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Pennington Symposium Supplement: The role of melanocortin neuronal pathways in circadian biology - a new homeostatic output involving melanocortin-3 receptors?

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Abstract

Obesity, insulin resistance and increased propensity for type 2 diabetes and cardiovascular disease result from an imbalance between energy intake and expenditure. The cloning of genes involved in energy homeostasis produced a simple feedback model for the homeostatic regulation of adipose mass. Serum leptin secreted from adipocytes signals nutrient sufficiency, curbing appetite and supporting energy expenditure. A rapid decline in leptin during nutrient scarcity instigates adaptive mechanisms, including increased appetite and reduced energy expenditure. Hypothalamic melanocortin neurons are important mediators of this response, integrating inputs of energy status from leptin with other peripheral signals. While this feedback response prolongs survival during fasting, other mechanisms allowing the prediction of nutrient availability also confer a selective advantage. This adaptation has been commonly studied in rodents using restricted feeding (RF) paradigms constraining food intake to limited periods at 24h intervals. RF rapidly elicits rhythmic bouts of activity and wakefulness anticipating food presentation. While the response exhibits features suggesting a clock-like mechanism, the neuromolecular mechanisms governing expression of food anticipatory behaviors are poorly understood. Here we discuss a model whereby melanocortin neurons regulating the homeostatic adaptation to variable caloric availability also regulate inputs into neural networks governing anticipatory rhythms in wakefulness, activity and metabolism.

Keywords

Melanocortins; Hypothalamus; Rhythms; Energy Homeostasis

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Introduction

Energy homeostasis involves mechanisms that balance calorie intake with the energy requirements associated with growth, organ and tissue maintenance, and physical effort associated with nutrient acquisition [1]. At the tissue level, it also involves regulating metabolite levels in key storage organs (e.g. triacylglycerol in adipose tissue, glycogen in liver) as well as in the blood (e.g. glycemia, lipidemia) [2, 3]. A failure to rigorously maintain energy homeostasis with time can adversely affect health, leading to either to obesity [1] or cachexia [4]. Of particular concern is the increased prevalence of the cluster of metabolic disorders commonly associated with obesity and insulin resistance, including type 2 diabetes and cardiovascular disease. Collectively, if unchecked the increased incidence of these disorders may negate the achievements in public health observed in the 20th century and reduce life expectancy in the 21st century [1, 5].

The increased occurrence of obesity and type 2 diabetes linked to abundant calories and reduced physical activity has motivated interest in determining the molecular mechanisms associated with energy homeostasis [1]. That the hypothalamus has a critical role in maintaining energy homeostasis by controlling appetite and metabolism has been known for nearly a century [6]. More recently, our understanding of the neuromolecular mechanisms involved in the hypothalamic regulation of energy homeostasis has been facilitated by studies describing the interactions between central nervous system (CNS) sites controlling behaviour and peripheral metabolism with signals received from peripheral organ systems [7–9]. Neurons residing in the hypothalamus and brain stem receive a continuous flow of information about energy status from the gastrointestinal tract and peripheral organs, with the adipocytokine leptin having a predominant and non-redundant role as an indicator of energy status [10]. In turn, the CNS rapidly responds to regulate ingestive behavior and the autonomic nervous system and neuroendocrine axes that exert a tight control on the metabolic activity of peripheral tissues to maintain energy homeostasis [11].

The ability to adapt to non-caloric environmental cues, including photoperiod (day length), temporal shifts in food availability and ambient temperature is also an important component of systems involved in energy homeostasis. Most life forms on Earth, including prokaryotes and plants, have evolved intrinsic clock mechanisms that synchronize their physiology with environment cues, allowing for adaptations anticipating changes in the environment [12, 13]. Indeed, we are all subject to an internal clock mechanism that compartmentalizes the circadian day into periods dominated by sleep or motor activity that in the past was centered on foraging for food, or preparing food stores for periods of the annual cycle when food is less abundant. Many aspects of mammalian behavior and physiology including sleep/wake cycles, locomotor activity, blood pressure, body temperature, hormone secretions and metabolic pathways exhibit daily rhythmicity under the control of circadian clocks [14]. Emerging evidence indicates that disturbances of circadian rhythms due to either mutations in clock genes [15] or lifestyle modifications [16] increases risk of developing metabolic disorders. Recent data also suggest a fundamental link between the cellular timekeeping mechanisms responsible for maintaining circadian rhythms with the regulation of essential metabolic processes including gluconeogenesis and lipogenesis [14, 17–22].

During periods of nutrient scarcity, the availability of food can become the dominant zeitgeber over riding the intrinsic tendency toward diurnal or nocturnal behavior [23]. The location and mechanisms of this “food entrainable oscillator” (FEO) has been the subject of much debate and controversy [24, 25]. At one level, the central nervous system must be involved, as the process involves the expression of complex behaviors including sleep cycles, the stimulation of appetite and motivation to seek food. The hypothalamus has been proposed to play an important function as a conduit for peripheral signals in this process [26, 27]. On the other hand, every cell in the body exhibits the potential for autonomous regulation of clock activity by energy status, suggesting a distributed response that is independent of a “central” site of control [20, 21, 28].

