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PERIPHERY

Peripheral Circadian Oscillators in Mammals: Time and Food

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Abstract Peripheral cells from mammalian tissues, while perfectly capable of circadian rhythm generation, are not light sensitive and thus have to be entrained by nonphotic cues. Feeding time is the dominant zeitgeber for peripheral mammalian clocks: Daytime feeding of nocturnal laboratory rodents completely inverts the phase of circadian gene expression in many tissues, including liver, heart, kidney, and pancreas, but it has no effect on the SCN pacemaker. It is thus plausible that in intact animals, the SCN synchronizes peripheral clocks primarily through temporal feeding patterns that are imposed through behavioral rest-activity cycles. In addition, body temperature rhythms, which are themselves dependent on both feeding patterns and rest-activity cycles, can sustain circadian, clock gene activity in vivo and in vitro. The SCN may also influence the phase of rhythmic gene expression in peripheral tissues through direct chemical pathways. In fact, many chemical signals induce circadian gene expression in tissue culture cells. Some of these have been shown to elicit phase shifts when injected into intact animals and are thus candidates for physiologically relevant timing cues. While the response of the SCN to light is strictly gated to respond only during the night, peripheral oscillators can be chemically phase shifted throughout the day. For example, injection of dexamethasone, a glucocorticoid receptor agonist, resets the phase of circadian liver gene expression during the entire 24-h day. Given the bewildering array of agents capable of influencing peripheral clocks, the identification of physiologically relevant agents used by the SCN to synchronize peripheral clocks will clearly be an arduous undertaking. Nevertheless, we feel that experimental systems by which this enticing problem can be tackled are now at hand.

Key words circadian clock, peripheral oscillators, phase entrainment, synchronization, feeding time, suprachiasmatic nucleus, body temperature rhythms

A WEB OF CELL-AUTONOMOUS CIRCADIAN OSCILLATORS

It has been known for centuries that the generation of daily rhythms in physiology does not require a brain or a complex nervous system. In fact, the first circadian behavior was discovered in mimosa plants in 1729 by the French astronomer de Mairan. He noticed that his plants continued to display regular daily leaf movements in constant dim light. Since then, circa-

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dian clocks have been found in a large variety of prokaryotes, unicellular eukaryotes, fungi, plants, and animals (Dunlap, 1999). For all of these organisms, the solar light-dark cycle is the most important zeitgeber adjusting the approximately 24-h period of these endogenous timekeepers to the exact 24-h period of the day.

In green plants and in unicellular organisms, it has been known for some time that this phase adjustment is cell autonomous. For example, antiphasic free-running oscillations of gene expression can be entrained in different areas of the same plant when these areas are entrained by light-dark cycles with opposite phases (Thain et al., 2000, and references therein). Nevertheless, the demonstration of circadian gene expression in severed body parts of *Drosophila* (Plautz et al., 1997), explants of zebrafish (Whitmore et al., 1998), hamster and rat organs (Tosini and Menaker, 1996; Yamazaki et al., 2000), and immortalized mammalian tissue culture cells (Balsalobre et al., 1998) came as a big surprise to most researchers. For decades, it had been assumed that during the evolution of complex metazoans, timekeeping had been delegated to specialized brain structures—pacemaker neurons in insects, pineal glands and/or retinas in cold-blooded vertebrates and some birds, and suprachiasmatic nuclei in mammals (Schibler and Lavery, 1999). This notion of specialized clock tissues in complex metazoans had been supported by superb lesion and transplantation studies (Ralph et al., 1990, and references therein) and is of course still valid. However, the existence of oscillators in many peripheral tissues incites us to reevaluate the hierarchical position of the so-called master pacemakers in driving overt rhythms in physiology and behavior. Such a question is particularly relevant for organisms such as *Drosophila* and zebrafish, in which peripheral oscillators can be directly entrained by light. For example, isolated *Drosophila* antennae kept in tissue culture display circadian chemosensitivity and can be independently entrained to LD cycles (Krishnan et al., 1999). Moreover, *Drosophila* Malpighian tubes transplanted into a host entrained to an antiphasic LD cycle keep the phase of the donor for several days if the host is kept in constant darkness (DD) after the transplantation (Giebultowicz, 2001). Thus, any zeitgeber emanating from the brain's central pacemaker neurons to these tissues must be weak at best.

In sharp contrast to this behavior, other circadian outputs, such as locomotor activity in *Drosophila* and egg laying in silkmoths, are strictly dependent on diffusible factors emanating from the brain (Renn et al., 1999; Sauman and Reppert, 1998). Therefore, at least in insects, circadian physiology is influenced both by neuronal master pacemakers and by autonomous, light-responsive oscillators in peripheral organs.

