Circadian Entrainment of *Neurospora crassa*

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The circadian clock evolved under entraining conditions, yet most circadian experiments and much circadian theory are built around free-running rhythms. The interpretation of entrainment experiments is certainly more complex than that of free-running rhythms due to the relationship between exogenous and endogenous cycles. Here, we systematically describe entrainment in the simplest of the traditional eukaryotic model systems in circadian research, *Neurospora crassa*. This fungus forms a mass of spores (bands of conidia) each day. Over a wide range of photoperiods, these bands begin to appear at midnight, suggesting integration of neither dawn nor dusk signals alone. However, when symmetrical light/dark cycles (T cycles, each with 50% light) are applied, dusk determines the time of conidiation with a uniform, period-dependent delay in phase. This “forced” synchronization appears to be specific for the zeitgeber light because similar experiments, but using temperature, result in systematic entrainment, with bands appearing relatively later in shorter cycles and earlier in longer cycles. We find that the molecular mechanism of entrainment primarily concerns posttranscriptional regulation. Finally, we have used *Neurospora* to investigate acute effects of zeitgeber stimuli known as “masking.”

INTRODUCTION

One of the key properties of circadian clocks specified by Pittendrigh (1961) 47 years ago at Cold Spring Harbor was that of a self-sustained, free-running, approximately 24-hour rhythm. This is a surprising characteristic on which to focus, considering that circadian systems evolved under cycles of light and darkness, as well as those of temperature, wind, humidity, and many other environmental factors. Although the Earth’s rotation frequency has changed over the course of evolution, circadian rhythms hardly ever had the possibility to free-run. Thus, the experimentally generated free-run is a consequence of how circadian systems *must* be put together to function as the adaptive timers that they are. Nevertheless, Pittendrigh’s list of properties overwhelmingly concerned circadian rhythms under constant conditions, dealing explicitly with entrainment in only one item. We propose that an understanding of the clock under entrained conditions may reveal mechanisms and genes relevant to clock functions in the real world beyond those discoverable under constant conditions.

Circadian entrainment describes a biological rhythm that is synchronized with the external physical environment (Johnson et al. 2003; Roenneberg et al. 2003). The most dominant zeitgeber appears to be light, not surprising, as it is the most dependable representation of time of day (compared with temperature or humidity, for example). The coordination of the two oscillators (the Earth’s rotation and the circadian clock) obeys well-established rules, long known from the example of two physical oscillators entraining each other (like two pendulum clocks on the same wall, which can influence each other) (Huygens 1673). In the case of the circadian system, the physical cycle is obviously inflexible, so the biological oscillation must find the appropriate, stable phase relationship within the external cycle. A longer circadian rhythm will settle

later in the cycle than a shorter one (Pittendrigh and Daan 1976; Duffy et al. 2001). A very strong zeitgeber may synchronize by driving or forcing the clock, rather than by entraining it. In this case, the phase will remain fixed relative to the zeitgeber despite changing cycle structure (e.g., length or photoperiod). Thus, comparison of cycles with different structures is one of the tricks to reveal circadian entrainment (Bruce 1961). This can involve changing either the length of the cycle (T) or the length or strength of, for example, the photoperiod within the cycle (often at $T = 24$ hours). Cycles can consist of full zeitgeber periods (e.g., a continuous signal throughout the photoperiod and scotoperiod) or skeletons, whereby a relatively short pulse of a signal is applied to simulate both on and off transitions.

Besides light, there are a multitude of other signals in the environment that can influence entrainment or that, under certain conditions, even can override entrainment by light. Combined light and temperature cycles can show synchronization to either zeitgeber depending on their respective amplitudes (Liu et al. 1998; Roenneberg and Merrow 2001), but without titration experiments, a single observation cannot distinguish entrainment versus masking by either zeitgeber. Similar to the situation of light and temperature in *Neurospora*, rhythmic gene expression in the mouse liver is indirectly controlled by light, via the suprachiasmatic nucleus (SCN), when food is administered ad libitum, but when food is restricted antiphase to normal activity, gene expression in the liver shifts accordingly (Damiola et al. 2000; Stokkan et al. 2001). Presumably, feeding, or rather circulating nutrients, can override the SCN signals for the clock in liver cells. Understanding entrainment on these levels—using multiple zeitgebers that show varied temporal structure—will be relevant to designing healthy work schedules in our 24/7 society, as well as understanding response characteristics to entrainment in the different seasons.

