Circadian waveform and its significance for clock organization and plasticity

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Chapter Outline

Introduction
- Parameterizing circadian rhythmicity

Multiple oscillators comprise mammalian circadian timing systems
- The complex circadian pacemaker and waveform plasticity
  - Photoperiodic regulation of circadian waveform
  - Changes in circadian waveform under constant light
  - Photoperiodic non-responsiveness and arrhythmicity in Siberian hamsters
  - Transient changes in circadian waveform
  - Waveform Bifurcation
  - Role of dim light in waveform modulation

Waveform variations in the SCN

Consequences of waveform manipulation

Summary

Chapter Overview

The daily rotation of the earth creates a strong selection pressure for the evolution of endogenous circadian clocks that, at least in mammals, are generally phase-shifted slowly and incrementally by light. Because the earth's axis of rotation is tilted relative to the revolution around the sun, there is an additional selection pressure for clocks to adjust their waveform (i.e., shape of the daily oscillation) to match seasonal variation in daylength. With a focus on rodents, this chapter reviews protocols demonstrating circadian waveform plasticity and its relationship to the functional organization of the suprachiasmatic nuclei (SCN) of the anterior hypothalamus. Manipulation of waveform uncovers additional novel and unanticipated effects on the lability of circadian timing systems.

Introduction

It is commonly appreciated that circadian clocks evolved to maximize fitness in a world where there is recurrent and predictable change in the environment. In particular, a circadian clock enables organisms to anticipate the changing environmental conditions – from light to dark or from warm to cool, for examples – that result from the rotation of the earth around its axis. A key discovery in chronobiology has been the demonstration of endogenous timing mechanisms across biological taxa (e.g., cyanobacteria, plants, invertebrates, mammals etc). In the absence of any environmental timing cue, organisms continue to express near 24 h rhythmicity in various outputs. Because the period of these rhythms does not match any known environmental cue or the rhythms of other organisms held under identical conditions, their endogenous origin is indisputable. And indeed, the molecular mechanisms, commonly involving transcription/translation negative feedback loops, have been characterized for a number of model systems. A second notable achievement is the increasing understanding of how these not-quite 24 h rhythms can be adapted, or entrained, to 24 h conditions. The most common entrainment signal, or Zeitgeber, is light which when presented acutely, can cause the phase of the rhythm to advance or delay. With a similar pattern observed across many species, the endogenous clock mechanism generates a circadian rhythm in the resetting effects of light. A light pulse presented early or late in the night will differentially reset the phase of the rhythm with the effect of promoting synchronization to the 24 h day. Finally, disruption of clocks, whether by pharmacological, genetic, or environmental means, commonly disadvantages organisms. Indeed, a close match between period of the clock and the environmental cycle improves survival.

Despite an impressive understanding of the mechanisms that generate and entrain circadian rhythms, it remains difficult to practically manipulate mammalian, and particularly human, rhythms in ways that have obvious utility. People asked to work during the night, for instance, are generally unable to use light effectively to realign their circadian clock to promote alertness during their night shift-work. And after rapidly crossing multiple time-zones, jet travellers require several days to realign the phase of their endogenous clocks to match local time. Thus, there is a remarkable robustness apparently built into the design of the mammalian circadian pacemaker that resists rapid adjustment in the face of abrupt environmental change.

Whereas circadian rhythmicity is arguably necessitated by the earth's rotation, the axis of rotation is tilted relative to the revolution about the sun. The geophysical consequence of this is that, except at the equator, the relative durations of day and night vary systematically throughout the year. To the extent that anticipation of an oscillating light environment is a strong selection pressure, circadian clocks of organisms should be well-adapted to such changes. Indeed, plasticity with respect to entrainment to these seasonal photoperiods (i.e., daylengths) should be an expected counterweight to the rigidity considered above.

Parameterizing circadian rhythmicity

As time is cyclical, the study of circadian rhythmicity has relied heavily on principals and tools of circular mathematics and statistics. Any recurrent rhythm can be readily characterized in terms of a) its *period* – the average time interval required to complete one daily cycle, typically measured from one instance of a phase marker to the next; b) its *phase* – one point in the ~24 h

oscillation and *phase angle* -- the temporal relation of the oscillator phase to some other marker; and c) its *amplitude* – a measure of deviation from high and low values of the oscillation. As trigonometric functions have the same specifications, simple sine/cosine curve-fitting techniques are frequently used to estimate and rigorously analyze these variables. While very useful, this approach omits one fundamental dimension of rhythmic organization of interest here – namely the *waveform* of the oscillation. The waveform is simply the shape of the rhythm over its cycle.