In the mammalian timing system, in which peripheral clocks are unresponsive to LD cycles, the master pacemaker has a more dominant role over peripheral clocks. Although many peripheral tissues are capable of generating circadian fluctuations in gene expression when kept as organ explants, these oscillations dampen after a few days (Yamazaki et al., 2000). Damping is likely the effect of both a gradual attenuation of the amplitude of clock gene expression in every cell and a loss of synchronization between cells. The induction of rhythmic gene expression in cell culture by serum probably does not resynchronize out-of-phase cellular oscillators but rather jump starts dormant clocks. For example, in serum-shocked immortalized rat-1 fibroblasts, circadian mRNA accumulation cycles can be monitored for 3 to 4 days (Balsalobre et al., 1998). If this treatment synchronized already existing, but desynchronized cycles of gene expression in individual cells, one would expect that before synchronization all circadian transcripts should be present at intermediate levels. Alternatively, if the serum shock reanimated dormant cellular clocks that are arrested at a given phase of the cycle (e.g., when *Rev-Erbα* mRNA levels are high and *Bmal1* mRNA levels are low; see Preitner et al., 2002), antiphasic accumulation cycles (such as those of *Rev-Erbα* mRNA and *Bmal1* mRNA) should start at zenith and nadir levels, respectively. Indeed, after a serum treatment, the concentrations of some circadian transcripts (e.g., *Rev-Erbα* mRNA, *Dbp* mRNA, *Per1/2* mRNA) start at peak values and then fall to nadir values after 12 h. Yet antiphasic transcripts (e.g., *Bmal1* mRNA) are present at nadir levels in untreated cells but increase to zenith levels within the same time span. Such a behavior is consistent only with a de novo initiation of rhythms that were frozen at a fixed circadian phase.

THE LOGIC OF PERIPHERAL OSCILLATORS

Before discussing how peripheral oscillators may be synchronized, it may be useful to address their utility. It could be argued that conservation of resources is the major purpose of restricting the expression of certain genes to the time when their products are needed.

However, cells are not particularly economical when it comes to gene expression, and most gene products are synthesized in excess of what is needed for survival. It is more likely that circadian gene expression in peripheral tissues serves to optimize cellular physiology by sequestering chemically incompatible reactions to different time windows. Transcriptome profiling studies using DNA microarray and differential mRNA display technologies support this hypothesis, as discussed below.

In different reports, the fraction of cyclically expressed transcripts amounted to 2% to 10% of the mRNA population, depending on the cell types investigated and the methods used. Even the most sensitive transcriptome profiling technologies reveal signals for only about 50% to 60% of the mRNA species accumulating in a given tissue (F. Gachon, O. Schaad, and U. Schibler, unpublished observation). Moreover, circadian transcripts are probably underrepresented due to their instability. Thus, it is likely that the reported fractions of circadian genes are underestimates and that 1000 or more genes may be clock controlled in a given peripheral cell type. The vast majority of these genes appear to be regulated in a tissue-specific fashion. For example, only 37 of the 575 and 462 circadian transcripts detected in liver and heart, respectively, by Affymetrix oligonucleotide microarray hybridization are shared by both tissues (Storch et al., 2002). Hence, as might be expected, circadian oscillators serve different purposes in different cell types. In the liver, a large fraction of circadian transcripts encodes proteins engaged in metabolic pathways related to food processing and detoxification (Akhtar et al., 2002; Duffield et al., 2002; Kornmann et al., 2001; Panda et al., 2002; Storch et al., 2002).

Transcriptome profiling studies have also unveiled another important facet of cyclic gene expression, namely, that transcripts produced by clock-controlled genes accumulate in many different phases (Akhtar et al., 2002; Duffield et al., 2002; Kornmann et al., 2001; Panda et al., 2002; Storch et al., 2002). This suggests that different cellular functions must be performed at different times. The myriad different possible phases of circadian gene expression might also help to explain why independent peripheral clockworks evolved. If each of these differently phased processes were independently regulated from the brain via a discrete substance secreted by the master clock, a very large number of signals would be required to set these different phases. The existence of cellular peripheral oscillators simplifies the setting of different phases by a small number of entrainment cues. At least in principle, the SCN would have to entrain only a single phase in peripheral clocks—say that of *Per* gene expression—and all the other phases would be timed automatically by the interlocked clockwork circuitry. Based on existing evidence, we propose that expression cycles with different phases are wired, as indicated in Fig. 1. In this scenario, the molecular oscillator directly drives the cyclic accumulation of circadian transcription factors (Reppert and Weaver, 2002; Ripperger et al., 2000). In turn, clock-controlled activators and repressors control the rhythmic expression of downstream genes. Some of these downstream genes also specify regulatory proteins. Each step in this cascade has a somewhat retarded phase with respect to the previous one, since the accumulation cycle of a protein is delayed by about 4 to 6 h from the transcription cycle of its gene. Obviously, different phases can also be obtained by the synergistic regulation of genes carrying multiple cis-acting elements binding circa-