ENTRAINMENT PROPERTIES OF *NEUROSPORA*

For several reasons, we have chosen the fungal genetic model system, *Neurospora crassa*, to systematically investigate entrainment properties: It is haploid, thus untangling genetic principles is simpler compared to diploid organisms. *Neurospora* lends itself to high-throughput experiments and is inexpensive to grow and easy to handle (it can “sit” on the lab bench for weeks without apparent injury); both temperature and light have been used as zeitgebers, enabling comparison of entrainment characteristics with multiple zeitgebers. The power of this system is supported by several other contributions to this volume (Jay Dunlap #95 NYR; Jennifer Loros #10 NYR; Querfurth et al.; de Paula et al.; Cha et al.; all this volume).

The circadian clock in *Neurospora* has been elucidated largely from assays concerning asexual spore (conidia) formation as it grows along the surface of agar media. Mutant screens have revealed a number of *Neurospora* clock genes (genes in which a mutation changes fundamental clock properties). The first to be identified was *frequency* (*frq*) (McClung et al. 1989). FRQ is part of a genetic network that was the prototype for demonstrating the principle of the negative feedback loop (Hardin et al. 1990; Aronson et al. 1994), the molecular mechanism that is thought to be fundamental to the molecular “clockwork.” Other components of this loop include *white collar-1* and *-2* (*wc-1*, *wc-2*), which encode a blue light photoreceptor and its essential cofactor, respectively. The WHITE COLLAR complex regulates transcription of *frq*. Considering their dominant effects on clock function, FRQ and the WHITE COLLAR complex are generally thought to be the core clock components, forming the “oscillator” that receives inputs and regulates outputs. Traditionally, the circadian system is separated into input–oscillator–output. In *Neurospora*, input and oscillator appear to be fused into one set of components. This principle was already suggested by Menaker, Takahashi, and Eskin (Menaker et al. 1978).

Mutations in *frq* alter the free-running period of spore formation in *Neurospora* (Fig. 1a) (Feldman and Hoyle 1976). The correlation of free-running period under constant conditions and the entrained phase under zeitgeber conditions (Pittendrigh and Daan 1976; Duffy et al. 2001) can be used to investigate fundamental principles of entrainment: How does a single-base-pair change (in the entire genome) lead to altered daily timing? As expected, *frq*¹, a short-period allele of the *frequency* locus, shows an advanced entrained phase in all entrainment protocols performed, regardless of zeitgeber modality (e.g., light or temperature, see Fig. 1) (Merrow et al. 1999; Tan et al. 2004), whereas a long-period allele, *frq*⁷, always shows a delayed phase. In *Neurospora*, we have looked at T cycles that use equal lengths in “on” and “off” times within the zeitgeber (e.g., 50% light and darkness [photoperiod and scotoperiod] or warm and cold [thermoperiod and cryoperiod]; Fig. 1b,c) as well as cycles of the same length (*T* = 24 hours) with changing length in photoperiods (Fig. 1d). In all cases, phase and period correlate.

A closer inspection of entrainment in light/dark cycles reveals that phase appears driven and not entrained in symmetrical T cycles of different lengths (Fig. 1c) (Merrow et al. 1999): For all *Neurospora* strains tested, conidiation occurs after a constant strain-specific delay following dusk, much like a reset that occurs upon release from constant light into constant darkness. For such a driven response, a self-sustained rhythm would not be necessary! Interestingly, the phase moves away from the theoretical driven line in cycles about 24 hours at least for the wild-type strain. In contrast to T cycles, in 24-hour cycles with different photoperiods, conidiation starts at about midnight for the wild-type strain and proportionally earlier or later for the short- and long-period mutants, respectively (Fig. 1d) (Tan et al. 2004). This series of experiments shows that conidiation in *Neurospora* is not simply driven by light.