The sine wave is one very specific waveform that is particularly useful mathematically. Most oscillations in nature, however, are not sinusoidal. For example, Figure 1A depicts the daily rhythm in light intensity averaged over one summer month at a temperate latitude. With nearly undetectable levels throughout the night, the light signal would be very poorly fit by a sinusoid. But more significantly, many rhythm waveforms *change* as a function of season of the year. Figure 1B depicts the same variables over a one-month period in winter. Whereas the rhythm *amplitude* is clearly reduced in winter, the *period* of the environmental oscillation does not differ seasonally – it is still 24 h. And considering peak light intensity as a phase marker, neither does the *phase* of the oscillation change with the seasons. But what changes markedly is the relative number of hours of light and dark. Further, relative to the peak phase of the rhythm, the *phase* of these critical environmental transitions (e.g., dawn and dusk) is altered by several hours. Were one to align these two waveforms with respect to the abrupt increase in light intensity (e.g. dawn), they are rendered misaligned with respect to peak intensity and to dusk. Common physiological and behavioral rhythms likewise exhibit deviations from sinusoidal waveforms and may vary seasonally (Figure 1C-F).

Insert Figure 1 approximately here

Thus, it becomes clear that waveform is a central and critical dimension of circadian organization. Nevertheless, waveform is far less commonly studied than other parameters of rhythmicity. As a crude example, in the Pubmed database, a search for the conjunction of "Circadian" with "Waveform" returns only 2% of the hits as those with "Phase" or "Period." By juxtaposing behavioral studies from early dates of chronobiology with those employing modern neurobiological methods, this chapter aims to highlight the importance of circadian waveform for understanding circadian organization and flexibility in mammalian systems.

Multiple oscillators comprise mammalian circadian timing systems

As described in greater detail in subsequent chapters, the circadian timing system of mammals can be considered a hierarchical, multi-oscillator system strongly governed by a dominant pacemaker in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus (1,2). As assessed by any number of rhythmic measures (electrophysiology, metabolism, gene expression) monitored *in vitro* or *in vivo*, the SCN is a robustly and indefinitely self-sustaining circadian clock. The SCN receives photic input through the retino-hypothalamic tract, which phase-dependently resets the SCN through induction of immediate early genes that transiently perturb the dynamic interactions of clock genes until they achieve a new steady state with altered phase. Organs throughout the body also demonstrate circadian oscillations in the same set of clock genes as well as in tissue-specific outputs, but unlike the SCN, these tissue-level rhythms are not

sustained indefinitely. Instead, they depend on persistent rhythmic inputs from SCN-outputs such as body temperature, locomotor activity, hormone secretion, neural innervation, or other factors to sustain coherent rhythmicity. In the absence of persistent Zeitgebers, cells that comprise peripheral organs remain individually rhythmic, but become desynchronized as a population.

In the absence of a complete knowledge of clock mechanisms, it is often necessary to use observable measures that may allow inferences about underlying circadian clocks. It is a relatively sure inference that in constant conditions, the period of a measured rhythm must reflect that of its underlying clock. However, with waveform, this need not be the case. Waveform may be the additive product of multiple rhythmic processes that jointly affect the same measure. For example, clocks entrained by light and by feeding, respectively, can jointly shape the daily waveform in locomotor activity (3): In nocturnal rodents, restriction of feeding to a few hours during the afternoon light phases induces daytime activity just prior to time of food availability. The SCN continues to drive night-time activity, but a separate food-entrainable oscillator renders the activity rhythm bimodal by virtue of its distinct regulation of activity. On the other hand, any given waveform may be determined by a combination of clock and non-clock processes. A classic example is the two-process model of sleep (4), whereby an endogenous circadian rhythm in alertness interacts with a homeostatic mechanism that accumulates and dissipates sleep debt, to govern the timing and duration (waveform) of sleep and wakefulness. Although undoubtedly pervasive and physiologically important, rhythms from the last two categories will not be emphasized here. Finally, a given waveform may indeed appear because it is programmed specifically by a single clock. Although proof of such control may be elusive, it is these types of waveform rhythms that will be the focus in this chapter because of their direct relevance to circadian clock mechanisms.