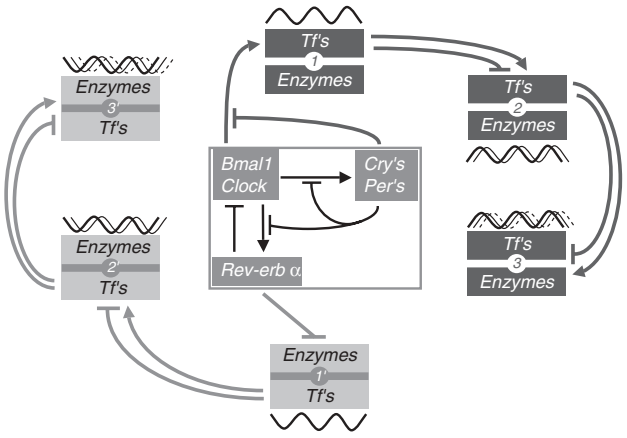

Figure 1. The generation of different phases inside the same cell. The cellular oscillator is composed of 2 feedback loops of clock gene expression, one within the negative limb (*Cry* and *Per*) and one within the positive limb (*Clock* and *Bmal1*). These are interconnected by the repressor REV-ERBα, an orphan nuclear receptor (Preitner et al., 2002; Reppert and Weaver, 2002). The molecular oscillator directly drives the cyclic expression of clock-controlled genes in box 1 via E-box elements interacting with CLOCK and BMAL1 (Ripperger et al., 2000), and clock-controlled genes in box 1′ via RORE elements binding REV-ERBα (Ueda et al., 2002). The transcription factors specified by the *Tf* genes in boxes 1 and 1′ govern the rhythmic transcription of the genes in boxes 2 and 2′, and the *Tf* genes in boxes 2 and 2′ impose cyclic transcription on the genes in boxes 3 and 3′. The phases of the circadian expression profiles (shown in a cumulative fashion below the boxes) differ by 4 to 6 h between genes in 2 consecutive boxes. The model shows that once the phase of the molecular oscillator is entrained, this oscillator can readily synchronize a large number of different phases of clock-controlled output genes (see text).

dian transcription factors accumulating with different phases. Such a combinatorial mechanism has recently been established for the circadian transcription of *Cry1*, which is mediated by both REV-ERBα and the PER/CRY complex (Etchegaray et al., 2003; Preitner et al., 2002).

Many of the results obtained in these transcriptome profiling studies help us to understand the utility of circadian oscillators in peripheral tissues. For example, glycogen stores in liver and muscle are built up during the absorptive phase and broken down to glucose during the postabsorptive phase. Clearly, it would not make sense to express the antagonistic enzymes glycogen synthase and glycogen phosphorylase at high levels throughout the day. Similarly, the circadian regulation of detoxification pathways may limit collateral damage of certain biochemical reactions to a minimal time span. In hepatocytes, cytochrome P450 enzymes play an important role in the detoxification of certain food components; however, these enzymes also generate reactive oxygen species (ROS) as hazardous by-products. Because ROS production may be even higher in the absence of cytochrome P450 substrates, it may be advantageous to accumulate high levels of these enzymes only when they are needed (Bondy and Naderi, 1994).

FEEDING TIME IS THE DOMINANT ZEITGEBER

As already mentioned, long-term circadian gene expression in peripheral tissues depends on a functional SCN pacemaker because no robustly cycling transcripts could be detected in SCN-lesioned mice (Hara et al., 2001). Moreover, in rats exposed to an experimental phase shift (the equivalent of a human jet lag), the SCN first shifts its phase and then imparts the new time schedule to peripheral clocks (Yamazaki et al., 2000). It probably accomplishes this entrainment primarily in an indirect way, namely, by imposing the timing of rest-activity cycles and thus of feeding. Indeed, feeding time is the dominant zeitgeber for peripheral circadian clocks, at least in laboratory rodents. Because many major organs must adapt their physiology to food and water absorption, the responsiveness of peripheral oscillators to feeding is physiologically plausible. The gastrointestinal tract absorbs food metabolites, the pancreas secretes digestive enzymes, skeletal muscles must regulate glycogen synthesis and utilization, and the kidney controls glomerular filtration and urine production, all food- and beverage-dependent processes.

