Journal of Neuroendocrinology **19**, 127–137

YOUNG INVESTIGATOR PERSPECTIVES

Circadian Clocks: Setting Time By Food

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Journal of Neuroendocrinology

In mammals, daily rhythms in behaviour and physiology are driven by a circadian timing system comprised, in a hierarchical way, of a master pacemaker in the suprachiasmatic nuclei (SCN) of the hypothalamus and of peripheral oscillators in most body cells. At the molecular level, in both the SCN and peripheral oscillators, the circadian clock mechanism is built from interconnected feedback loops in gene expression that operate in a cell-autonomous and self-sustained fashion. The SCN clock is mainly entrained by light/dark cycles. By contrast, peripheral oscillators can be strongly affected by daily feeding cycles, which have little effect on the phase of the SCN. However, when feeding schedules are coupled with a caloric restriction, behavioural and physiological circadian rhythms and gene expression in the SCN are shifted and/or entrained to meal-time. Moreover, the reward and motivational value of food can also be a potent synchroniser for the SCN clock. This suggests that energy metabolism and motivational properties of food can influence the clock mechanism of the SCN. Food-related cues may entrain clock genes of the SCN with a direct effect, or be mediated indirectly by another neural or peripheral site. In addition, there may be one or more oscillator sites that would play an integral role as a food-entrained oscillator (FEO), responsible for anticipation of meal-time. The site housing, or the network underlying, this putative FEO is still unknown. The aim of this review is to summarise our current knowledge of the central and peripheral circadian clocks and how they can be entrained by feeding at the physiological and molecular levels.

Key words: suprachiasmatic, circadian, food-entrainment, calorie restriction, clock genes.

doi: 10.1111/j.1365-2826.2006.01510.x

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The idea of having an internal biological clock might sound weird at first sight, but if we consider the number of times that we wake up automatically before the alarm clock rings, or we experience all the feelings of hunger, even without seeing food, at a precise time, it is because our body is strictly controlled by an internal timing system. The earth's daily rotation around its axis has imposed potent selective pressures on organisms. The temporal environment is so predictable and has such a pervasive effect on vital processes; thus, living organisms have evolved complex biological systems to keep up with daily time. Therefore, the daily rhythms of physiology and behaviour, such as the sleep/wake rhythm, body temperature or hormone release, cannot be considered as a passive response to environmental changes (1).

Circadian clocks generate cycles close, but not exactly equal to 24 h. Actually the word circadian means about a day (from the Latin: *circa diem*). Remarkably, circadian clocks are autonomous and do not require exogenous influences to keep time and, when deprived of external cues, their expression continues (free-runs) to organise our biology appropriately, with a period of slightly less or slightly more than 24 h (2). Circadian clocks are present in almost all the living organisms, from plants to humans. In mammals, including humans, they are located not only in the central nervous system, but also in several peripheral tissues. All these circadian clocks are kept in synchrony to maintain stability within the circadian system (3). Circadian clocks contribute to the regulation not only of daily, but also of seasonal rhythms. Nowadays, numerous social and commercial influences, such as jet-lag and shift work, disrupt the internal temporal order by an altered synchronisation with the environment. These disruptions lead to several health problems, such as cardiovascular and metabolic diseases, cancer or some mental disorders (3, 4). The importance of the study of human circadian biology, as well as its consequences on health and disease treatment, needs to be considered.

The suprachiasmatic nuclei as the principal circadian pacemaker in mammals

The suprachiasmatic nuclei (SCN) in the ventral part of the anterior hypothalamus are the master circadian pacemaker in mammals (5). The SCN clock mechanism is cell-autonomous (i.e. it can sustain itself in isolated SCN neurones). This has been demonstrated by recording circadian rhythms of electrical firing from individual SCN cells, Ca^{2+} concentrations, metabolic activity and gene expression (5) (Fig. 1). The SCN are composed of several sets of small and densely packed neurones (approximately 10 000 per nucleus) in which different peptides are expressed. Two principal subdivisions have been revealed in the SCN, the dorsomedial region in which cell bodies express arginine vasopressin (AVP), and the ventrolateral region in which vasoactive intestinal peptide (VIP) is synthesised (6). VIP signalling and its receptor ($VPAC_2$) may be a particularly important factor in maintaining robust coupling between SCN neurones and molecular timekeeping within individual SCN neurones (7). In addition, SCN cells are coupled by intercellular communication via GABA-ergic signalling (8).

Disruption of the SCN leads to arrhythmicity in almost all behavioural and physiological circadian processes (9). SCN grafts in previously SCN-ablated animals restore the rhythm of locomotor activity (10). This recovery of rhythmicity may be driven by diffusible factors released by the SCN, such as transforming growth factor α, neuropeptide prokineticin-2 and, recently, cardiotrophin-like cytokine (11–13). All these factors act on their respective receptors in the periventricular hypothalamus to inhibit locomotor activity (11–13).

Clock genes and circadian feedback loops

The generation and maintenance of circadian clock function depend on clock genes and their protein products in autoregulatory transcriptional feedback loops, consisting of both positive and negative elements (14). The positive arm of the clock depends on the protein products of the *Clock* and *Bmal1* genes. *Clock* is expressed constitutively in the SCN, but *Bmal1* expression cycles with a circadian period. CLOCK and BMAL1 are basic helix–loop–helix factors that form heterodimers capable of activating the transcription of the *Period* (*Per1-3*) and *Cryptochrome (Cry1-2)* genes (14, 15) (Fig. 1).