Here we discuss our recent data suggesting that the melanocortin system is required for expression of rhythms that anticipate food presentation [29]. Hypothalamic melanocortin neurons are devoted to nutrient sensing and the integration of many signals of energy homeostasis that, in turn, influences a distributed network in the CNS that affects satiety and peripheral metabolism [30]. We posit that these neurons also coordinate feeding-related inputs into a distributed network that governs expression of rhythms in activity during period of restricted feeding.

Neuroanatomy and molecular composition of the mammalian biological clock

The intent here is not to provide an exhaustive review of the circadian literature; for this the reader is directed to recent reviews [14, 31, 32]. Hypothalamic structures involved in maintaining circadian rhythms have been the subject of investigation for nearly 4 decades since the publication of Stephan and Zucker reporting the results from studies examining the effect of lesions in the suprachiasmatic area in rats [33]. Overall, the hypothalamus acts as a relay center to process information not only from the environment but also from systemic inputs to affect the multiple systems controlling behavior and homeostasis (Fig. 1). The master clock responsible for maintaining a robust circadian rhythm in the absence of photic cues (i.e., constant dark) resides in the suprachiasmatic nucleus of the hypothalamus (SCN) [14]. The SCN receives information concerning photoperiod from the retinohypothalamic tract, and sends projections to the dorsomedial hypothalamus (DMH) through the subparaventricular zone of the paraventricular nucleus (spPVNz) [26, 27]. The DMH functions as a relay center, sending projections to areas involved in regulating neuroendocrine systems (the paraventricular nucleus), thermoregulation (medial preoptic area) and sleep cycles (the lateral hypothalamus, ventrolateral preoptic nucleus) [26, 27]. The DMH may also be involved in the integration of photic cues with other signals, including signals of food intake (Fig. 1) [26, 34].

At a molecular level, a clock mechanism involves a core oscillator comprised of nuclear transcription factors and transcriptional repressors that establishes a rhythm of approximately 24h. Directly or indirectly, these transcription factors affect the activity of a large proportion of the transcriptome (~3000 genes) [35]. Circadian oscillators are comprised of transcriptional activators and repressors assembled in an autoregulatory loop that

generates rhythmic patterns of gene expression that varies by tissue type (Fig. 2). Brain and muscle ARNT-like protein 1 (Bmal1), circadian locomotor output cycles kaput (Clock) and neuronal PAS domain protein 2 (Npas2, a Clock homolog) are transcription factors that form a positive limb. Bmal1-Clock or Bmal1-Npas2 heterodimers bind to E-box enhancer elements within the promoters of the *Period* (*Per*) and *Cryptochrome* (*Cry*) genes to activate transcription. The reason for the existence of two heterodimers containing Bmal1 and either Clock or Npas2 is unclear at this time. However, the analysis of circadian rhythms in single and double *Clock−/− Npas2−/−* mutant mice suggests a partial redundancy of function [36]. In the SCN, Npas2 can compensate for the absence of Clock to maintain a rhythm as assessed by measurement of wheel running activity, and measurement of activity of the *Per2* promoter as a clock output [36, 37]. However, Npas2 is unable to compensate for the loss of Clock in peripheral tissues, resulting in the loss of rhythm of *Per2* reporter [37].

The negative limb of the clock involves the Period (Per1, Per2) and Cryptochrome (Cry1, Cry2) proteins. As the levels of cytosolic Per and Cry proteins increase, they form heterodimers that translocate to the nucleus where they act to repress their own transcription through direct interaction with Bmal1-Clock or Bmal1-Npas2 heterodimers. In addition to regulation at the level of transcription, Per and Cry proteins are also regulated at the post-transcriptional level through phosphorylation by the casein kinase epsilon (CKIε). An additional feedback loop involves the nuclear receptors Rev-erbα and RORα [38.] The transcription of Rev-erbα is activated by Bmal1-Clock or Bmal1-Npas2 heterodimers bind to E-box enhancer elements. Then Rev-erbα represses the transcription of *Bmal1* through shared ROR binding elements (RORE) within the *Bmal1* promoter. With the repression of *Bmal1* transcription, the expression levels of Rev-erbα also collapses, thus removing an inhibitor of *Bmal1* transcription and facilitating reinitiation of the cycle (Fig. 2).

Biological clocks and energy homeostasis

A role for the circadian clock in energy homeostasis has been suggested by several studies reporting that voluntary or genetic disruption of rhythms of behavior and physiology impacts health. Clinical studies have demonstrated that sleep restriction, shift work and night eating conditions increase risk for obesity, hypertension, cardiovascular disease and other components of the metabolic syndrome [39]. Furthermore, some reports suggest that a loss of circadian rhythms of glucose metabolism may contribute to the development of type 2 diabetes [40, 41]. Circadian rhythms in both insulin secretion and sensitivity are altered in patients with type 2 diabetes [42]. Gene expression profiling studies have also identified a large number of transcripts (approximately 5–15% of the transcriptome) that exhibit rhythmic expression in different active metabolic organs such as liver, skeletal muscle, brown and white adipose tissue [43–45]. Many of the identified genes are involved in different metabolic processes such as glucose and lipid metabolism, oxidative phosphorylation and detoxification pathways, suggesting again that clock system might have an essential role in the regulation of the major metabolic pathways.