When nocturnally active rats and mice are allowed to eat only during the day, the phase of gene expression of all of these organs is inverted in the course of about 1 week. Yet the SCN oscillator is completely refractory to the signals associated with feeding time (Damiola et al., 2000; Stokkan et al., 2001). This does not simply reflect that the food-associated phase-resetting signals cannot traverse the blood-brain barrier, since circadian gene expression in brain regions other than the SCN can easily adapt to altered feeding regimens. Rather, the unresponsiveness of SCN neurons suggests that these cells lack one or more signaling components required for the interpretation of feeding cues. The glucocorticoid receptor (GR) was one candidate for such a cue: It accumulates in most body cells but is undetectable in SCN neurons of adult rats and mice (Balsalobre et al., 2000a; Rosenfeld et al., 1993). However, glucocorticoid signaling inhibits rather than promotes food-induced phase shifting in peripheral organs, so it is not itself the resetting signal. In fact, food-induced phase changes proceed with much faster kinetics in GR-deficient hepatocytes or in livers and kidneys of adrenalectomized mice when compared to intact wild-type animals. It is thus tempting to speculate that the SCN attempts to counteract the food-induced uncoupling of peripheral clocks via the pituitary gland-adrenal axis (Le Minh et al., 2001). Such interference could slow the phase-shifting process so that transient shifts in feeding patterns would not immediately produce lasting circadian physiological consequences. In any case, the active conspiracy of the SCN in this process could be examined by comparing the kinetics of food-induced phase resetting in intact and SCN-lesioned animals. Already, a recent study has shown that restricted feeding can reactivate and phase entrain circadian liver gene expression in SCN-lesioned mice, which are otherwise completely arrhythmic (Hara et al., 2001).

Sassone-Corsi and coworkers have recently established an interesting and promising approach for studying the entrainment of peripheral oscillators. They grafted mouse embryonic fibroblasts (MEFs) encapsulated in a collagen disk under the skin of mice. A few days after implantation, these MEFs displayed circadian *mPer2* expression that was in phase with *mPer2* expression recorded in the host organs (Pando et al., 2002). Moreover, cyclic MEF gene expression can be food entrained, similar to normal peripheral host tissues. Hence, the timing cues controlled by the SCN

and/or feeding time must be freely circulating diffusible signals, such as hormones, cytokines, salts, or small-molecular-weight food metabolites (see below). The MEF implantation assays revealed another noteworthy feature: By entraining the phase of peripheral oscillators, the timing cues circulating in the intact body also entrain their period length. Thus, in tissue culture, the period length of cyclic *mPer2* expression is only 20 h in MEFs isolated from *mPer1*-deficient mice. Yet after the implantation of these cells into a wild-type host, the period length of *mPer2* mRNA accumulation was found to be identical to that of host tissues (Pando et al., 2002).

WHAT IS THE NATURE OF THE ZEITGEBERS?

The molecular nature of physiologically relevant zeitgebers for peripheral mammalian clocks is not yet known. Therefore, this section is bound to center on "how it could be" rather than "how it is." The identification of putative timing cues can be approached from 2 directions: first, what the SCN might secrete to serve as a peripheral zeitgeber and how it might work, and second, what substances might synchronize circadian rhythms in peripheral cells.

Many signaling pathways have been identified that induce circadian gene expression in tissue culture cells or provoke phase shifts when pharmacologically activated in animals. Substances activating such pathways include butyryl cAMP, forskolin, tumor promoters (e.g., PMA), endothelin, fibroblast growth factor, calcium ionophors, glucocorticoids, and retinoic acid (Akashi and Nishida, 2000; Balsalobre et al., 2000a, 2000b; McNamara et al., 2001; Yagita et al., 2001). Some, but not all, of these chemicals trigger signaling cascades that converge after a few steps. For example, the release of intracellular Ca^{++} stores by calcium ionophores, the activation of protein kinase C by tumor promoters, and the activation of G-protein-coupled receptors by endothelin and receptor tyrosine kinases by FGF all result in the activation of mitogen-activated protein kinases (MAPKs) and probably in the phosphorylation of cyclic AMP response element-binding protein (CREB), a transcription factor involved in the immediate early gene response. Forskolin, butyryl cAMP, and endothelin increase the intracellular concentration of cAMP, which in turn leads to CREB phosphorylation via protein kinase A (De Cesare et al., 1999). The phosphorylation of this transcription factor at serine 142 has been shown to be required for efficient photic phase shifting of circadian locomotor activity (Gau et al., 2002). Taken together, these observations suggest that at least some downstream components of signaling cascades (e.g., MAPKs and CREB-related transcription factors) participate in both the photic entrainment of the SCN pacemaker and the chemical entrainment of peripheral oscillators.