The information obtained in T cycles versus those recorded in photoperiods does not appear to be consistent; we still do not fully understand the driven phase angles of *Neurospora* in T cycles with light as a zeitgeber. The midnight lock of the entrained phase demonstrates that this simple system takes information either from dawn and dusk or from the entire structure of the light/dark cycle, rather than simply responding to either dawn or dusk. Dawn and dusk signals *could* be transduced by different photoreceptors, from different kinetic properties of the same photoreceptor signaling pathway, or from divergent consequences of the same light signal on distinct oscillators. So far, two blue light photoreceptors have been shown to be involved in circadian rhythms in this system (WC-1 and another flavin-binding protein, VIVID; Froehlich et al. 2002; He et al. 2002; Schwerdtfeger and Linden 2003), and more photoreceptors are indicated by sequence in the *Neurospora* genome and by functional analyses (Bieszke et al. 1999a,b; Heintzen et al. 2001; Froehlich et al. 2002, 2005; He et al. 2002; Galagan et al. 2003).

In contrast to T cycles with light, temperature cycles yield systematic entrainment (Fig. 1b), with all strains showing a later phase in a short cycle and an earlier one in a long cycle (Merrow et al. 1999). Furthermore, the expected phase relationship between *frq* strains with short and long free-running periods is evident.

ENTRAINMENT OF THE CLOCK AT THE MOLECULAR LEVEL

In view of the different strategies that the *Neurospora* clock uses for entrainment in temperature (T cycles) and light (photoperiod cycles), we looked for differences in the regulation of clock components at the molecular level. We followed the level of *frq* RNA, as its regulation by light was one of the first mechanisms proposed for how light phase-shifts the clock (Crosthwaite et al. 1995). In temperature T cycles, the phase of expression of *frq* RNA correlates with the phase of conidia formation (Fig. 2) (Merrow et al. 1999). In entraining light cycles (*T* = 24 hours), however, neither accumulation nor disappearance of *frq* RNA correlates with entrained phase (Fig. 3a) (Tan

Figure 1. Characteristics of *N. crassa* in circadian protocols. In all panels: (open circles) *frq*⁺; (closed triangles) *frq*⁷; (closed squares) *frq*¹. (a) The free-running period in constant darkness is 22 hours for the *band* (*bd*) strain (*frq*⁺), 16 hours for *bd frq*¹, and 29 hours for *bd frq*⁷. (b) Circadian entrainment of *Neurospora* in T cycles using temperature as a zeitgeber. The onset of conidiation is plotted, in hours, according to external time (*ExT*₀ = midnight) (Daan et al. 2002). (Open circles) *frq*⁺; (closed triangles) *frq*⁷; (closed squares) *frq*¹; (open diamonds) *frq*⁰. (Dotted line) Theoretically derived plot for a nonentrained but driven output (see c). (Gray area) Cold phases of the cycles. (c) T cycles using light as a zeitgeber. The onset of conidiation is plotted, in hours, according to external time. (Dotted lines) Theoretically derived plots for a nonentrained but driven output, using 5.5 hours following lights off for *frq*¹, 8 hours following lights off for *frq*⁺, and 11 hours after lights off for *frq*⁷. (d) Light cycles of T = 24 hours with varying photoperiods. The onset of conidiation is plotted, in hours, according to external time. (Gray area) Darkness; (open areas) light period; (dotted line) midnight.

et al. 2004). *frq* RNA is rapidly induced whenever the light comes on, and it decreases when lights are turned off. Although the light responsiveness of the *frq* transcript level was confirmed in these results (and extended to its immediate degradation in darkness), *frq* appears to have lost its role as a clock component in entrainment by light/dark cycles. Our results address the difficulties in extending results from light-pulse/release experiments to entrainment with light/dark cycles. We also investigated other light-regulated RNAs that show different kinetics relative to *frq* (*wc-1*, *vvd*, *al-1*, and *con-10*), some of which are considered clock genes or clock-regulated output genes. None of these genes showed transcriptional kinetics that could be associated with the respective entrained phases of conidiation.

In contrast to the results found in light cycles concern-

ing transcriptional regulation, the kinetics of FRQ protein did correlate with the entrained phase. The daily initiation of conidiation always coincided with the point of half-maximal decline of FRQ levels (Fig. 3b). These results suggest that posttranscriptional regulation is more important for entrainment by full zeitgeber cycles than transcriptional control.