Further evidence linking the circadian system with energy homeostasis is demonstrated from the analysis of mice with disrupted clock gene function [17, 46]. Turek *et al.* reported that mice which are homozygous carriers of the *ClockΔ19* mutation mice not only exhibited

dampened diurnal rhythms in locomotor activity, but also an altered feeding rhythm with increased food intake during the day. These mice are hyperphagic, and exhibit a more severe diet-induced obese phenotype compared to wild type controls. The development of many characteristics of the metabolic syndrome, including hyperglycemia, hyperlipidemia, hyperleptinemia and hepatic steatosis is more severe in *ClockΔ19* mutants [15]. The involvement of the internal clock in regulating the rhythm of food intake was also demonstrated recently in a report examining *Per2* knockout mice, which also exhibit abnormal circadian rhythms of food intake, particularly when fed a high fat diet [47]. *ClockΔ19* mutant mice also exhibit adipocyte hypertrophy and reduced expression of hypothalamic peptides associated with energy balance such as ghrelin, orexins and CART (cocaine and amphetamine regulated transcript).

Interestingly, an important metabolic role for peripheral clocks has also been reported in a context of normal feeding behavior and locomotor activity. Global *Bmal1* knockout mice display abnormal feeding behavior and locomotor activity, altered daily oscillations in glucose and triglycerides plasma levels, reduced gluconeogenesis, reduced subcutaneous fat and decreased lifespan [48–50]. The analysis of mice with specific hepatic disruption of the *Bmal1* gene indicates that this transcription factor is crucial for circadian regulation of hepatic glucose regulatory genes and for systemic glucose homeostasis [50]. Bmal1 also has an important role in adipocyte function, promoting adipocyte differentiation and lipogenesis in mature adipocyte [51, 52]. There are other components of the core clock machinery that have been reported to have a crucial role in physiologic process. For example, Rev-erbα has been demonstrated as an important regulator of lipid and lipoprotein metabolism, adipogenesis, and vascular inflammation [38].

These studies have suggested a link between the systems that maintain circadian rhythms with energy homeostasis, and established a causal relationship between clock disorders and energy imbalance. However, the reciprocal relationship is also true, with mutations or challenges disrupting normal metabolic regulation affecting the activity of the circadian clock. For example, mice fed a high fat diet display altered circadian behavioral in feeding, locomotor activity, sleep/wake cycle as well as altered oscillations of clock gene expression in peripheral metabolic tissues [53, 54]. Collectively, these studies suggest that clock genes regulate energy homeostasis either directly through the regulation of gene expression, or indirectly by influencing the timing of food intake (Fig. 3).

How do clocks monitor calories?

The clock machinery is linked to energy metabolism pathways at multiple levels, and clock oscillators are regulated by factors that influence the supply of energy. In vitro studies have demonstrated that nutrient signals (glucose, fatty acids, and sterols) or circulating hormones (insulin, leptin, and glucocorticoids) are involved in the entrainment of clock gene expression [55–58]. Moreover, the activity of clocks in tissues involved in energy metabolism, including the liver and adipose tissues, rapidly entrains to meal time independently of clocks in the SCN [59]. These observations have led to efforts to understand how clocks in the periphery respond to caloric cues. The regulation of clock activity by calories may occur at two levels. The analysis of clock activity in cultured cells suggests autonomous regulation

by altered energy status. At the level of the whole organism, hypothalamic structures residing outside the SCN may be involved in integrating inputs from signals of food intake and energy reserves with signals of photoperiod received from the retina.

Nuclear receptors that sense metabolites are a potential input by which energy metabolism can regulate clock activity autonomously [60]. Nuclear receptors were initially perceived as acting as core transcriptional regulators that control major aspects of energy metabolism in response to activation by diverse intracellular metabolites including fatty acids, steroids or xenobiotic compounds [61]. Interestingly, of the 45 known nuclear receptors 25 exhibit marked rhythms of expression [62]. The dual role of nuclear receptors in coordinating energy metabolism and circadian clock has been illustrated by the studies on Rev-erbα and RORα. As previously described, Rev-erbα and RORα are components of the core clock circuitry that regulate *Bmal1* expression. They are also important regulators of lipid metabolism, adipocyte differentiation and vascular inflammation [38, 63]. Other nuclear receptors may also regulate clock activity. Recent studies have observed that transcription factors involved in regulating carbohydrate and lipid metabolism including Peroxisome proliferator activated receptor-alpha (Pparα), Pparγ coactivator 1 (Pgc1α) and Sirtuins regulate clock activity. Liu *et al.* reported that Pgc1α, a transcriptional coactivator that regulates adaptive energy metabolism, is essential not only for regulation of metabolic gene expression but also for normal clock function [19]. *Pgc1α* null mice display both metabolic and circadian abnormalities including impaired diurnal rhythms of activity, body temperature and metabolic rate [19, 64]. Pgc1α regulates transcription of *Bmal1* and *Rev-erbα* through coactivation of the ROR receptors [19]. Pgc1α is thus not only important for regulating metabolism and clock activity, but may also be a key component of the circadian oscillator that integrates the mammalian clock and energy metabolism [19].