Per1 and/or *Per2* are among the immediate early genes induced by the above-mentioned chemicals. Conceivably, it is the activation of *Per* gene expression that actually triggers circadian gene expression in vitro and phase shifts clocks in vivo. Unfortunately, this hypothesis will be difficult to verify by loss-of-function genetics, since *mPer1* and *mPer2* have partially redundant functions and since *mPer1/mPer2* double mutant mice are devoid of a functional clock (Zheng et al., 2001). However, the question of whether PER induction is sufficient for phase shifting or for de novo initiation of circadian rhythms in cultured cells could be addressed using mouse strains or cell lines carrying an *mPer1* or *mPer2* transgene, whose expression can be induced by chemicals that, by themselves, do not affect the circadian timing system.

A "transient" phase response curve (PRC) has been established for the liver clock by injecting the GR agonist dexamethasone into mice at different times during the day (Balsalobre et al., 2000a). As shown in Fig. 2, the shape of this PRC is quite different from the PRCs recorded for light-pulse-induced phase shifts of locomotor activity (Daan and Pittendrigh, 1976). While SCN neurons display a "dead time window" of nearly 12 h during which no phase shifts can be provoked, circadian liver gene expression can be delayed or advanced throughout the 24-h day, except during the short transition periods during which the sign of the phase shift changes from positive to negative and vice versa. As slave oscillators would be expected to obey signals controlled by the SCN or feeding throughout the 24-h day, their constant responsiveness to entrainment cues may be physiologically meaningful.

SIGNALS EMANATING FROM THE SCN

Silver and coworkers provided the first convincing demonstration that the SCN can govern overt circadian rhythms via diffusible humoral timing cues. In

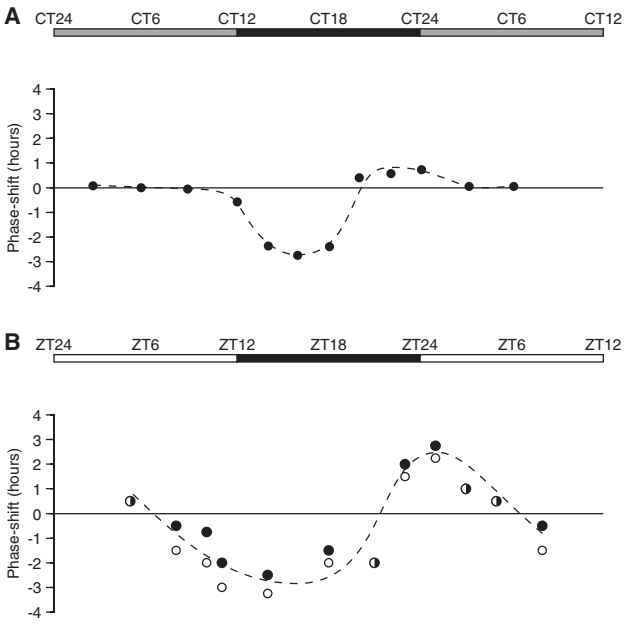

Figure 2. Gated and nongated phase response curves. (A) The photic phase response curve (PRC) shown in panel A was obtained by recording actograms from mice exposed to a 15-min light pulse (for details, see Daan and Pittendrigh, 1976). Note that light pulses delivered at ZT 3, 6, and 9 did not shift the phase. (Adapted with permission from Daan and Pittendrigh, 1976. A functional analysis of circadian pacemakers in nocturnal rodents. J Comp Physiol 106:267–290). (B) A transient PRC of circadian liver gene expression was obtained by recording the circadian accumulation of *Dbp* mRNA (open circles) and *Rev-erbα* mRNA (filled circles) after intraperitoneal dexamethasone injections at the indicated times into mice. Reprinted with permission from Balsalobre A, Brown SA, Marcacci L, Tronche F, Kellendonk C, Reichardt HM, Schutz G, and Schibler U (2000a) Resetting of circadian time in peripheral tissues by glucocorticoid signaling. Science 289:2344-2347. Copyright 2000 American Association for the Advancement of Science.

their experiments, fetal SCN tissue could rescue rhythmic locomotor activity in SCN-lesioned hamsters even when encapsulated in a porous plastic that does not permit axon/dendrite outgrowth (Silver et al., 1996). In very recent experiments, 2 candidates for diffusible timing factors were identified as transforming growth factor alpha (TGFα) (Kramer et al., 2001) and prokineticin 2 (PK2) (Cheng et al., 2002). Both of these signaling proteins inhibit (nocturnal) locomotor activity when injected into the cerebral ventricle, and both are secreted cyclically by the SCN. Unexpectedly, glial rather than neuronal cells appear to be the major factories of TGFα in the SCN (Li et al., 2002). Whether or not SCN-derived signals such as TGFα and PK2 can also serve as phase-shifting agents in distal peripheral organs has not been investigated.