The complex circadian pacemaker and waveform plasticity

In the early days of formal chronobiology, the study of rhythm waveform was a central concern, and two major phenomena – *photoperiodism* and *splitting* induced by constant light – inspired a highly influential model of the circadian pacemaker.

Photoperiodic regulation of circadian waveform

As was well-appreciated in plants for decades, photoperiod was shown to be a potent modulator of many aspects of vertebrate physiology and behavior including reproduction, metabolism, thermoregulation, social behavior and migration to name a few (5–7). Daily rhythms in locomotor activity, likewise, were markedly influenced by daylength: under long summer days, the night-time wheel-running behavior of nocturnal rodents was of short duration and the interval of non-activity was proportionally long. Under long nights of winter, the relative durations of activity and inactivity were reversed. Moreover, when rodents from these conditions were subsequently exposed to constant darkness, these patterns of rest/activity persisted for weeks, definitively ruling out acute effects of the light:dark cycle as the proximate basis for these different waveforms (Figure 2A). Mirroring the photoperiodically-entrained waveforms in activity, the length of the photosensitive phase of the circadian cycle (as determined by light-induced phase shifts) was extended following winter entrainment and the interval of elevated

melatonin secretion was correspondingly longer (8). Across species it emerged that there was a suite of diurnally- and nocturnally-phased events the duration of which mirrors the length of the light and dark portion of the LD cycle, respectively, and could be conceptualized as distinct states of "biological day" and "biological night" (9). Moreover, the correspondence between multiple measures extended even to non-steady state conditions such as when activity duration gradually increased after release from long days to DD (10).

The fact that numerous circadian waveforms are modulated in concert suggested that the central pacemaker itself is sensitive to photoperiod. To account for these seemingly adaptive and flexible features of the circadian clock, Pittendrigh and Daan (11) proposed a model of a complex circadian pacemaker (reviewed in (12)). Here, "complex" is used in the sense of made up of more than one unit, rather than the sense of difficult to understand – perhaps an infelicitous word choice from the perspective of encouraging deep engagement with the model. According to the model, changes in circadian waveform were said to derive from adjustments in the phase relationships between two distinct populations of clocks. Based on the differential control of activity onset and offset, respectively, these two clock populations were designated Evening (E) and Morning (M) oscillators. When E preceded M by 8-12 h, the duration of subjective night (i.e., α) was shorter than subjective day as is the case in summer conditions. To account for the increase in activity duration after transfer from long days to short days or to DD, the two oscillators were modeled to have different free-running periods such that $\tau_E < \tau_M$. Under this scenario, the phase difference between E and M (φ_{EM}) would grow in the absence of entraining light to yield an increasing duration of subjective night. It is further proposed that during entrainment, the E oscillator, with $\tau < 24$ h, is routinely phase-delayed by light at dusk, and the M oscillator, with $\tau > 24$ h, is phase-advanced daily by light at dawn.

If two independent oscillators with different periods were jointly programming the activity rhythms in DD, one would expect the activity waveform to show activity bouts that periodically diverged and converged to produce a "beating" pattern when monitored over many cycles. Indeed, this is rarely the case, and typically the duration of subjective night increases and then becomes fixed under DD. In fact, DD-induced increases in α and melatonin secretion are typically proportional to the length of the scotophase under the previous LD cycle (10), with little to no further expansion occurring after release from very short days. Thus, if two oscillators (a pacemaker complex) underlie the rhythm waveform, then these two oscillators must be coupled to one another. In other words, they interact so that they do not merely free-run with respect to one another.

But simple models that do not posit the existence of multiple oscillators may just as easily account for the waveform phenomena considered above. Consider, for example, a simple sinusoidal clock mechanism paired with a threshold that initiates and terminates biological night. If the threshold is gradually lowered, night duration will increase gradually and τ_E will appear shorter than τ_M. More subtle differences in phase markers can easily be modeled if the underlying oscillation is postulated to deviate from a pure sinusoid. Such a model, moreover, does not require coupling mechanisms.