On one hand, this is no surprise in light of the elaboration of the importance of posttranslational chemistry such as phosphorylation (Kloss et al. 1998; Liu et al. 2000; Yang et al. 2002; Nakajima et al. 2005; Schaffmeier et al. 2005). On the other hand, in all systems, a transcriptional feedback loop is absolutely essential for a normal clock function. What are we missing? For instance, there are at least two *frq* transcripts, and it could be that only one of them is light-induced and is simply masking a functional

Figure 2. *frq* RNA is entrained in temperature cycles. The *frq* RNA levels are double-plotted after normalization within each time series according to ribosomal RNA levels, followed by adjustment of the maximum value to 1.0. (a) The 16-hour T cycle; (b) the 22-hour T cycle. (Gray area) Cool phase (22°C); (open area) warm phase (27°C). The arrows show where the conidial band is developing relative to the RNA profiles.

and entrained expression of another less-abundant transcript, the kinetics of which could be associated with entrainment. However, as we understand the system now, the (predominant) light-induced *frq* transcript(s) can clearly be ruled out as a state variable of the *Neurospora* clock under entrained conditions, as much as it appears crucial for self-sustained rhythmicity of conidiation in constant darkness (Aronson et al. 1994).

Figure 3. *frq* RNA and FRQ protein levels in photoperiod cycles, with $T = 24$ hours. The highest value in each individual profile is normalized to 1.0, and the rest of the values are adjusted accordingly. The time series are double-plotted, with dotted lines indicating midnight ($ExT = 0/24$ on the x axis) or half of the peak value of normalized expression (0.5 on the y axis). (a) *frq* RNA levels reflect the photoperiod. (Thick gray line) Data points corresponding to incubation in the light phase; (thin black lines) data points corresponding to the dark phase incubation. $ExT0$ = midnight (Daan et al. 2002). (b) FRQ protein levels reflect conidiation in entrainment with light. The lines are drawn according to a.

REVEALING A MULTIOSCILLATOR SYSTEM WITH ENTRAINMENT OF “CLOCK-NULL” STRAINS

Most clock genes have earned their status based on their loss of free-running circadian rhythm, implying a central and critical function in this behavior. However, modeling reveals how an input component upstream of an oscillator can drastically change circadian properties, leading even to arrhythmicity (Roenneberg and Merrow 1998). The fact that many circadian rhythms are arrhythmic in constant light demonstrates the strong impact of inputs on the entire clock system. So, any interpretation of arrhythmicity resulting from a genetic mutation should be extended to the input components of the clock as well as to components that may couple the oscillator to the output pathway (Roenneberg and Merrow 2001).

There are several ways to test whether a clock component is on an input pathway versus being central to the oscillator mechanism. One of them compares different constant conditions (e.g., constant light of different intensities or constant darkness) to see if rhythmicity is restored in one of them. When *mPeriod2* and *mClock* mouse mutants that are arrhythmic in constant darkness are kept in constant dim light, they show free-running activity rhythms. They also respond to changing fluence rate (Aschoff's rule; Aschoff 1979), by systematically changing their circadian period (Spoelstra et al. 2002; Steinlechner et al. 2002). In *Neurospora*, clock-null mutants can show rhythms when incubated on media containing different nutrient concentrations (Dragovic et al. 2002; Granshaw et al. 2003).

An alternative approach to discern input from oscillator is to look for features of circadian entrainment in clock-null mutants that are otherwise arrhythmic under constant conditions. In light cycles, the *frq* null mutant strains cannot be synchronized, but temperature cycles yield rhythmic conidial bands that are nearly antiphase to those found in wild-type strains (Merrow et al. 1999). In a series of T cycles, the phase of conidiation in these mutants advances with increasing cycle length, reflecting entrainment of a circadian oscillator (or of a multioscillator network). This indicates that free-running rhythmicity in constant darkness may utilize different components than entrainment in temperature cycles. The *frq* gene is a key clock component for the former but apparently dispensable for the latter and for free-running rhythms in constant light (de Paula et al. 2006).

