)(Hsu, et al., 2010; Table 3.2).

In Chapter 3 we also thoroughly investigated how long it took mice to show FAA and alterations in ghrelin concentrations, and determined if entrainment of these measures actually occurred in mice given a palatable snack everyday. Our findings show that ghrelin levels did not positively correlate with the development of FAA or the maintenance of FAA to the scheduled snack. Given ghrelin's role in reward based feeding (Egecioglu et al., 2010; King et al., 2016) and its role in the development of FAA in the restricted feeding paradigm (Blum et al., 2009) it was expected that terminal ghrelin levels would be elevated in anticipation of the treat during the development and the maintenance. However, that was not the case. There were, however, changes in GHSR mRNA fold increases in the cookie dough access group in the ARC in anticipation of the scheduled treat

suggesting that sensitivity to ghrelin increases in the ARC. There was, however, no association between the increase in GHSR expression and changes in NPY/ AGRP mRNA levels within the ARC. Thus it remains unclear if increased GHSR expression is driving the activity of these neurons. Alternatively, increased GHSR expression and signaling may result in increased release of GABA from these cells which, independent of NPY or AGRP, could increase appetite (Cowley, et al., 2003). Alternatively, it is important to note that these animals were not in a negative energy balance and while NPY and AGRP neurons are necessary for homeostatic feeding (Luquet, Perez, Hnasko, & Palmiter, 2005) they may not be necessary for the drive of palatable food (Denis, Joly-Amado, Webber, Langlet et al., 2015). In addition, the GHSR is expressed in astrocytes located in the ARC, where ghrelin stimulates its glutamate transporter expression and glutamate uptake (Fuente-Martín, García-Cáceres, Argente-Arizón, Díaz et al., 2016). These astrocytes are considered to be apart of the rapid reorganization of the hypothalamic circuitry in response to ghrelin (Fuente-Martín, et al., 2016) and are involved in inhibiting AGRP neurons.

Work from our lab showed the potential for ghrelin in mediating the entrainment of RER to the timing of meal presentation, and the entrainment of locomotor activity to a daily palatable snack (Blum et al., 2010). Indeed, ghrelin meets many of the criteria required for a circulating signal conveying information to the neural circuitry believed to form the FEO. Ghrelin levels rise in anticipation of a meal and are correlated with the presentation of FAA (Blum et al., 2009), whereas, animals that are non-responsive to ghrelin exhibit attenuated levels of FAA (Blum et al., 2009; Waddington-Lamont et al., 2014). Ghrelin also affects energy utilization by promoting the utilization of carbohydrates as a source of fuel, while sparing the use of fat (Tschöp, et al., 2000). Our data, however, does not support the idea that ghrelin or ghrelin receptor signaling play a critical

role in these processes. In our studies, GHSR KO mice showed robust entrainment of RER to restricted feeding schedules, and they also showed FAA to a palatable snack that was comparable to FAA shown by WT mice. Thus, GHSR signaling is not required for these processes.

Our results demonstrate that the overall entrainment of RER rhythmicity to restricted feeding paradigm is not dependent on intact ghrelin signaling. Ghrelin responsiveness did however, influence energy utilization patterns at the initiation of the food restriction, where the GHSR-KOs displayed lower RER levels signaling a higher ratio of fat utilization. However, by the end of experimental day 5, the rhythms of the two groups were indistinguishable from one another.

Taken together these results highlight that ghrelin signaling is not a critical modulator of the energy utilization under a restricted feeding paradigm, rather it facilitates the gradual utilization of stored fats. It may be postulated that under a chronic and/or unpredictable food restriction, that circulating ghrelin levels may position the organism to be more metabolically flexible. Ultimately aiding in the consumption of the energy substrate that is readily available versus their fat stores.

Our results describe that the CD-1 male mice consistently exhibited entrained FAA behaviours while our GHSR-KO/WTs male mice, which were backcrossed on C57bl/J6 strain, exhibited FAA but did not entrain to it. These results support previous literature (Gallardo et al., 2012), in that entrainment of FAA may be strain dependent. Furthermore, our results also highlight that the type of scheduled snack may influence the development of FAA, in our studies the HF snack did not elicit FAA behaviours whereas our cookie dough snack did. This is consistent with results in the literature in which different diets invoked different behavioural responses (Table 3.1, Table 3.2).