An important question, however, is whether the SCN can generate a concentration of these signaling proteins in body fluids that is sufficient to activate their cognate receptors. Based on the following feasibility calculation, this seems formally possible but unlikely. The SCN of an adult rat contains about 10,000 cells (Guldner, 1983)(equivalent to 20,000 alleles for each single copy gene). The circadian accumulation of signaling peptides demands that both mRNA and protein decay with a relatively short half-life (e.g., 3 h for both). Thus, at a maximal transcription rate $S_R \times 3'600$ mRNA molecule/h/gene (Schibler et al., 1978) and a maximal translation rate $S_P \times 600$ proteins/h/mRNA (Palmiter, 1975), the SCN would be capable of producing approximately 8×10^{11} molecules of a specific protein at steady state ($[20{,}000\ S_R S_P T_{1/2R} T_{1/2P}]/[\ln 2]^2$, where $T_{1/2R}$ and $T_{1/2P}$ are the mRNA and protein half-lives, respectively, which were assumed to be 3 h each). This corresponds to a protein concentration in the extracellular fluid of approximately 2×10^{-11}M (= 20 pM, assuming that an adult rat contains about 60 ml of extracellular body fluid). As the affinity between signaling proteins and their membrane receptors are generally in the picomolar to low nanomolar range (e.g., Kd = 5 pM and 227 pM for the high and low affinity PK2 receptor, respectively; Li et al., 2001), the concentration of 20 pM would be just sufficient to allow for some receptor binding. However, most genes are transcribed at rates that are 1 to 3 orders of magnitude lower than the one assumed in our calculation. Moreover, only a fraction of SCN cells may actually secrete a given signaling protein. Hence, it is unlikely that secreted SCN factors reach a body fluid concentration that is sufficient for the activation of receptors on all peripheral cells. Instead, such factors may operate preferentially in brain regions proximal to the SCN. A local action of SCN signals is also suggested by transplantation experiments, which have shown that the SCN can rescue behavioral rhythms efficiently only when grafted into the third ventricle (Serviere et al., 1994) and in the proximity of its normal position (LeSauter et al., 1997). Hypothalamic cells producing hypophysiotropic hormones are likely targets of signaling molecules secreted by the SCN factors. These hormones (e.g., CRF) then activate the production and secretion of hormones in the pituitary gland (e.g., ACTH), which in turn control hormone production in other endocrine glands (e.g., glucocorticoids in the adrenal gland). Although we feel that hypothalamic brain regions are the likely targets of SCN factors, it should be mentioned that signals produced by an

immortalized SCN cell line kept in tissue culture have been reported to influence circadian gene expression in co-cultured fibroblasts (Allen et al., 2001).

Glucocorticoid hormones, whose cyclic secretion is controlled by the SCN through the hypothalamus-pituitary gland-adrenal axis, are likely candidates for signals entraining peripheral clocks. However, they cannot be the only zeitgeber for peripheral oscillators since the steady-state phase of circadian gene expression is the same in the presence and absence of glucocorticoid signaling (Balsalobre et al., 2000a). These hormones nonetheless contribute to the synchronization of peripheral clocks, as indicated by restricted feeding experiments. As already mentioned, the phase uncoupling of peripheral and central oscillators is much faster in the absence of glucocorticoids or that of their receptor (Le Minh et al., 2001). Whether this process is SCN dependent remains to be investigated. Furthermore, the precise role of other cyclically secreted hormones, such as thyroid hormones and growth hormones, in the phase entrainment of peripheral clocks has not yet been examined.

Another possible entrainment cue directed by the SCN are body temperature rhythms. These circadian fluctuations can influence circadian gene expression in vitro and in vivo (Brown et al., 2002). In rodents, body temperature oscillations are controlled by the SCN, by feeding, and by rhythmic locomotor activity, which are themselves outputs of the SCN. These small physiological temperature changes are capable of sustaining the rhythms of cultured cells, and altered environmental temperature cycles can phase shift clocks in peripheral organs. Because feeding behavior is dominant over temperature in the entrainment of peripheral organs, temperature is unlikely to serve as a feeding-induced zeitgeber. Rather, it might be one of a suite of SCN-directed signals that could help maintain peripheral rhythms.

SIGNALS GENERATED THROUGH FEEDING AND FOOD PROCESSING

Different types of signals can be considered by which feeding sets the phase in peripheral oscillators. These include food metabolites themselves, food-induced metabolites, and hormones whose secretion is controlled by feeding or the lack of it. Before discussing the possible relevance of these pathways, we wish to emphasize that none of the timing cues involved in the food-dependent entrainment of peripheral oscillators have yet been identified. Hence, the ideas presented in the following paragraphs are of an entirely speculative nature.