The more we probe circadian systems under different conditions, the more it becomes apparent that we are dealing with a network of feedback loops and oscillators (Roenneberg and Merrow 2003). This is supported by the findings that certain combinations of mutations in independent clock genes can result in the restoration of clock properties as was shown in *Drosophila*, when the *cry^b* mutation was crossed into a *per⁰* fly, and in mice, when *mCry2mPer2* animals were generated (Oster et al. 2002; Collins et al. 2005). In *Neurospora*, temperature-compensated molecular oscillations were revealed in *frq* knockout strains (Correa et al. 2003). Collectively—rhythms and evidence of entrainment without key clock genes—

should make us reevaluate the role of the transcriptional feedback loop in both entrained and free-running circadian systems. Clearly, in all model organisms studied to date, it is critical for proper timing, but evidence is accumulating to support the idea that there are clock components which are difficult to identify using the traditional genetic tools and the protocols that have brought us so far (Lakin-Thomas 2006).

MASKING IS PART OF ENTRAINMENT

Stable entrainment is typically conditional. It depends on the length of the zeitgeber cycle being within the range of entrainment (Aschoff and Wever 1962) and on sufficient zeitgeber strength. If a zeitgeber is too weak, it will not stably entrain and if it is too strong, it will simply drive the system. Furthermore, zeitgebers can have a number of additional effects on the system that do not strictly concern timing. The term “masking” describes the regulation of a circadian behavior by a zeitgeber in a manner that is not consistent with circadian entrainment (Mrosovsky 1999). A common example is the effect of light on nocturnal rodents, which—at least in the laboratory—remain inactive until dark (Mrosovsky et al. 2001). Release from light/dark cycles to constant darkness often reveals that the clock would have initiated activity somewhat earlier and that it was masked by light. This is presumably an adaptive function, an override of the circadian system.

We have characterized masking in *Neurospora* in several ways. First, we tested for frequency demultiplication: When an entraining cycle length is approximately half of the free-running period, a robust circadian clock entrains to every other cycle, rather than to the short cycle that lies well outside its range of entrainment (Bruce 1961). In 12-hour temperature cycles, one band of conidia appears every 24 hours when the *Neurospora* wild-type strain is used, whereas two conidial bands per 24 hours are produced by the *frq* null strain (Morrow et al. 1999). This is reminiscent of the driven conidiation of the wild-type strain in T cycles when light is used as a zeitgeber. With closer inspection, however, the two bands produced by the *frq* null strain in 24 hours appear to be asymmetrical, suggesting that one is a masking response and the other circadian (Roenneberg et al. 2005). Dose-response titration of the zeitgeber confirms this explanation, showing increasing masking with higher mean temperatures and a higher-amplitude temperature cycle, in both wild-type and mutant strains.

A second exploration of masking made use of air pulses and their induction of conidia (perhaps by not allowing CO₂ to accumulate in the race tube or, alternatively, due to the physical stimulation of air flow over hyphae) (Roenneberg et al. 2005). Under otherwise constant conditions, air pulses once per 18 hours yield a conidial band, whereas circadian conidiation continues with its endogenous period of 22 hours (in wild type). When the masking conidiation and the circadian conidiation coincide, the amplitude of the signal increases. The *frq* null mutant strain shows a similar pattern except that the masking response is larger relative to the clock mutant's endogenous conidiation pattern; the free-running period in this

case is shorter than the wild-type strain, and once the endogenously and exogenously stimulated conidiation come together, they do not immediately separate. These observations also show how the circadian clock affects the amplitude of the masking response.

Finally, in a multilab repetition of temperature cycles in *Neurospora*, a data set was generated that, at first analysis, did not show circadian entrainment of *frq* null strain (Pregueiro et al. 2005). Through comparison with the original data set, the waveform was interrogated to extract masked elements of the synchronization (Roenneberg et al. 2005). Retrospectively, the zeitgeber conditions (temperature transitions, relative humidity) were different enough to result in the differences in conidiation patterns through masking.

These experiments suggest that entrainment will always show some degree of masking. In understanding entrainment, on the one hand, we have to understand how to demask entrainment data (to uncover the pure circadian components of entrainment). On the other hand, we have to appreciate more the role of masking itself in contributing to entrainment in the real world.