In negative feedback, the cyclic translation of *Per* and *Cry* mRNA leads to cyclic levels of PER and CRY proteins. These proteins form complexes and accumulate in the nucleus where they inhibit expression of their genes by acting on CLOCK/BMAL1 heterodimers (15). The orphan nuclear receptors REV-ERBα and

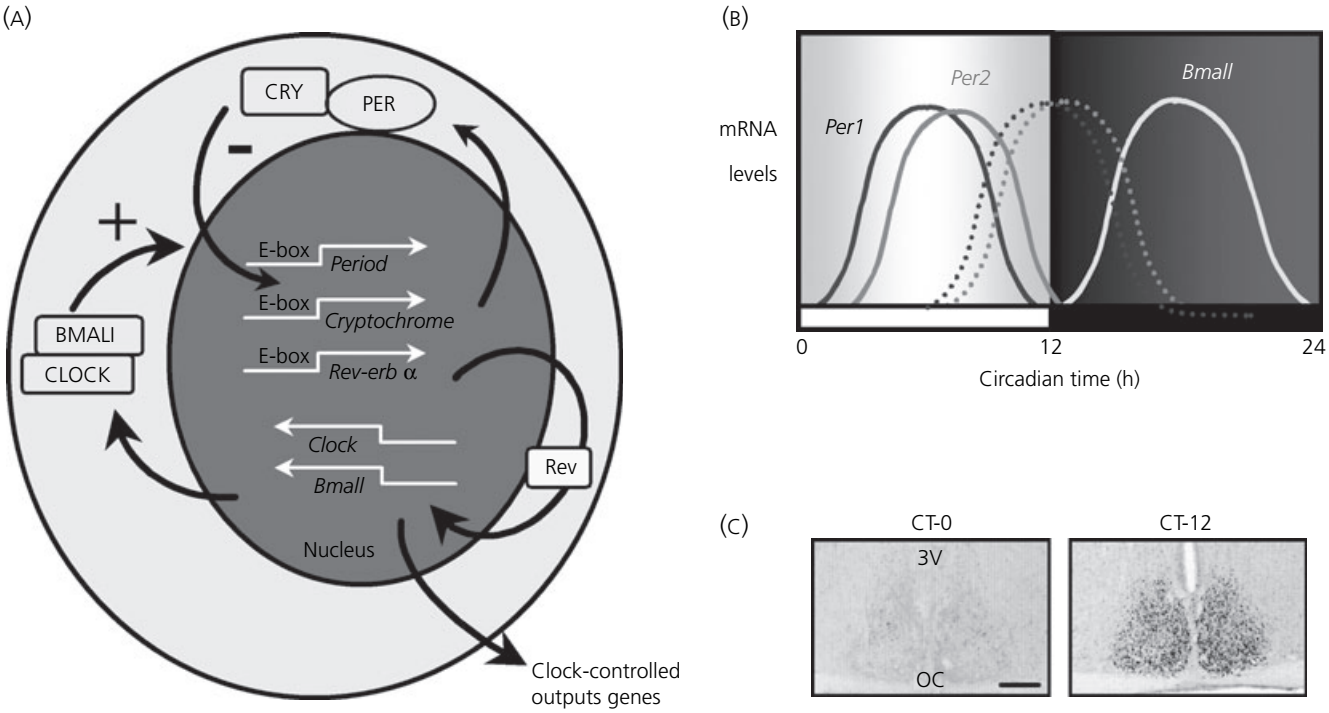

Fig. 1. (A) The suprachiasmatic nuclei (SCN) clock mechanism comprises positive and negative feedback loops. The activation of *Per* and *Cry* genes is driven by CLOCK-BMAL1 heterodimers through E-box enhancers. As the levels of PER proteins increase, they form heterodimmers with CRY proteins. In the nucleus, the CRY-PER complexes associate with CLOCK-BMAL1 heterodimers to shut down the transcription forming the negative feedback loop. *Rev-erb*α, which is also activated by *Clock/Bmal1* heterodimers, is a negative regulator of *Bmal1* expression and is expressed in phase with *Per* and *Cry*. In addition, there is a circadian expression of clock-controlled genes (CCGs) responsible for imposing temporal order given by the clock to the whole body. (B) Circadian expression of *Per1 and Per2* mRNA in mid circadian day (continuous lines) and their protein expression at the end of circadian day (dotted lines) in the SCN. *Bmal1* mRNA is in antiphase with *Per* expression. (C) Immunoreactivity for PER-1 protein in the mouse SCN at CT-0 (activity offset) and CT-12 (activity onset). 3V, Third ventricle; OC, optic chiasm. (scale bar = 200 μm).

RORα are also driven by CLOCK/BMAL1, and thus accumulate during circadian day (16, 17). The REV-ERBα protein translocates into the nucleus to bind to a ROR element in the *Bmal1* promoter inhibiting *Bmal1* transcription. Disappearance of REV-ERBα during circadian night releases this inhibition, and renews expression of *Bmal1* driven by RORα cues for the next cycle of circadian gene expression (16, 17).

The expression of circadian rhythms are coordinated by the expression of another set of genes called clock-controlled genes (CCGs) (3, 18). Some of these genes are expressed rhythmically in phase with *Per* and *Cry* and are considered as the molecular outputs from the core clock mechanism. The most notable CCGs are those encoding the putative peptidergic outputs of the SCN, such as AVP (3). The transcription factor albumin D element-binding protein (*Dbp*) gene is another strongly rhythmic CCG. DBP is endogenously highly rhythmic, with an amplitude comparable to that of *Per1*, and its expression is not directly influenced by environmental light (19).