Another nuclear receptor implicated in clock gene regulation is PPARα, which regulates lipid synthesis, storage and fatty acid oxidation [65, 66]. PPARα has been reported to be involved in the adaptive response to feeding cues, and is required for adapting fatty acid metabolism during fasting [67]. Rhythmic expression of PPARα has been observed in peripheral tissues such as liver, heart, kidney [43]. PPARα has been shown to bind directly to the *Bmal1* promoter by chromatin immunoprecipitation [68]. Reciprocally, Bmal1/Clock heterodimers can regulate PPARα expression by binding to functional E-box element in PPARα promoter 68–70. Treatment of mice with fibrates, which are agonists of PPARα, increases expression of *Bmal1* in the liver, an effect which is not observed in *Pparα−/−* mice [68.]

Sirt1 is a NAD^+ protein deacetylase linked to metabolism and aging [71], and has recently been also shown to interact directly with clock genes and to regulate their expression by inducing chromatin remodelling [18, 72]. Sirt1 binds with Clock-Bmal1 heterodimers in a circadian manner and promotes the deacetylation and degradation of Per2 [18]. Genetic ablation of the *Sirt1* gene or pharmacological inhibition of Sirt1 activity is associated with abnormal circadian rhythms of *Bmal1* expression [72].

Clock oscillators can themselves act directly as sensors for feeding related signals and the cellular oxidative level. The transcriptional activity of Clock-Bmal1 heterodimers has been

shown to be strongly influenced by the ratio of NAD^+/NADH, which is closely tied to cellular energy metabolism [28]. Indeed, Clock, Npas2, Bmal1 and Per2 oscillators all contain a PAS domain (PAS comes from the first letters of Per, Arnt and Sim), that can detect redox state, hypoglycaemia, oxygen balance, and xenobiotic metabolism. NADH enhances DNA binding of Clock-Bmal1 and Npas2-Bmal1 heterodimers, whereas NAD^+ inhibits binding [28].

Several studies have reported that peripheral tissue oscillators can function independently, and even become uncoupled from the SCN in response to hormonal signals [55] or RF [59, 73]. Restricting food intake to the day time results in a phase shift in the rhythmic expression of clock genes in the peripheral tissues of mice. Some studies indicate that the activity of the master clock in SCN remains locked to the light-dark cycle [59, 73], however RF when combined with calorie restriction does alter the circadian clock in the SCN [74]. Interestingly, clock gene expression in the liver is rapidly entrained by RF within two days, prior to the expression of FAA [73]. The ability of the RF to rapidly entrain peripheral oscillators indicates that food is a very potent synchronizer (or zeitgeber) for peripheral clocks. This nutritional regulation of clock genes in peripheral tissues may play a direct role in coordinating metabolic oscillations suggesting then that peripheral oscillators are directly responsive to cellular energy status [75].

Central nervous system and expression of anticipatory behavior

A growing body of literature has described in detail a mechanism for the regulation of clock activity by cellular energy status. However, it is also evident that the expressions of rhythms in activity involve areas of the central nervous system involved in complex behavior. At this time, it is not clear how the cellular response to altered energy status is translated to coordinate rhythms with nutrient availability. In addition, whether the adaptive response to restricted feeding is indeed a decentralized response, or is coordinated by a distinct group (or groups) or neurons in the CNS, is unclear. Certainly, the master clock required for maintaining rhythms resides within the hypothalamus. In mammals, lesioning and transplantation studies demonstrate that SCN is necessary for daily rhythms in locomotor activity and feeding [76–78]. The SCN receives signals of light from the retina via the retinohypothalamic tract. Under conditions of continuous food availability, the SCN is thought to entrain clocks in peripheral tissues indirectly, either through behavioral rhythms (rest/activity or fasting/feeding cycles) [79] or through secreted hormones displaying a circadian rhythm [31] (Fig. 2). However, there is also evidence suggesting that areas of the hypothalamic outside the SCN are required for maintaining the expression of rhythms in behavior associated with restricted feeding. Moreover, the SCN is connected to regions of the hypothalamus thought to regulate feeding behavior and energy metabolism.

Destruction of specific hypothalamic areas using neurotrophic toxins has been instrumental in the identification of neuronal populations outside of the SCN critical for maintaining circadian rhythms of arousal, core temperature and locomotor activity under conditions of *ad libitum* feeding or during restricted feeding. The subparaventricular zone (SPZ) and dorsomedial nucleus of the hypothalamus (DMH) may be necessary for organizing circadian rhythms of body temperature, sleep/wake cycle, locomotor activity, feeding and corticoid

production [34] (Fig. 1). Specifically, neurons within the dorsal and ventral SPZ are necessary for circadian rhythms of body temperature and sleep/waking, respectively [80]. The DMH, a region involved in the regulation of food intake and satiety, has been also implicated in the ability of the organisms to express food anticipatory activity [81, 82]. When food access is limited to defined periods outside of the normal feeding period, animals rapidly develop food seeking behavior and wakefulness that anticipate food presentation [83]. It has been reported that this adaptive response is blocked in animals with cell-specific lesions in the DMH. Indeed, some reports have suggested that DMH lesions resulted in severe impairment of feeding, sleep/wake cycles, locomotor activity and corticoid secretion [84, 85]. Projections from DMH to other regions of the brain include the ventrolateral preoptic nucleus, the paraventricular nucleus, and the lateral hypothalamus which regulate sleep, corticoid release, and wakefulness/feeding respectively (Fig. 1). The ventromedial hypothalamus (VMH) has also been hypothesized to be linked with the FEO [86], while VMH activation has been observed to be associated with increased arousal in anticipation of food presentation [87]. As the DMH appears to be a key site for the expression of food-entrainable circadian rhythms, it has been suggested that this area may be a source of inputs in the food entrainable oscillator (FEO) and the expression of food anticipatory activity (FAA) [81].