With the discovery of the role of the SCN as a dominant circadian pacemaker, it became possible to test whether photoperiodic regulation of waveform inhered in the SCN itself (i.e., did the SCN

have the properties of a complex oscillator) or whether it derived from the interaction of a waveform-invariant SCN with other extra-SCN rhythmic or homeostatic processes. Supporting the former conclusion, direct measures of SCN function indicate that it does indeed encode photoperiod. SCNs of animals entrained to long or short photoperiods show different waveforms of rhythms in electrical firing, in endogenous c-fos expression, and in rhythms of clock genes and their protein products (13,14). Moreover, these differences persist for multiple cycles in LD and there is a gradual transition upon transfer from long to short daylengths (15).

Changes in circadian waveform under constant light

Simultaneously informing this early model of a complex oscillator were findings that radical changes in waveform could be induced by exposure to constant light. In hamsters, for instance, such exposure induced gradual reductions in the duration of wheel-running behavior similar to that seen in very long photoperiods, except also free-running. In a substantial fraction of animals, however, this single short activity bout devolved into two components, each of which might free-run with its own period until the two bouts reached anti-phase and from there adopted a common free-running period. Although study of melatonin was precluded by the required exposure to constant light, as with photoperiodism, the two split bouts of behavior were shown to be accompanied by other clock outputs (e.g., the LH surge) suggesting an origin in a central clock mechanism. Unlike the findings related to photoperiodism, however, a simple threshold mechanism did not easily explain this phenomenon, termed "splitting." Rather, the simultaneous appearance of two oscillations with different periods strongly suggested the existence of at least two distinct clock mechanisms. As in the case of photoperiodism, the fact that they eventually (and universally) adopted a common phase relationship again suggested that they were not independent, but were instead coupled.

As with photoperiodic variations in waveform, electrophysiological and lesion studies strongly suggested that splitting reflected altered circadian organization intrinsic to the SCN. More recently, splitting has been convincingly related to anti-phase oscillations of the left and right SCN (16), which are connected by contralateral projections. Finer-grained analysis in hamsters additionally shows anti-phase oscillations between cellular compartments *within* each of the two SCNs (17). As lateral asymmetries in SCN cycling are uncommon under any other conditions, it can be concluded that LL alters the coupling, or interactions, between the left and right SCNs, by mechanisms that remain to be understood.

Photoperiodic non-responsiveness and arrhythmicity in Siberian hamsters

In many rodent species, some individuals fail to adopt a complete short-day phenotype in response to winter photoperiods. In Siberian hamsters, *Phodopus sungorus*, this short-photoperiod non-responsiveness has a basis in circadian entrainment. Non-responsive Siberian hamsters permanently express both a short interval of locomotor activity and a short melatonin signal despite prolonged exposure to long nights, with each rhythm phase-locked to dawn in the large majority of animals (Figure 2B) (18). The incidence of non-responsiveness is highly sensitive to artificial selection establishing a strong genetic basis (19). However, its expression is additionally photoperiod-history dependent: If never exposed to long daylengths, artificially

selected strains will show typical winter responses. Conversely, unselected strains can be induced to become non-responsive if exposed to very long daylengths (20). Thus there appears to be genetic polymorphism in the environmental conditions for inducing the non-responsive phenotype. This model system represents a unique example in which there are genetic differences in the regulation of circadian waveform by ambient light. Unfortunately, the genetic basis of this trait remains unanalyzed, and comparable circadian patterns of non-responsiveness have not been reported in other species. A second waveform phenomenon apparently unique to *Phodopus* is the induction of permanent behavioral arrhythmicity following a single-phase advance and a subsequent phase delay. Rhythmicity is not restored even under regular light dark cycles (21).

Both behavioral phenotypes are associated with alterations in SCN function. In the former case, *in vivo* light sensitivity of the SCN and *in vitro* electrical activity rhythm of hypothalamic slices are both markedly delayed in the non-responsive phenotype (22). In the latter case, rhythms of clock gene expression in SCN are eliminated and expression values are markedly suppressed (23).