DISCUSSION

It is remarkable how extensively one can describe entrainment of circadian clocks by rules that are based on the synchronization of simple mechanical oscillators. This was already known when the pioneers of the field met at Cold Spring Harbor in 1960: Woody Hastings had published the first light-pulse PRC 2 years before (Hastings and Sweeney 1958). Forty-seven years later, the apparent power of this simple explanatory approach is even more remarkable, given that the overwhelming theme of the 2007 Cold Spring Harbor Meeting on Clocks and Rhythms was the complexity of circadian systems. In addition, complexity derives from the temporal structure of the entraining environment in nature, which includes multiple zeitgebers whose precise qualities and quantities vary from day to day. Furthermore, the impact of environmental signals on the circadian system is modulated by the clock itself (zeitnehmers; Roenneberg and Morrow 1998). Thus, it is surprising that PRCs can be classified by a relatively small number of forms as an indication of how entrainment works.

Here, we have described our first attempts to apply the holistic approaches of systems biology on understanding entrainment. Using a relatively simple and inexpensive system, we are generating a “surface” of circadian entrainment by light (all combinations of endogenous period, zeitgeber period, photoperiod, and zeitgeber strength). We are currently generating the same circadian surface for temperature and plan to construct surfaces with different combinations of zeitgeber modalities. Eventually, the effects of different nutrients on surface characteristics can be investigated. We expect this approach will identify hitherto unknown properties of circadian entrainment. Although some of these may be special for *Neurospora* spore formation, others will be common to all clocks. Protocols developed in this manner will also help to identify new clock genes that may not be

able to be discovered under standard circadian protocols, such as 12:12 light/dark cycles or constant conditions.

These protocols, so far, operate at the level of the organism (entrainment of conidiation) or of the gene (entrainment at the molecular level). In addition, there are intermediate levels within systems. Clocks in cells, tissues, and organs are entrained by endogenous zeitgeber signals that may not strictly correlate with (represent) exogenous zeitgeber signals. The result is a set of internal phase angles of which we know little but which will be critical to the functioning of the system as a whole. Understanding entrainment at all of these levels will eventually help us to treat the pathologies of entrainment in humans associated with shift work, jet lag, or weak zeitgeber exposure in modern city life.

Our results have already changed how we view entrainment by light in *Neurospora*. Although former experiments suggested that release from light resets the clock, we find—when looking at the entire surface—that the clock integrates more features of the zeitgeber structure. Nevertheless, the simple mechanical oscillator rules apparently often still apply. The strong correlation between free-running period and phase of entrainment (chronotype) is systematically present, regardless of (period) mutant and zeitgeber conditions. Yet, there are apparently exceptions, as demonstrated in *Neurospora* by the *vivid* mutant. Although they show a wild-type-like free-running period, their phase is delayed, either on release from light to constant darkness (Heintzen et al. 2001; Shrode et al. 2001) or under entrained conditions (C. Boesl et al., unpubl.). This is probably not an isolated case but a rather common case regarding the principles of entrainment. The identification of genes that influence both phase of entrainment and period versus those that influence either period or phase alone will be an excellent phenotyping tool for understanding the molecular mechanisms of the clock.

Our systems approach to entrainment has so far concentrated on the physiological outputs of the system. In the future, this must be extended to the molecular level with high-throughput methods (i.e., reporter genes or sensitive physicochemical methods such as LC/MS). In addition, we have to find ways to assess “small effects” that are a critical aspect of how complex traits, such as circadian clocks, are regulated. Entrainment of circadian clocks—with genetic control mechanisms that extend from big to small effects—is an excellent systems biology question. A post hoc analysis of microarray data showed that essentially all transcripts in mammalian cells, as was previously shown for *Synechococcus* (Liu et al. 1995), are oscillating with a circadian period (Ptitsyn et al. 2007). The oscillating cellular machinery will likely include many zeitnehmer feedbacks with small (sometimes very small) effects. To identify these is a challenge that will benefit from experiments with constant and entraining conditions. We have begun to attack this question by creating surfaces involving all parameters influencing entrainment and by measuring physiological outputs. Now, this has to integrate transcriptome, proteome, and phosphorylome data in the context of various entraining conditions.

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