However, controversy remains concerning the role of DMH. Landry *et al.* have reported that DMH-lesioned rats can still anticipate mealtime, and that this anticipation persists during total food deprivation [88]. Two other reports have also suggested that the DMH is not required for the expression of food entrainment [89, 90]. However, other studies have produced results suggesting that the DMH (and the VMH) are important for maintaining circadian rhythms and entrainment of food seeking behavior [82, 87]. These hypothalamic nuclei are also crucial in the regulation of appetite control, energy expenditure, and metabolism, suggesting an interconnection between systems regulating metabolism and circadian rhythms [6]. Due to the difficulty to identify a distinct anatomical location for the FEO, it has been suggested that it may be distributed or non-local system [24].

The melanocortin system integrates systemic signals of energy status to regulate energy homeostasis

While the hypothalamus has been proposed to contain sites where caloric cues are integrated with outputs from the SCN to regulate the expression of rhythms, the actual identity of these neurons is unknown. Neurons in the lateral hypothalamus expressing the neuropeptide orexin have an important role in regulating sleep state [26], and may also be involved in increasing wakefulness in anticipation of food presentation [91, 92]. Hypothalamic melanocortin neurons are another well defined system involved in the regulation of energy homeostasis [9, 93]. In the brain, the melanocortin system is composed of primary neurons expressing proopiomelanocortin (Pomc), agouti related peptide (AgRP), and secondary neurons expressing melanocortin receptors [9, 93]. The activity of Pomc and AgRP is regulated by many peripheral signals of energy status, including leptin, insulin, glucose, cholecystokinin, PYY3-36, and ghrelin [30]. Orexin may also regulate the activity of AgRP neurons, suggesting a connection with a system involved in regulating arousal. Recent studies using scheduled feeding protocols have also indicated that melanocortin neurons

respond rapidly to meal ingestion [94, 95]. Collectively, the published data support the hypothesis that Pomc neurons transmit an anorectic signal, while the output of AgRP neurons is primarily orexigenic, and that these neurons respond rapidly to signals of caloric intake and energy status.

Five melanocortin receptors have been cloned, and all are 7 transmembrane g-protein coupled receptors [9]. Of these, the Mc3r and melanocortin-4 receptor (Mc4r) have been identified as important regulators of energy homeostasis. These receptors can be activated by α melanocyte-stimulating hormones (αMSH), a peptide derived from post-translational processing of POMC in the arcuate nucleus of the hypothalamus. Both Mc3r and Mc4r are expressed in brain areas known to be involved in regulating energy balance, including the hypothalamus. Disruption or blockade of Mc3r and Mc4r activity using central administered non-selective antagonists is associated with hyperphagia and weight gain [96–99], as well as altered lipid metabolism and nutrient partitioning in peripheral tissues [100, 101]. The results from studies investigating the phenotype of *Mc3r* and *Mc4r* gene in knockout mouse models indicate that the acute regulation of satiety, energy expenditure and glucose disposal by melanocortins in mediated by the Mc4r, and not Mc3r [97–99, 102–105].

While the importance of the Mc4r in maintaining appropriate metabolic homeostasis has been intensively studied, the role of the Mc3r remains unclear. Mc3r knockout mice (*Mc3r−/−*) exhibit an obese phenotype characterized by modest increase in fat mass and reduced lean mass as well as increased metabolic efficiency and sensitivity to high fat diet [97, 98]. The role of the Mc3r in the regulation of food intake is complex. Feeding studies with *Mc3r−/−* mice have reported varied results including no difference in food consumption, modest hypophagia, and even a modest hyperphagia during the lights-on phase [93, 98]. On the B6 background, the measurement of food intake using an automated system suggests that *Mc3r−/−* mice may be mildly hyperphagic when first provided a purified high fat diet. This hyperphagia is primarily due to an increased food intake during the daytime, suggesting that Mc3r might function in the circadian regulation of ingestive behaviour [93]. Further evidence of reduced amplitude of night-time (peak) versus day-time (nadir) feeding was also observed in a separate cohort of mice fed a purified low fat diet [29]. Interestingly, dense expression of *Mc3r* mRNA has been observed in VMH and DMH [106]. As previously mentioned, these are two hypothalamic sites are involved in regulating food intake and that may also function to maintain circadian rhythms [81, 82, 86].

MC3R are required for entrainment to restricted feeding

During our interrogation of the phenotype of *Mc3r−/−* mice, we developed an interest in how organisms adapt anticipatory rhythms to nutrient intake. The provision of a limited amount of food on a recurring basis rapidly elicits food anticipatory behavior, a phenomenon that displays features suggesting the involvement of a clock or hour glass mechanism [23]. Based on observations from studies examining daily rhythms in rats with various hypothalamic lesions [82, 84, 86], we developed the hypothesis that the melanocortin system acts through the melanocortin-3 receptor (Mc3r) to link signals of energy status with system expressing rhythms of food anticipatory behavior [29]. The original unpublished studies used *Mc3r* knockout mice (B6.129S4-*Mc3r*^{tm1Cone}/J) on the original out bred C57BL/6J (B6) and

129/Sv backgrounds developed in Dr Roger Cone's laboratory at the Vollum Institute in Portland, Oregon [98]. More recently, studies have used mice back crossed >10 generations onto the B6 background [29].