Synchronisation of SCN by light and behavioural cues

Because the endogenous period of the SCN is close to, but not exactly 24 h, these nuclei need to be synchronised (entrained) to daily external signals (1). The most powerful synchroniser or *zeitgeber* (from German Zeit, 'Time'; geben, 'to give') known is the light/dark cycle. Light stimulates a group of photosensitive retinal ganglion cells that contain the photopigment melanopsin (20), and project to the SCN through the retinohypothalamic tract (21). Current evidence demonstrates that melanopsin plays a critical role in the circadian photoentrainment (20). Glutamate and pituitary adenylate cyclase activating polypeptide (PACAP) are the primary neurotransmitters responsible for mediating the synchronising properties of light (22). Their postsynaptic effects on NMDA, AMPA/kainate receptors for the glutamate and the PACAP-specific receptor (PAC1) for PACAP lead to an increase of the intracellular concentrations of both Ca^{2+} and cAMP that induce the phosphorylation of the Ca^{2+}/cAMP response-element binding protein (CREB) (23). This ultimately leads to the transcription of several immediate early genes, such as *c-fos* or NGFI-A (24), and the clock genes *Per1* and *Per2* (25, 26).

The behavioural response induced by a nocturnal light pulse depends on the clock's temporal state (27). Light perceived during the early night elicits phase delays, while light perceived during the late night evokes phase advances (27).

Although light is the principal *zeitgeber* to the SCN, afferent projections to the SCN from different parts of the brain, such as the neuropeptide Y system from the intergeniculate leaflets and the serotonergic input from the midbrain raphe, act together to mediate the nonphotic entrainment process that depends mainly on a behavioural arousal (28, 29). Different from light resetting, nonphotic stimulation leads to a circadian time-dependent reduction of *Per1* and *Per2* mRNA in the SCN (30, 31).

Peripheral circadian clocks

Subsequent to the discovery of several mammalian clock genes and the use of molecular biology tools, the presence of circadian clocks in peripheral tissues and also in other brain regions, outside the SCN, has been demonstrated (32–34). In the periphery, a circadian clock gene expression in mammals has been detected in the liver, heart, muscle, kidney, pancreas, adipose tissue and lung (32, 33, 35, 36). Moreover, there is circadian gene expression in mouse and rat fibroblasts when these cells are treated with a serum shock (a serum-rich medium; 50% of the serum concentration in some species) suggesting that fibroblasts contain circadian oscillators (37).

As the SCN clock expresses CCGs, peripheral clocks may directly modulate some of these CCGs (38, 39). Some genes are cycling in a circadian manner in peripheral tissues, such as the liver and heart (39). *Dbp* is a CCG that controls the circadian expression of several digestive enzymes in the liver that are relevant to metabolism (40).

An important characteristic of mammalian peripheral clocks is that their oscillations were initially shown to dampen very rapidly, and its circadian gene expression is delayed (from 7 h to 11 h) compared to the gene expression observed in the SCN (32–34, 41).

However, sustained oscillations in some peripheral tissues have now been reported in mice. In an elegant experiment using *mPer2* luciferase transgenic mice, Yoo *et al.* (42) showed robust and persistent circadian oscillations of *Per2* gene expression in several tissues for up to 20 days independently of the SCN input. In SCN-ablated mice, peripheral tissues are still rhythmic but they do not work in a coordinated way, with phases differing widely from tissue to tissue (42).

Although, at the central nervous system level, the SCN is perhaps the only clock nuclei relevant for the generation of circadian rhythmicity, recent studies show that circadian rhythms in firing rate or clock gene expression can be found in other brain regions, such as the striatum, the septum, the medial preoptic region, the bed nucleus of the stria terminalis, the pineal and pituitary glands, the amygdala, the olfactory bulbs and in many hypothalamic nuclei (34, 43–47). Interestingly some of these brain structures can sustain circadian rhythmicity for some days independently of the SCN (34, 47).

The molecular loops that generate circadian oscillations within peripheral cells have been partially characterised and are approximately similar to those expressed in the SCN. However, oscillators in some sites of the forebrain, but not in the SCN, appear to use NPAS2 (neuronal PAS domain protein 2; also called MOP4), a member of the basic helix–loop–helix-PAS class of transcription factors that are homologues of the transcription factor CLOCK, which is an important component in the SCN (48).

In short, even if some of these circadian oscillators, in and out of the brain, need the SCN to sustain circadian rhythmicity, or for synchronisation between peripheral clocks, they can be entrained to other cues independently of the SCN.

Entrainment of peripheral clocks by feeding and other cues

The SCN control the phase of peripheral tissues mainly by imposing a rest/activity cycle, which in turn determine a daily feeding cycle (42, 49). But, what are the timing signals that entrain peripheral oscillators? One possibility is that the SCN communicate with

peripheral oscillators through sympathetic and parasympathetic neurones of the autonomic nervous systems (in part via catecholamine release). This hypothesis was proposed because electrical stimulation of the sympathetic nerves or adrenaline injection produces an increase of *Per1* expression in the liver. Moreover, sympathetic denervation and SCN lesions abolish the rhythms of *Per1, Per2*, and *Bmal1* genes and also the noradrenaline content in the liver (50, 51). Moreover, in a recent fascinating experiment, Ishida *et al.* (52) revealed that light induces gene expression in the adrenal gland and an increase in corticosterone secretion via the SCN-sympathetic nervous system pathway.

In nocturnal rodents, feeding occurs principally during the night. In experimental conditions, when access to food is restricted to a few hours during the day, animals become active in anticipation of meal-time. There are also phase advances of the circadian rhythms of gene expression in the liver, kidney, heart, pancreas and other tissues, and in some brain structures, uncoupling them from the control by the SCN whose entrainment to the light remains intact (53–55, 56). *In vitro* experiments of liver and lung tissues from animals that have been submitted to restricted feeding show differential phase advances of the spontaneous circadian cycle of *Per1* activity (53). Thus, feeding–fasting signals might be involved in the entrainment of the peripheral circadian oscillators (53, 54). Glucocorticoids have been proposed as a good candidate signal (57). Indeed, the light-induced increase in corticosterone secretion may also participate in the phase resetting of peripheral oscillators throughout the body (52).