Recent biochemical experiments performed in McKnight's laboratory have suggested a direct and attractive route through which food may influence peripheral clocks (Rutter et al., 2001). CLOCK and its cousin NPAS2 are major actors of the positive limb of the rhythm-generating feedback loop in gene expression. Both of these transcription factors bind as heterodimers with BMAL1 to E-box sequences in promoters and/or enhancers of *Per*, *Cry*, and *Rev-erbα*, 3 negatively acting genes of this feedback loop. CLOCK and NPAS2 can bind efficiently only to BMAL1 and consequently to their E-box cognate sequences in the presence of the reduced nicotinamide adenine dinucleotides NADH and NADPH. Conversely, NAD+ and NADP+, the oxidized versions of these electron carriers, strongly inhibit the interaction of CLOCK and NPAS2 with their DNA elements. The NAD(P) redox equilibrium depends on the metabolic state of the cell. For example, if glucose is abundant in the cell and oxygen is limited, a substantial fraction of pyruvate—the end product of glycolysis—gets reduced to lactate in the cytosol, instead of being burned to H_20 and CO_2 in mitochondria. This reduction is accompanied by the oxidation of NADH to NAD+, which in turn might inhibit the binding of CLOCK/NPAS2 to E-boxes in essential clock genes. The transient repression of *Per*, *Cry*, and *Rev-erbα* genes (provoked by the inhibition of CLOCK/NPAS2 activity) might then result in the phase shifting of cyclic clock gene expression. This scenario is in keeping with a recent study demonstrating that glucose attenuates *Per* gene expression in cultured fibroblasts and thereby activates the circadian oscillator in these cells (Hirota et al., 2002).

Yet another novel finding from the McKnight laboratory points toward a role of NPAS2 as a gas sensor (Dioum et al., 2002). In vitro, each NPAS2 molecule can bind 2 heme groups, and when these interact with carbon monoxide, DNA binding of NPAS2 is dramatically impaired. It has not yet been investigated whether CLOCK is also a heme-binding transcription factor whose activity can be affected by carbon monoxide. If so, processes that modulate carbon monoxide levels in peripheral cells might be involved in phase resetting. Inside liver cells, carbon monoxide can be generated through the catabolism of heme by heme oxgenase (Dioum et al., 2002) or through microsomal

lipid peroxidation, a process assisted by the cytochrome P450 2B4 (Archakov et al., 2002). It is not yet clear whether feeding influences intracellular CO production, but given the above mentioned findings, such a hypothesis is conceivable.

As mentioned above, hormones whose secretion depends on feeding—or fasting—may also participate in the entrainment of oscillators in peripheral cell types. In fact, the gastrointestinal tract is the largest endocrine organ of the body, and endocrine cells in the gut express about 30 different genes encoding peptide hormones. These genes can specify more than 100 bioactive peptides through alternative splicing and differential protein processing (Rehfeld, 1998). Indeed, secretin, gastrin, and cholecystokinin (CKK), 3 members of this family, were among the first hormones to be discovered. Interestingly, and of relevance for this review, many gastrointestinal peptide hormones are also expressed by neuroendocrine cells in the central nervous system, and in particular in the hypothalamus. The structure and function of the numerous bioactive gut peptides have been discussed in other reviews (Dockray, 1999; Rehfeld, 1998), and we will here only speculate on their potential as timing signals for peripheral oscillators.

Even before the food is ingested, the smell or sight of food components triggers the release of gastrointestinal hormones via the vagus nerve. This leads to a short-term secretion of acid into the stomach. The arrival of food components in the stomach then elicits a long-term response of gastrin and acid secretion. Gastrin and CKK signal through the CKK-B/gastrin receptor, a 7-transmembrane domain G-protein coupled receptor that is present in several cell types inside and outside of the gastrointestinal tract. The activation of this receptor results in the release of intracellular Ca^{++} stores, the activation of PKC, MAPK, and PKA, and, as a consequence the phosphorylation of transcription factors, such as CREB and Jun (Yassin, 1999).