Recent studies in our laboratory suggest that Mc3r may regulate behaviour via effects on circadian rhythms in the brain [29]. Our findings indicate that Mc3r are involved in the expression of adaptive entrainment of behaviour in response to feeding cues [29]. By using scheduled feeding protocols associated with a caloric restriction, we have demonstrated that the *Mc3r* gene is necessary for the coordinated development of anticipatory activity and increased wakefulness associated with restricted feeding. Moreover, analysis of cortical gene expression revealed severe abnormalities in rhythmic expression of clock genes such as *Bmal1, Npas2* and *Per2* in *Mc3r −/−* at both RF and *ad libitum* feeding. These observations suggest that Mc3r containing neurons may regulate inputs maintaining proper cyclic oscillation of clock genes in the forebrain. This alteration in oscillation in *Bmal1, Npas2* and *Per2* gene expression could play a role in the impaired entrainment of *Mc3r−/−* mice to RF.

Studies have reported altered arousal rhythms in mice with mutated Bmal1/Mop3 gene expression [107]. Moreover, delayed expression or loss of FAA has been also reported in animals with Npas2 or Per2 disruption [108, 109]. However, a recent study has raised questions about the interpretation of those results, reporting that clock mutant mice with total disruption of *Bmal1* or *Per1/Per2* still exhibit normal FAA both in a light dark cycle and in a constant darkness conditions [90]. Involvement of the known clock genes in daily rhythms of FAA is controversial as reports are conflicting [85, 109]. In a recent study, Storch *et al.* emphasized the importance of the proper evaluation of the FAA. In particular, they have demonstrated that reduced FAA observed in *Bmal1* knockout mice during an abrupt shift from constant food availability to restricted food availability is secondary to a lethargic and moribund state [90]. Studies examining the response of mutants to restricted feeding thus require a careful assessment of the health and metabolic status of the animal.

Decreased FAA in *Mc3r−/−* mice is likely not a consequence of any disability, as we have not observed increased mortality in *Mc3r−/−* mice subject to restricted feeding. Moreover, the assessment of spontaneous locomotory activity and energy expenditure in *Mc3r−/−* mice and wild type animals is not consistent with illness-like behaviour in the former [29]. It is possible that Mc3r are involved in transmitting information regarding energy status into the DMH (Fig. 1). However, *Mc3r* mRNA is expressed in nearly 30 areas in the central nervous system that may also be involved in expression of complex behaviours [106]. More precise studies that will clearly define the role of Mc3r in anticipatory and reward-seeking behaviour are warranted. This unique function of the Mc3r to signal calorie intake to clocks governing circadian rhythms in arousal, food seeking behaviour and metabolism also suggests a novel output of the Mc3r for regulating energy homeostasis. It will also be of interest to determine whether Mc3r in the central nervous system synchronize the activity of peripheral clocks, which have a critical role in maintaining energy homeostasis, with food intake.

Conclusions

Biological clocks promote the synchronization of behavioural and physiologic processes with environmental cues, and facilitate the expression of rhythms that anticipate changes in the environment. During the past decade, significant advances have been made in determining how the circadian clocks function, and have contributed to a better understanding how circadian rhythms are involved in energy homeostasis. Clocks integrate cues from environment and systemic signals of energy status to regulate diverse cellular and physiological function. The integration of the circadian clock and energy metabolism is controlled by biological signals at multiple levels. Our results imply that Mc3r could be important mediators in transmitting feeding related inputs into neurons governing the development of anticipatory activity especially during periods of nutrient scarcity.

It is also important to acknowledge the significant controversy associated with this field of research, and in the interpretation of data [24, 25]. The results from recent studies suggesting a critical role for the known clocks in the expression of food entrainable rhythms [85, 108, 109] have been questioned by other experiments suggesting an alternative interpretation of food anticipatory activity [25, 90]. Significant questions concerning the restricted feeding protocol have been raised, leading to a re-interpretation of published data. It also appears likely that an abrupt phase shift to limited nutrient availability is too severe for clock mutants to adapt too, and that the failure to express behavioral rhythms is due to metabolic distress. The known clock genes may thus not be required specifically for the expression of rhythms, but may have a critical role in metabolic adaptation. In this context, the *Mc3r* knockout mouse may provide a unique genetic model for investigating mechanisms responsible for the expression of rhythms in food anticipatory activity.