A single administration of dexamethasone, a glucocorticoid receptor agonist, induces circadian gene expression in rat-1 fibroblasts and phase-shifts circadian gene expression in mouse peripheral tissues, such as the liver, kidney and heart, without affecting SCN neurones (57). Moreover, the synthetic glucocorticoid prednisolone induces expression of *Per1* in cultured hepatic cells (58). However, mice lacking the glucocorticoid receptor show circadian gene expression in the peripheral clocks, suggesting that glucocorticoids are not essential for circadian rhythmicity (59). Moreover, restricted feeding can entrain gene expression rhythm in the liver of adrenalectomised or glucocorticoid receptor deficient mice. Synchronisation to restricted feeding is faster in knockout mice than in wild-type mice (57, 59). Therefore, these data suggest that the ability of restricted feeding to entrain peripheral oscillators depends on different factors.

In addition to glucocorticoids, other compounds, such as forskolin, some agonists of the adenylate cyclase and some growth factors have been shown to affect the expression of *Per* genes in peripheral tissues and cultured cells (60–62). On the other hand, a serum shock given to cultured fibroblasts can induce the oscillation of clock genes by the activation of many intracellular kinases, such as protein kinase A, protein kinase C and mitogen-activated protein kinase; many of those act through the CRE sequences in *Per* genes (37, 61).

Restricted feeding causes periodic availability of many circulating macronutrients (63), which can reset or induce clock gene expression in peripheral oscillators. For example, glucose can initiate circadian gene expression in fibroblasts and insulin can also induce *Per* gene expression in the liver *in vivo* (64, 65). Thus, the changes in some metabolic parameters by feeding are also relevant for entrainment.

The changes in the metabolic status of a tissue affect its cellular redox state (66). The cofactor nicotinamide adenine dinucleotide (NADH), and its phosphorylated form, NADPH, can increase the affinity of CLOCK/BMAL1 and NPAS2/BMAL1 complexes for their target DNA *in vitro*. Whereas the reduced forms of nicotinamide adenine dinucleotide NAD(P)H stimulate DNA binding of CLOCK and NPAS2, the oxidised forms NAD(P)+ inhibit it (48, 66, 67). Thus, the anabolic/catabolic cycle, in which animals are under food restriction, may affect the circadian feedback loop in clock gene expression in peripheral tissues.

Changes in body temperature by restricted feeding also can modify circadian gene expression in peripheral oscillators. Fibroblasts incubated in a low-amplitude temperature cycle show longer circadian gene expression than those incubated at a constant temperature (68).

Although the reduction of body temperature in animals under restricted feeding could be a relevant factor to reset the clock, the mechanism by which temperature cycles might entrain peripheral clocks is still unknown. Moreover, as mentioned above, metabolic status and/or the autonomic nervous system might be involved.

When we look at the brain, we can see that food restriction is also able to entrain the activity of these central circadian oscillators. In the hypothalamus, multineuronal activity in the lateral and ventromedial hypothalamic nuclei from animals exposed to restricted daily feeding schedules shows a peak of activity entrained to the time of feeding (69). In addition, *Per1* and *Per2* expression in the cerebral cortex, hippocampus and striatum from mice entrained to restricted feeding also shows a phase shift with peaks at meal-time, which is different from the nocturnal peak expression in animals fed *ad libitum* (56).

In another brain structure, the oval region of the bed nucleus of the stria terminalis, the rhythm of PER2 expression is shifted by restricted feeding and becomes uncoupled from the PER2 expression rhythm in the SCN (70).

All these data suggest that peripheral clocks within and outside of the brain are affected by restricted feeding schedules.

The food entrainable oscillator: a mysterious circadian clock

In the Introduction to this review, I mentioned that we can feel the physiological signals of hunger at specific times of the day (even in the absence of food), in anticipation of meal-time. This phenomenon has been observed at the behavioural level in many laboratory animals (71, 72). When animals are entrained to a daily food restriction, there is an increase in locomotion, corticosterone secretion, body temperature and several metabolic parameters, in anticipation of meal-time (71, 72). The increase of arousal for optimal food ingestion, and for facilitation in the use of nutrients, leads to the assumption that having a food clock ensures that organisms obtain sufficient energy sources even when food becomes restricted

to being given at a specific time of the day. Is there a clock responsible for this anticipation of daily meals?

The SCN (which drive almost all behavioural and physiological rhythms) were the first candidates proposed to be responsible for the generation or entrainment of food-anticipatory circadian rhythms. However, Stephan (72) showed that food anticipatory activity (FAA) is still present in SCN-ablated animals. FAA is expressed in wheel running, general activity, feeder approaches and unreinforced bar pressing in an operant chamber. Moreover, some physiological parameters entrained to restricted feeding are still present after SCN lesions, suggesting the presence of an additional circadian oscillator. The food-entrainable oscillator (FEO) displays clear circadian characteristics. One of the most important of these is that its behavioural output (FAA) persists in the absence of food, suggesting that the FEO is able to generate a sustained free-running rhythm (71, 72).

Despite many years of research, the existence of the FEO remains putative. A long list of lesions affecting neural, neuroendocrine tissues and peripheral organs have failed to fully eliminate FAA (71, 72). Ablations of specific brain nuclei as a possible site of the FEO, or part of it, have demonstrated an alteration in FAA, suggesting that the FEO might be a network of scattered brain structures connected to peripheral organs implicated in metabolism.

Lesions of the parabrachial nucleus of the brainstem produce the almost total elimination of FAA (73). In view of its anatomical localisation and physiological role, it was proposed to be an input relay that transmits all the metabolic information from the digestive system to the FEO in the brain, rather than the FEO itself (73).