The latter part of this signaling cascade, from Ca^{++} onward, is already well documented to precede the stimulation of circadian gene expression in tissue culture cells and the photic phase shifting in SCN neurons. Thus, it is tempting to speculate that some of the gastrointestinal peptide hormones not only regulate physiological processes related to food processing (e.g., acid, bicarbonate, bile acid secretion, delivery of pancreatic enzymes into the gut, intestinal peristaltic), but also serve as feeding-induced zeitgebers of peripheral oscillators. Although not all cell types that harbor peripheral oscillators are known to contain

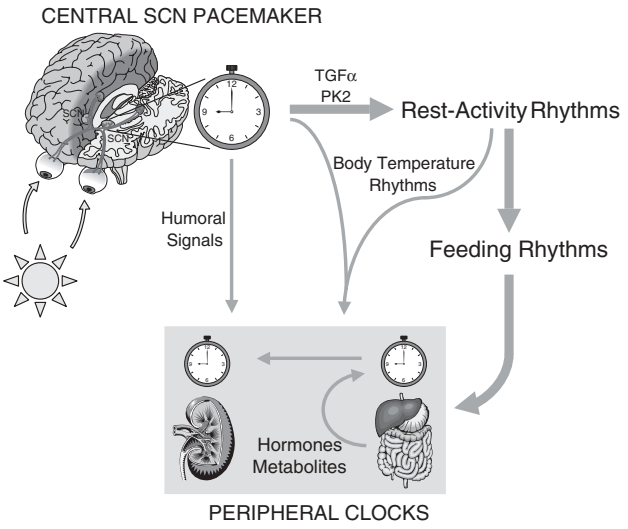

Figure 3. Phase entrainment of peripheral oscillators. See text for explanations.

receptors for gastrointestinal peptide hormones, it is still possible that these substances operate indirectly, by triggering the secretion of other bioactive substances. For example, gastrin can mediate the release of heparin-binding epidermal growth factor–like growth factor (HB-EGF) and amphiregulin (Dockray et al., 2001). TGFα, another member of the EGF family, has already been identified as a possible timing cue of the mammalian circadian timing system (see above). PK2, the second SCN-derived signaling protein found to have a zeitgeber function for behavioral rhythms, and prokineticine 1 (PK1) are also expressed in various regions of the gastrointestinal tract (Li et al., 2001). It is conceivable that these 2 PK isoforms may also act as communication signals between different visceral organs. As insinuated by their name, prokineticines influence the gastric motility by acting on the contraction of smooth muscle cells.

The pathways by which the SCN master clock may synchronize circadian oscillators in peripheral tissues through direct and indirect zeitgebers are schematically depicted in Fig. 3. The SCN master clock, which is synchronized via ocular photic cues, synchronizes peripheral oscillators via direct and indirect timing cues. The most dominant entrainment pathway involves feeding time, which in turn is determined by SCN-driven rest-activity cycles. Feeding time sets the phase of the oscillators of most peripheral tissues. This might be accomplished through signal metabolites, such as glucose, food-induced changes in cellular

metabolism such as changes in NAD(P)H/NAD(P)+ ratios, and food-dependent hormone secretion (see above). Body temperature rhythms, controlled by the SCN and locomotor activity rhythms, may also contribute to the phase setting of circadian oscillators. In addition, the SCN appears to control the entrainment of peripheral oscillators through more direct signaling pathways, such as cyclically secreted hormones (e.g., glucocorticoids).

PERSPECTIVES

How might one dissect the mechanisms by which the master clock and feeding time set the phase of peripheral oscillators? In the previous sections, we have speculated about possible candidate mechanisms, but we are still at a stage where "your guess is as good as ours." In fact, we are ignorant not only of the extracellular signals implicated in the entrainment but also of their intracellular targets. Clearly, the importance of many of the above-mentioned signaling pathways can be verified only by loss-of-function genetics. To this end, the analysis of already available mutant mice with lesions in various signaling pathways will prove invaluable. For example, it has been shown that mice with a CREB transcription factor version that cannot be phosphorylated at serine 142 show a significant deficiency in photic phase shifting (Gau et al., 2002). The same mice can now be examined for their phase-shifting capability with regard to peripheral oscillators or for the entrainment of the clock responsible for food anticipatory activity (see Stephan, 2002). To date, a large number of mutant mouse strains already exist that carry null or hypomorphic alleles of genes encoding transmembrane receptors, protein kinases, adaptor proteins, GTPases, transcription factors, and other signaling components. In most of these strains, the circadian timing system has not yet been examined. Nevertheless, many additional mutant mouse strains will have to be established for a comprehensive genetic analysis of signaling pathways involved in setting the time in peripheral clocks and in the master pacemaker. Moreover, one pervasive problem with the genetic analysis of intact animals will be the discrimination between systemic and cell-autonomous effects. The mouse embryonic fibroblast implant system offers a powerful approach to examine cell autonomous entrainment phenotypes of MEFs from mice carrying mutations in signaling constituents (Pando et al., 2002).

Obviously, genetic strategies can be used only for the identification of signaling components encoded by genes. However, as mentioned above, the food-dependent zeitgeber signals may include intracellular and extracellular metabolites, sugars, fatty acids, and even inorganic salts. The identification of such signals will demand a large amount of biochemical and physiological work. We thus feel confident that those working in the field will be rewarded with surprises and fun for many additional years.

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