The food entrainable oscillator is understood as a system of oscillators that respond to food as external cue and acts independently of the SCN (reviewed in Silver, Balsam, Butler & LeSauter,

2011). In our lab we have previously utilized the restricted feeding paradigm to illustrate the rise of ghrelin in anticipation of the FAA and how the GHSR may dampen the FAA (Blum et al., 2009; Waddington-Lamont et al. 2014). Using that particular paradigm as theoretical framework, we implemented the scheduled treat paradigm, to examine the importance of ghrelin and GHSR to the development of the FEO output, FAA.

Presenting a scheduled snack during the non-active phase is a unique paradigm of measurement of feeding and establishment of FAA. There is no homeostatic drive that acts as a survival cue to drive food intake and appetitive behaviours. A line of inquiry to further examine the role of ghrelin and the GHSR, would be to examine circulating ghrelin levels in response to a scheduled treat during the active-phase (dark-phase). If ghrelin levels are positively correlated with the FAA during the active-phase, it could be inferred the ghrelin is involved of the development or/and maintenance of FAA to a scheduled treat, only when not challenged with a circadian condition.

Venturing outside the hypothalamus, the hippocampal formation (HPC) has been recently implicated in feeding behaviour that is influenced by ghrelin (Hsu, Hahn, Konanur, Noble et al., 2015; reviewed in Kanoski & Grill, 2017). These neurons directly project on to the hypothalamus and the amygdala, directly influencing ghrelin controlled food intake (Russo, Russo, Pellitteri, & Stanzani, 2017). This area is of particular interest, considering its role in the animal “remembering” during the development of FAA (reviewed in Hsu, Suarez & Kanoski, 2016). Consequently, should be an area of interest in the further understanding the relationship of ghrelin to the FEO.

A factor to be considered with regards to the snack paradigm is the duration of the experimental period is incredibly short when compared to binge-models of hedonic feeding (Bake et

al., 2014; King et al., 2016), models of FAA in mice (Hsu et al., 2010) and rat models of FAA (Merkestien, et al., 2012). This is important to note, especially considering that Merkestien and colleagues have correlated terminal plasma ghrelin levels to FAA to chocolate, but that was after 5 weeks of exposure to the treat. Consequently, peripheral ghrelin levels may only be elevated after the animal becomes acclimatized to the treat and therefore is a secondary response.

Taken together, these results and those presented within the literature can conclude that the development of entrainment under the FEO is influenced by energy status and palatability and is differentiated between species and strain.

The understanding of the mechanisms of the FEO can aid in the understanding how circadian disruptions can influence and potentiate disorders in metabolism and mental health. Clinical epidemiological studies in humans have shown that circadian disruptions, such as sleep disorders and sleep disruptions (i.e. shift work) are correlated with the raise of metabolic dysregulation (reviewed in Sharma & Kavuru, 2010). Human genetic studies have reported genetic polymorphisms of clock components that are related to metabolic syndrome (Woon, Kaisaki, Bragança, Bihoreau et al., 2007; Scott, Carter & Grant. 2008; Garaulet, Lee, Shen, Parnell et al., 2009; Garaulet, Lee, Shen, Parnell et al., 2010) and mental health issues (Soria, Martínez-Amorós, Escaramis, Valero et al., 2010). These studies provide evidence that circadian clocks and metabolism are strongly associated and can lead to metabolic disruptions.

The association between sleep, metabolic syndrome and mental health has recently been on as priority in public health. An initiative that has been on the radar for Health Canada and the Public Health Agency is procuring research surrounding sleep and the consequences of disturbed circadian rhythmicity. Questions surrounding sleep duration, sleep hygiene and sleep quality (circadian

disruption) have been added to the Canada Community Health Survey alongside questions about mental health and physical activity. Just recently, the Public Health Agency of Canada has announced a partnership with the US Center for Disease Control and Prevention and the US Department of Health and Human Services with MaRS to collect data on sleep and physical activity. These data would aid in the development and implementation of policy recommendations at a federal level that would inform the public on the importance of valuing their circadian rhythmicity.

In the FEO, ghrelin is important but not critical, in the development and maintenance of appetitive behaviours once an organism is in a negative energy state. Furthermore, ghrelin's role in non-homeostatic feeding under a circadian challenge requires further examination. The results of this thesis further emphasize that FAA presented under a restricted feeding paradigm is not synonymous with FAA exhibited in response to scheduled snack. Studies are needed to address the overlap and intricacies between homeostatic and hedonic feeding pathways coupled with a circadian challenge.

This research provides a framework in which future studies can manipulate to further investigate the intricacies of the FEO. In particular, the development of a scheduled snack paradigm in mice will allow researchers to take advantage of the extensive transgenic models and biological techniques that are currently exclusive to mice models.

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