The reward value of food and its motivational properties are important in food entrainment. Lesions of the core, but not in the shell of nucleus accumbens, also produce a clear reduction of FAA without affecting the nocturnal rhythm of locomotor activity controlled by the SCN, suggesting that the effect of the lesion is specific to the output of FEO (74). Moreover, the importance of motivation in the FEO is demonstrated by the expression of FAA in animals fed *ad libitum* and entrained with a palatable diet (an appetitive and tasty food in nondeprived animals), suggesting that the FEO can be expressed without long fasting–short feeding conditions (75).

Recently, a relevant role of the dorsomedial hypothalamic nucleus (DMH) has been reported for the FAA expression. Circadian c-Fos expression in the DMH is shifted by restricted feeding schedules. Moreover, excitotoxic lesions of this nucleus ablates FAA and the premeal rise in body temperature (76). In addition, oscillations of *mPer1* and *mPer2* expression in the DMH are entrained to restricted feeding (77). Thus, these studies suggest that DMH is a key structure for the FEO expression (76, 77).

With respect to the anatomical localisation of the DMH and its projections to brain regions critical for the regulation of sleep, body temperature and its SCN projections, studies suggest that the DMH is a neural site necessary for entrainment to circadian feeding schedules (76, 77). The DMH integrates circadian and energy information to modulate physiological and behavioural processes, including the sleep/wake cycle, body temperature and locomotor activity (78). The attenuation, rather than total elimination, of FAA by DMH lesions raises the question of whether the DMH is the site of FEO. This is reinforced by another recent study in rats with electrolytic DMH lesions, which does not support the hypothesis that the DMH harbours the FEO (79).

The circadian mechanism of FEO at the molecular level is not yet clear (Table 1). Mice with mutations of clock genes are able to entrain activity rhythms to restricted feeding. *Clock* mutant mice show FAA to restricted meal-time and the persistence of this during food deprivation after 2 days of *ad libitum* feeding (80, 81). This suggests that the FEO does not require the normal CLOCK protein.

Food restriction also induces entrainment of locomotor behaviour in *Cry1–/– Cry2–/–* mice (82). Few differences in FAA were found between wild type and *mCry*-deficient animals. FAA in *Cry1–/– Cry2–/–* double-mutant mice demonstrated slow establishment with an unstable amplitude (82). However, the conclusion of the same study was that CRY proteins are not necessary for FAA.

The gene *NPAS2* forms heterodimers with BMAL1 for the activation of *Per* and *Cry* transcription in the forebrain (48). Molecular oscillations in the forebrain structures, such as the cortex and

Table 1. Phenotype of Food Anticipatory Activity (FAA) in Different Mutant Mice.

Gene	Circadian phenotype	Physiological alterations	FAA
$Clock^{\Delta 19/\Delta 19}$	Longer period/arrhythmic	Metabolic and sleep patterns Drugs sensitisation	Normal (80, 81)
$Per1^{-/-}$	Shorter period	Drugs sensitisation Cancer development	Normal (86)
$Per2^{-/-}$	Shorter period/arrhythmic	Drugs sensitisation and alcohol consumption Cancer development	Absent (86)
$Cry1^{-/-}/Cry2^{-/-}$	Arrhythmic	Sleep patterns	Less stable and gradual (82)
$NPAS2^{-/-}$	Shorter period	Sleep and memory patterns	Delayed (83)
$Bmal1^{-/-}$	Arrhythmic	Sleep and metabolic patterns Infertility	Absent (87)
Ear2 (*Nr2f6*)	Less stable free-run	Increased nociception	Delayed and reduced (90)
Orexin/hypocretin	Unknown	Sleep and feeding behaviour	Reduced (89)

striatum, can be entrained by restricted feeding (56). Moreover, *NPAS2*-deficient mice show delayed responses to restricted feeding schedules. Indeed, although wild-type animals only took 2 days to adjust to food regimen, *NPAS2*-deficient mice took several days and, in addition, lost weight and became sick during food restriction (83).

Despite the interesting data related to FAA expression in mice deficient for clock genes, the effect of gene mutations leads a reduced or deficient ability to entrain to feeding schedules. However, FAA is still present is all these mutant mice, indicating that the FEO is not dependent on one of these genes.

The *Per2* gene is a critical component of the molecular clock mechanism in the SCN and many peripheral oscillators (84, 85). *Per2*-mutant mice are arrhythmic in constant darkness and show altered responses to light resetting (84, 85). Feillet *et al.* (86) exposed *Per2*-mutant and wild-type mice to restricted feeding. FAA was expressed in food-restricted wild-type mice, but not in *Per2* mutant mice. Moreover, to avoid a masking effect of light, the *Per2* mutant mice were housed in constant darkness and FAA was still absent. These results indicate that the *Per2* gene is a key component in the molecular clock mechanism of FEO.

On the other hand, similar to *Per2*-mutant mice, FAA is almost absent in mice lacking the clock gene *Bmal1* (87). These results suggest that at least two principal actors from the SCN clock mechanism could also be implicated in the molecular mechanism of FEO (86, 87).

The orexin (hypocretin) peptides, orexin A and orexin B, are expressed exclusively in the neurones of the lateral hypothalamus. The orexins have been reported to be relevant in the control of feeding behaviour, energy homeostasis, the sleep/wake cycle and motivated behaviours (88). Mice and dogs lacking the orexin gene or the orexin-2 receptor show phenotypes similar to the human sleep disorder narcolepsy (88). Considering the role of orexins in promoting arousal and stimulation of food intake, their role in food-entrainment should be expected. Indeed, there is an attenuation of FAA in genetically orexin neurone-ablated mice, suggesting that orexinergic neurones, which promote wakefulness, are essential for the normal FAA expression (89).