Insert Figure 2 approximately here

Transient changes in circadian waveform

As described briefly above, photic entrainment is facilitated by the fact that light falling in early subjective night produces phase delays where light late in the subjective night produces phase advances. But acute light pulses may also cause transient perturbations in circadian waveform: Following late night light pulses, for example, the offsets of activity and melatonin secretion are readily advanced but onsets are not shifted commensurately for several cycles (24). The different resetting kinetics of distinct phase markers thus produces "transient" cycles where subjective night is compressed and which are resolved as activity onset shifts gradually over subsequent days. In extreme cases, transient cycles may be characterized by the complete loss of nocturnal events, such as melatonin secretion (24). In contrast, α compression is less pronounced during light-induced phase delays because both phase markers reset with similar kinetics following a light pulse applied during early night. Direction-dependent transients in circadian waveform also emerge following shifts of the LD cycle that simulate travel across time zones, although shifts in activity onset and offset can be masked by light under these conditions. Transients observed in melatonin regulation and behavior, and believed to reflect oscillator interactions, are closely mirrored by rhythms of light sensitivity in the SCN (13,24)**.**

A "skeleton photoperiod" is produced when a full, uninterrupted photophase is replaced with only two short light pulses simulating light transitions at dusk and dawn. The remaining portion of the day is left un-illuminated. Across a range of ecologically relevant conditions, entrainment under skeleton photoperiods generally resembles that elicited by full photoperiods. However, if the skeleton photoperiod simulates very long day lengths, a "phase jump" may result: Often occurring suddenly, activity traverses one of the entraining light pulses; the pacemaker re-entrains with activity phased to the longer of the two available scotophases and a longer α is adopted (Figure 2C) (25,26). As large phase jumps are not seen under comparable full

photoperiods, manipulation of the Zeitgeber waveform alone, without a change in period or phase, is sufficient to strongly modulate the stability of the pacemaker.

Waveform Bifurcation

Over the past 15 years, a new class of entrained variations in circadian waveform has been characterized in hamsters and mice. Coining the phrase "behavioral decoupling", Mrosovsky and Janik (27) demonstrated that the pattern of night-time locomotor activity could be altered by repeatedly transferring hamsters to novel wheel-running (NWR) cages during the middle of subjective day. The lights were turned off for the 3 h interval of NWR opportunity. With repeated exposure, the onset of the home-cage running at night progressively delayed, and running duration in the home cage was curtailed. Extending the study of this waveform change to steady-state conditions, Gorman and colleagues proceeded with daily NWR until the night-time was approximately half of its former duration. Hamsters were then left in the home cage but continued to receive a 3 h exposure to darkness during the subjective day, effectively exposing them to a 24 h LDLD cycle. Under such conditions, the hamsters exhibited stably bifurcated activity rhythms characterized by robust activity in the latter half of the long original night, and nearly equal duration activity in the second afternoon scotophase (28).

Although the role of novelty-induced wheel running appeared critical to hamsters under this protocol, it shortly became apparent that manipulations of the light environment alone were sufficient to induce a comparable entrainment state. Specifically, exposure to 24-h LDLD cycles could reliably induce "bifurcation" of activity rhythms in mice, Siberian hamsters and Syrian hamsters, provided that two conditions were met. First, the duration of the individual nights had to be short (< ~6 h) to induce bifurcation. Second, the twice-daily nights could not be completely dark, but needed to be very dimly illuminated (at an intensity comparable to that from the stars or a dim moon). Under such conditions, a majority of animals in each species can rapidly and reliably adopt the bifurcated entrainment pattern (FIGURE 2D) (29). Moreover, within these constraints, there is tremendous latitude in the duration and relative phasing of the LDLD components. For examples, the two scotophases may or may not be in anti-phase (e.g., 12 h apart or 9 h apart), and they may or may not be the same duration (e.g., both 5 h or one 5 h and one 3 h) (Figure 2E). Deviation of the LDLD pattern for one or more 24 cycles induced acute changes in behavior, but the stable bifurcation was immediately recovered upon restoration of the LDLD (Figure 2E) (30).

A critical question is to what degree masking by light contributes to the bifurcated entrainment state. Perhaps the animals are adopting a short photoperiod waveform that is merely interrupted by light falling in the middle of the long subjective night. To discount this possibility, hamsters were exposed to skeleton photoperiods of the LDLD cycle. For example, each original 7 h L phase was replaced by a two one hour light pulses with 5 h of darkness between, yielding a LD1:5 cycle repeating 4 times per 24 h. Under such conditions, bifurcated entrainment of hamsters is maintained with activity confined to alternate 5 h dark periods, discounting the role of negative masking (Figure 2E) (29).