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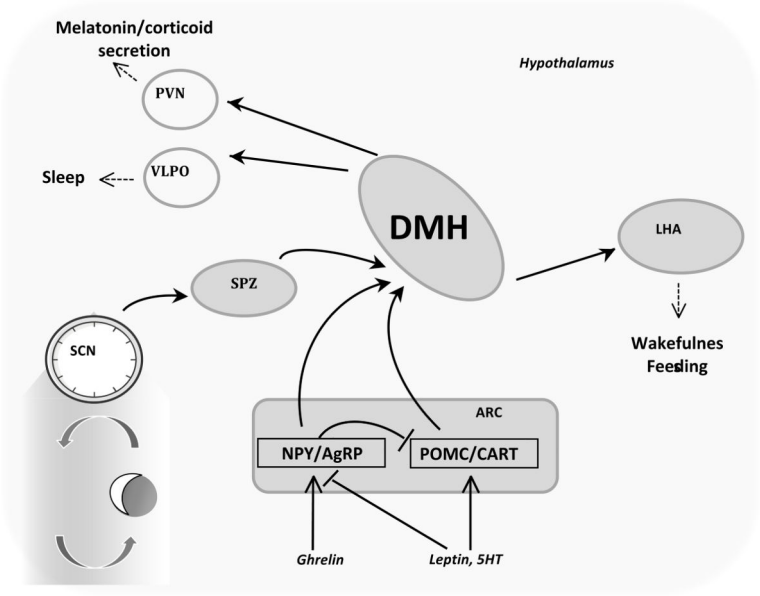

Figure 1. Neuroanatomic and neuroendocrine connections in the hypothalamus

The SCN is the master clock, and sends information through the subparaventricular zone (SPZ) and dorsomedial nucleus of the hypothalamus (DMH). An unbiased survey of clock activity in rats subject to restricted suggests the induction of a rhythm in the DMH that persists in the absence of feeding[79]. Lesions studies also suggest that the DMH is important for expression of circadian rhythms [80,81]. Projections from DMH extend to other regions of the hypothalamus such as the lateral hypothalamus (LHA), the ventrolateral preoptic nucleus (VLPO), and the paraventricular nucleus (PVN). These areas involved in sleep cycles (LHA, VLPO), neuroendocrine function (PVN) and feeding behavior (LHA). The arcuate nucleus (ARC) is a center involved in regulating feeding behavior. ARC neurons expressing the neuropeptides AgRP and neuropeptide Y (NPY) are orexigenic, while those expressing POMC (which is post translationally modified to produce α-MSH) and cocaine-and-amphetamine regulated transcript (CART) are anorectic. These neurons integrate many signals of energy status and food intake, regulating satiety and energy expenditure through Mc4r not shown in this diagram. NPY/AgRP and POMC/CART neurons may transmit signals of energy status into the DMH expressing Mc3r. Alternatively, neurons expressing Mc3r reside in other areas of the brain, such as the LHA and ventral tegmental area[102], involved in complex behavioral processes. An involvement of these neurons in the expression of rhythms in arousal and food seeking behavior is also possible.