The *Ear2* gene, an orphan nuclear receptor Ear2 (Nr2f6), is expressed in the locus coeruleus (LC), a structure of the mesencephalon that influences several physiological and behavioural processes implicated in the sleep/wake cycle, arousal, nociception, anxiety, stress, cognition and attention (90). *Ear2*-deficient mice lack almost 70% of LC neurones, and they show less efficient adaptation to daily feeding schedules. FAA is reduced in these *Ear2*-deficient mice compared to wild-type animals. These data suggest that LC and the *Ear2* gene also play an important role in the modulation of FAA (90).

Many of the attempts to localise a single structure housing the FEO have failed, and thus have not made its existence so clear (71, 72). Perhaps, it would be more suitable to try to conceptualise the FEO as a system composed of diverse cerebral and peripheral structures, and to define how they work or interact for the functioning of the FEO. Presently, the possible molecular mechanism implicated in the FEO clock suggests the relevance of some clock genes, such as *Per2* and *Bmal1* (Table 1).

Are the SCN also a food-entrainable oscillator?

We know that the SCN clock is principally entrained to the light/dark cycle, and also to many nonphotic factors (27, 28). However, the effects of restricted feeding schedules on the SCN are complex. At the molecular level, food restriction can synchronise peripheral clocks with or without an effect on clock gene expression in the SCN (53). The lack of an effect of food cues suggested that temporal restricted feeding is unable to alter the SCN function (53, 54).

However, in rats under a light/dark cycle and entrained to restricted feeding coupled with a hypocaloric diet (60% of animal's daily food intake), there is a phase advance for circadian rhythms of locomotor activity, body temperature and melatonin in comparison to *ad libitum* fed animals (91). These results suggested that a timed calorie restriction (T-CR) has an effect on the SCN physiology because all these rhythms are controlled by the SCN (Fig. 2).

Recently, we have reported that mice under a T-CR showed an altered entrainment to a light/dark cycle at both behavioural and molecular levels (92). In addition to the behavioural phase advances, timed calorie-restricted mice showed phase advances of *Per1* and *Cry2*, two key genes in the SCN clock machinery, and of the CCG *AVP* (92) (Fig. 3).

This effect is not limited to the transcriptional level because we also detected changes at the protein level, at least for PER1, PER2, CLOCK and the AVP protein expression in response to a T-CR (Mendoza and Challet, unpublished data) (Fig. 3). The alterations of mRNA and protein levels in the SCN, may result from the competition between food and light synchronising signals on the circadian molecular loops.

Behavioural light-induced phase-shifts and light up-regulation of *Per1 and Per2* are deeply modified in mice fed with a timed hypocaloric diet (92). These alterations may be due to the hypometabolic state of the animals in response to chronic calorie restriction, which could lead to a decreased sensitivity of the SCN clock to light-resetting properties. Modifications in circadian phase-shift responses to light have also been reported in animals under low glucose availability (93). In addition to the altered circadian responses to light, T-CR may also modify the circadian responses to nonphotic cues. Serotonergic input from the median raphe nuclei to the SCN conveys nonphotic information (31). (+)8-Hydroxy-2-(di-*n*-propylamino) tetralin [(+)8-OH-DPAT], a 5-$HT_{1A/7}$ receptor agonist, produces phase-advances on the mouse locomotor activity rhythm when injected around subjective midday (31). The behavioural response to (+)8-OH-DPAT injection in mice under T-CR is altered compared to mice under *ad libitum* food conditions (Mendoza and Challet, unpublished data).

The mechanism mediating the effects of T-CR on the SCN is unclear. At the neural level, nonphotic signals to the SCN are depending on the serotonergic and NPY-ergic projections from the raphe nuclei and the intergeniculate leaflets, respectively (28, 29). These pathways may also participate in transmitting metabolic cues associated with hypocaloric feeding signals to the SCN. Moreover, the ventromedial hypothalamic nuclei have been involved to some extent in mediating the behavioural phase-advance produced by

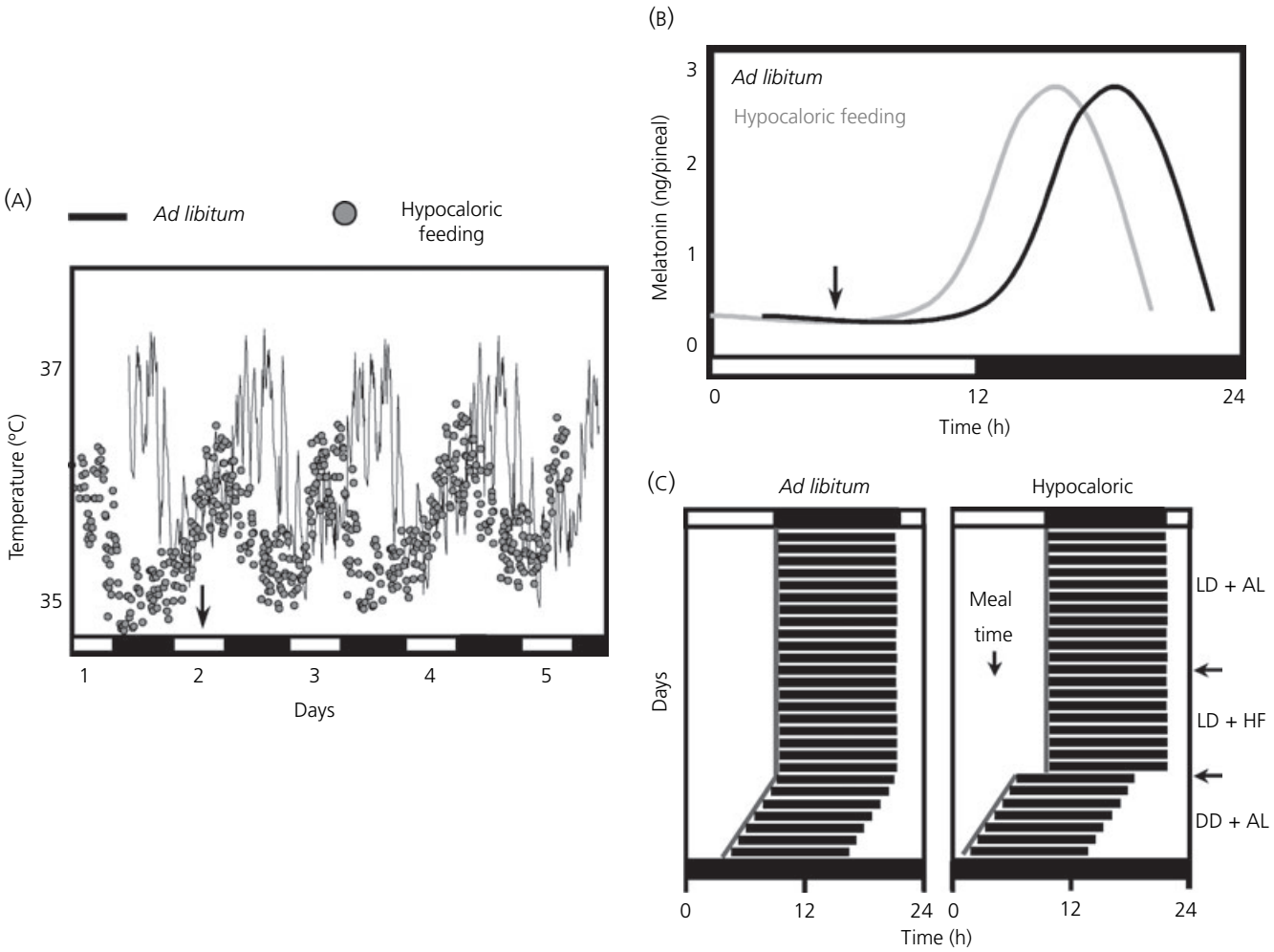

Fig. 2. Phase shift effects of daily hypocaloric feeding (vertical arrows) on circadian rhythms of body temperature (A), nocturnal rhythm of melatonin (B) and wheel-running activity rhythms (C). In these actograms, solid rectangles depict running-wheel activity under light/dark (LD) and constant dark (DD) conditions. In LD, the animal is initially synchronised to lights off (black top bar). Next, it receives a daily hypocaloric feeding (HF) during the middle of rest period (vertical arrow). Then, it is released into DD and food *ad libitum* conditions. Note the phase-advance effects of the circadian rhythms by the hypocaloric conditions compared to animals fed *ad libitum* (91–94).

T-CR (94). Alternatively, direct effects of metabolic cues on SCN cells also have to be considered.

CLOCK/BMAL1 heterodimers are part of the positive arm in the molecular clock mechanism in the SCN for the transcription of other clock genes (15). Alterations in the SCN of timed calorie-restricted mice may be due to some changes occurring in the transcription of *Clock and Bmal1*. The binding of CLOCK/BMAL1 to DNA is modulated by the cellular redox state (66, 67). Thus, as peripheral oscillators, it is possible that the effect of T-CR in the SCN may be due to an alteration in the redox state of the SCN cells.

Although it is not yet possible with the available data to fully understand how T-CR affects the SCN clock mechanism, one possibility is that both behavioural and molecular phase-advance in response to T-CR would be also due to all physiological changes implicated in metabolism and feeding, which could directly act on the SCN clock. Receptors to metabolic factors such as insulin, leptin and ghrelin are present in SCN cells, and these factors are possible candidates for resetting the SCN (95, 96). In addition, these receptors are also located in brain structures that project directly to the SCN such as the arcuate nucleus of the hypothalamus (97). Further studies are needed to assess the putative involvement of these circulating factors and their possible, direct or indirect, effects on the SCN.

As already mentioned, there is an altered photic entrainment when animals are supplied only with a timed hypocaloric diet. Furthermore, in the absence of the light/dark cycle (constant darkness), T-CR is sufficient to entrain behavioural rhythms, as well as the SCN molecular machinery (98). This suggests that a T-CR under constant darkness acts as a synchroniser for the SCN clock mechanism. In addition, rats fed *ad libitum* under constant light conditions become behaviourally arrythmic, and the rhythm of PER2 protein expression in the SCN is abolished (70). Restricted feeding re-induces a circadian rhythm of PER2 and also behavioural rhythmicity in these rats (70). Both studies suggest that, in the

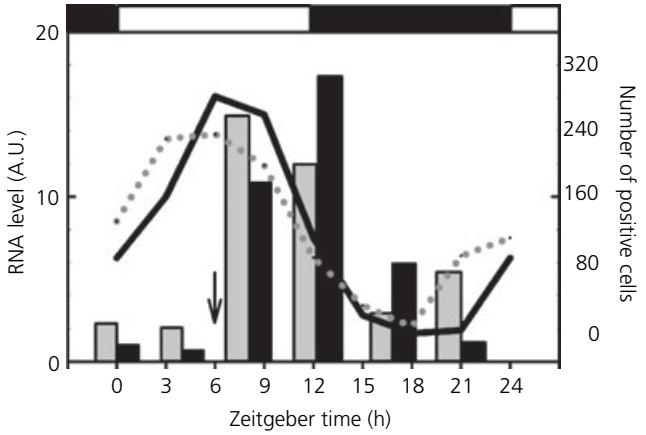

Fig. 3. Circadian profiles of *Per1* mRNA (lines) and PER-1 immunoreactive neurones (bars) in the suprachiasmatic nuclei of mice under food *ad libitum* conditions (solid line and black bars) and daily hypocaloric feeding (dotted line and grey bars). Note the phase advance of both circadian profiles by the hypocaloric regimen. The horizontal bars at the top indicate the light/-dark cycle. The vertical arrow indicates meal-time.