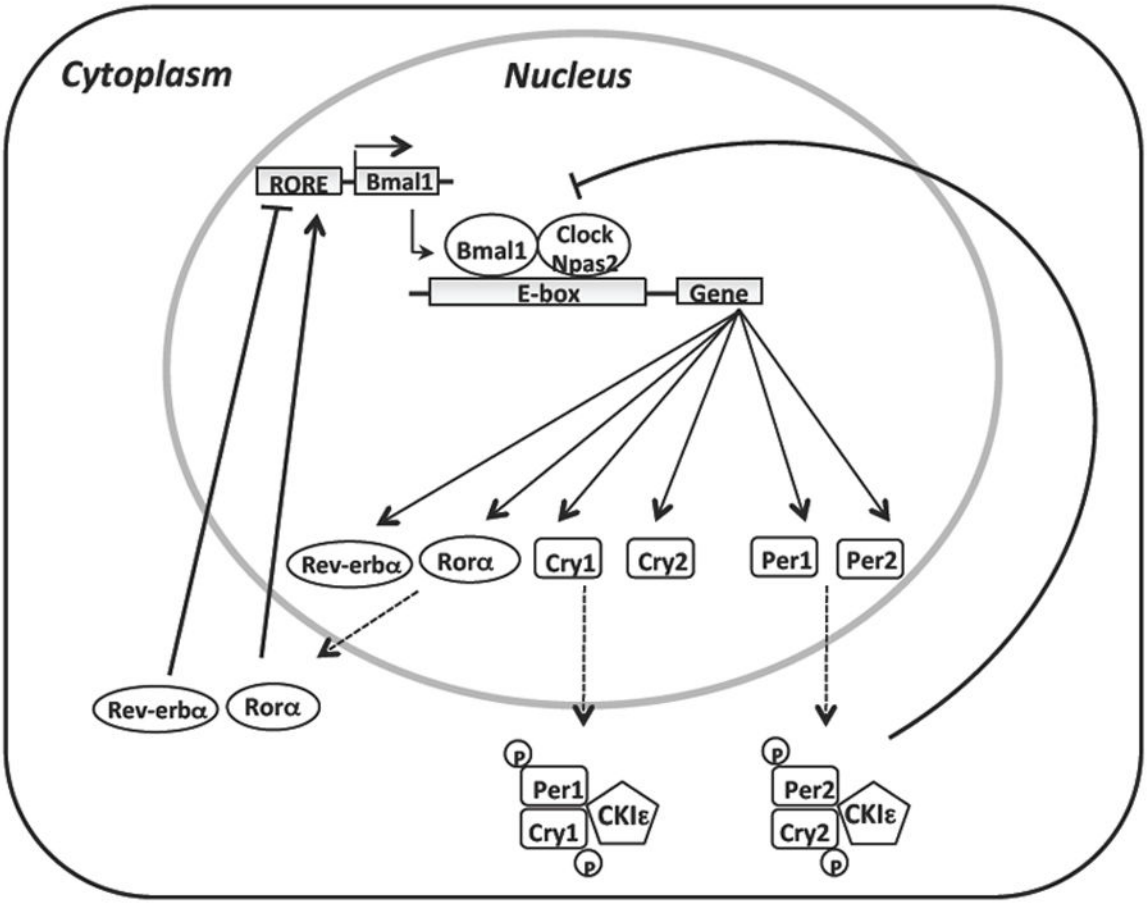

Figure 2. Molecular mechanisms of mammalian biological clock

The mammalian circadian clock consists of a network of transcription-translation feedback loops that oscillates with a periodicity of 24h. This core oscillator includes different transcription factors such as Brain and muscle ARNT-like protein 1 (Bmal1), circadian locomoter output cycles kaput (Clock) and neuronal PAS domain protein 2 (Npas2). These transcription factors can heterodimerize and activate transcription of downstream targets such as Period genes (Per1, Per2) and Cryptochrome genes (Cry1, Cry2), Rorα and Rev-erbα, which contain an E-box enhancer elements within their promoters. Upon translation, Per and Cry proteins heterodimerize and translocate to the nucleus to inhibit the action of Bmal1-Npas2/Clock complex. Casein kinase ε (CKIε) protein regulates expression of Per and Cry proteins through phosphorylation. A secondary inhibitory feedback loop composed of Rev-erbα and Rorα drives the rhythmic transcription of Bmal1. Rev-erbα represses *Bmal1* expression while Rorα activates its transcription.

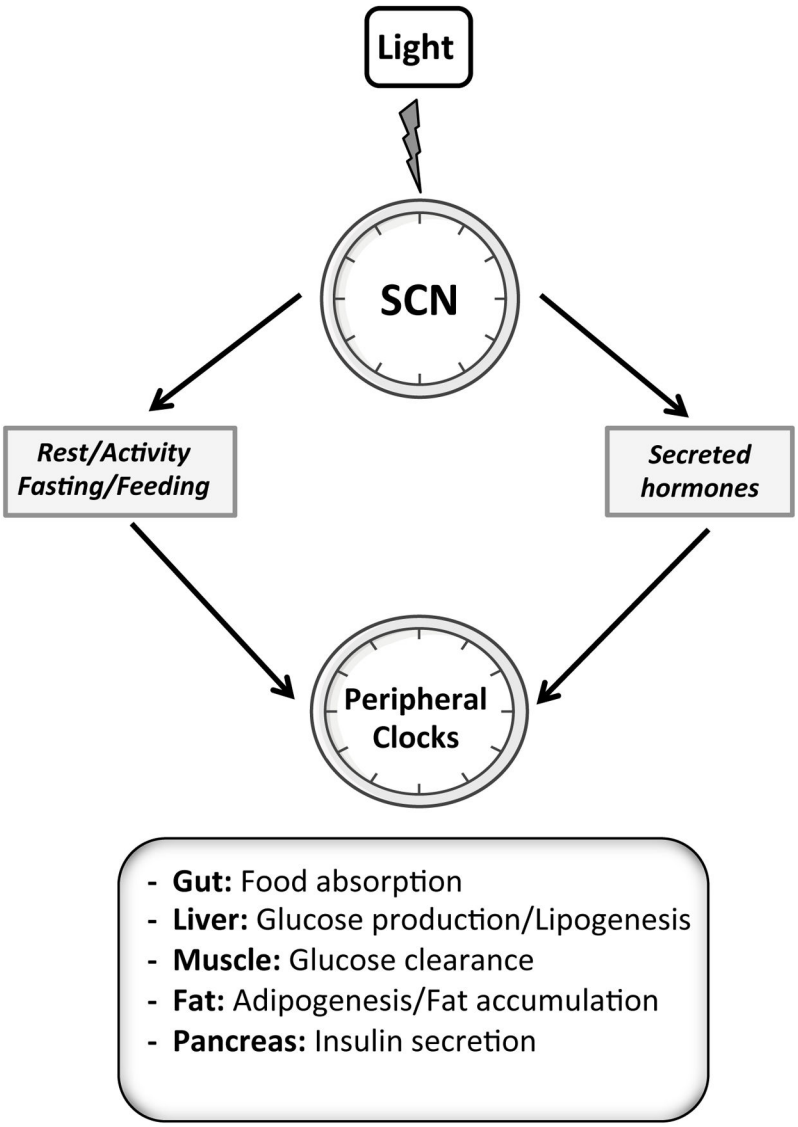

Figure 3. Entrainment of peripheral clocks by SCN

The master pacemaker encoding the mammalian clocks is located in the suprachiasmatic nucleus of the hypothalamus (SCN). Clocks residing in the SCN are entrained by light signals transmitted through the retinohypothalamic tract. The synchronization of other oscillators throughout the body by the SCN is presumably indirect *via* behavioral rhythms (e.g., feeding, physical activity) or rhythmically secreted hormones.