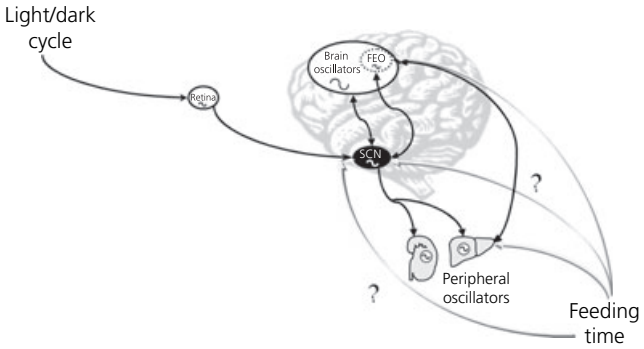

Fig. 4. A proposed model of the effects of food restriction on the mammalian circadian system. The suprachiasmatic nuclei (SCN) are principally entrained by the light/dark cycle through the retina stimulation. The SCN control the circadian activity of the other oscillators by neuronal and humoral pathways. Food restriction is able to entrain peripheral oscillators, such as the liver and kidney, and brain oscillators included the putative food-entrained oscillator (FEO) (possibly in the brain). Restricted feeding (by a hypocaloric diet) also has some effects on the SCN clock mechanism, as well as on their photic entrainment by a direct or indirect mechanism, which remains unknown.

absence of the principal *zeitgeber* (i.e. light) for the SCN, restricted feeding is able to entrain the SCN. On the other hand, food entrainment of mice SCN has been reported in a study using no calorie restriction (99). The entrainment effect was observable in terms of wheel running activity and in the expression of PER2 protein in the SCN (99). The difference in this study is the duration of food restriction (animals took almost 12 weeks to show a stable entrainment to scheduled feeding) and also these animals were previously entrained to a light/dark cycle when food restriction started.

A different way to entrain the SCN by food-related cues, other than food restriction itself, is to use a palatable diet in animals fed *ad libitum* with a standard diet. Recently, we reported that rats under constant darkness are able to entrain the locomotor activity rhythm to a daily palatable meal (100). In addition, in their SCN, the circadian rhythms of c-Fos and PER1 are also entrained (100). This suggests that the reward value of food is relevant for the entrainment of the SCN. The mechanism implicated in this entrainment is still unclear, and further studies on the anatomical and physiological relationships between the SCN and the limbic system are thus required.

Conclusion

The presence of circadian oscillators in almost all the tissues and the putative FEO leads to a large number of fundamental issues to understand the circadian physiology. Moreover, the effects of meal-time on SCN activity in certain food restriction conditions opens a new re-evaluation of research in the entrainment of the SCN by feeding (Fig. 4). One fundamental aim is to understand how the SCN synchronises other circadian oscillators, including the FEO, and to unravel the role of feeding, hormones, metabolic and neuronal signals during these processes. In addition, there is a need to analyse the interactions or coupling between these peripheral clocks and the SCN to know how metabolic and reward networks are implicated in the food entrainment of both SCN and peripheral oscillators.

The conflict between environmental signals and the circadian system (and thereby peripheral functions) is reflected in some circadian health problems in humans as experienced by shift workers, during jet-lag or seasonal depression (3). Altered sleep patterns, ageing, feeding schedules or lighting regimens can perturb the synchrony of the circadian system, showing marked changes in the phase, amplitude, precision of the over expressed rhythms (3, 4). This could reflect a desynchronisation of SCN with the peripheral oscillators.

The regular and daily use of regimens for helping to eliminate these disturbances is relevant to safeguard the well-being of our circadian system. A regular light/dark cycle or dietary meal programme might alleviate circadian disturbances. For example, in animals used as models of diabetes mellitus, a restricted feeding schedule can rescue normal peripheral clock gene expression and also reduce the activity of *Pai-1* (plasminogen activator inhibitor type 1), a primary regulator of the fibrinolytic cascade, in the liver of diabetic mice (101). Moreover, disrupted circadian coordination in SCN-lesioned animals or chronic jet lag exposure leads to an acceleration of the tumour growth (102). Furthermore, it accelerates the growth of tumours and flattens the rhythms of clock gene expression in the liver (102). Temporal restricted feeding slows down tumour growth and increases the amplitude of circadian clock gene expression (103).

Thus, the use of food restriction as a treatment in circadian and noncircadian pathologies should be considered as a tool to coordinate and entrain circadian clocks.

In addition, calorie restriction has been shown to reduce the incidence of age-related disorders, such as diabetes, cardiovascular

disorders, atherosclerosis and cancer (104). The mechanism underlying this effect depends on a reduction of oxygen radicals and cellular damage (104). Disruption of circadian rhythms and the increased incidence of disturbed sleep during ageing are correlated with alterations in the anatomical and temporal organisation and synchronisation of the SCN clock (105). Because a timed hypocaloric feeding affects the SCN pacemaker (92), the relationship between caloric restriction, the circadian system and the mitochondrial free radical theory of ageing should be considered in future investigations.

To examine the physiological benefits of the circadian timing system in humans, several behavioural, physiological, cellular and molecular approaches must be explored. Furthermore, understanding the mechanisms implicated should support the development of more precise therapies for the treatment of circadian pathologies.

Acknowledgements

I would like to thank Dr Etienne Challet for nominating me to write this review and for his critical reading of this manuscript. I would also like to thank Dr Paul Pévet, for his continued guidance and support, Dr Françoise Eclancher, for her critical comments on the manuscript, and two anonymous reviewers, who made valuable comments on this review. This work was support by l'Institut SERVIER and the Fondation pour la Recherche Médicale.

Accepted 2 November 2006

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