As would be expected on the basis of behavioral results, bifurcation also appears to represent alterations in SCN function. Thus in NWR-induced bifurcation of hamsters, each activity bout

was associated with melatonin secretion and light-sensitivity of the SCN as measured by c-Fos expression, and inactive periods with heightened per gene expression (31,32). After bifurcation induced in hamsters without timed NWR, we observed that rhythms of PER1 protein cycled in anti-phase in shell versus core regions of the SCN (33). A similar temporal reorganization of *Per1* and *Bmal1* mRNA of SCN compartments is seen in mice (34), and contrasted with global uni-modal expression patterns in these transcripts in LD12:12 mice. Unfortunately, neither the hamster nor mouse study was able to make comparisons with non-bifurcated rodents under the same LDLD cycle. Thus, it is still unclear to what extent these altered clock gene product rhythms relate to the entrainment status versus the LDLD exposure. Lateral asymmetries were absent in all studies, however, definitively distinguishing this entrainment phenomenon from LL-induced splitting.

Role of dim light in waveform modulation

The critical role of dim nighttime illumination in bifurcation was surprising as the irradiance fell far below putative thresholds of the circadian system sensitivity, and had been incorporated into activity recording chambers only to facilitate night-time experimental manipulations. Its biological significance was only suspected after a cohort of hamsters failed to bifurcate and it was determined that the dim lights had become unpowered. Controlled experiments confirmed its critical influence on the entrained waveforms of activity to LDLD cycles of Syrian hamsters (35) and subsequent experiments assessed its significance in additional plastic waveform paradigms. In Siberian hamsters, its facilitated role in bifurcation was replicated, but additionally, dim light accelerated elongation of subjective night after transfer to short photoperiods; prevented circadian non-responsiveness induced by long-day exposure; and promoted arrhythmicity in constant conditions (dim versus dark) (36,37). These results suggested a critical role of dim light on the coupling of multiple oscillators hypothesized to underlie waveform regulation. Indeed, in the outbred Siberian hamster, which is genetically suitable for studies of individual differences, there was significant correlation between the effects of dim light in multiple waveform paradigms suggesting a convergent effect on oscillator coupling (36).

Night-time light exposure has recently attracted great attention as a result of numerous documented adverse outcomes in humans and animal models (38–43). What other authors call "dim" (e.g., 5 lux) is orders of magnitude brighter than the "dim" light employed in our studies (0.01 – 0.1 lux). And whereas other authors aim to simulate light pollution or rodent-equivalents to artificial light exposure of humans, our night-time illumination does not exceed levels occurring under natural conditions of rodents.

Waveform variations in the SCN

The SCN functions as a network of multiple, coupled oscillators (2). Like cells throughout the body, individual SCN neurons are self-sufficient cellular clocks that continue to express circadian rhythms in clock gene expression and electrical activity even when synaptic communication is prevented by pharmacological blockade and/or physical dispersal (44,45). Alternatively, when oscillating independently in dispersed cell cultures, individual SCN neurons have period lengths that may differ from one another by several hours. Because cells in an

organotypic hypothalamic slice preparation adopt a common period, the cells must be functionally coupled to adjust their periods. The ability to maintain period synchrony at the tissue level under constant conditions appears to be a property that is not shared by other tissue clocks (46).

Although coupled SCN neurons generally adopt a common period, they do not all adopt a common phase. Indeed, as reported by bioluminescent reporting of Per1 mRNA or PER2 protein rhythms, the explanted SCN shows regional variation of 2-4 h in peak phase of each reporter (47,48). The critical role of synaptic communication in organizing these phase differences can be demonstrated by blocking Na^+-dependent action potentials with tetrodotoxin. Under such conditions the individual cells are free-running with a range of periods and thus their phases are progressively scrambled. Conversely, the phases of cells can be tightly synchronized by first inhibiting protein synthesis with cyclohexamide and then restarting cellular rhythms at a common phase upon washout of the drug. Regardless of how the original phase relationships are desychronized or synchronized, when allowed to resume coupled interactions, the SCN cells return to their prior phase relationships (48). Thus, phase organization across the SCN is a highly-regulated feature of the network.

How does this relate to waveform plasticity? Considerable work has characterized the spatio-temporal dynamics of the SCN network following entrainment to different photoperiods. In one approach, electrical activity of single units and of neuronal ensembles was monitored in SCN explants. Firing rhythms in slices from short versus long photoperiods were distinguished by the duration of elevated activity, in proportion to the length of the light phase. Across photoperiods, individual units were characterized by relatively short (~4-5 h) periods of increased electrical activity, suggesting that the population waveforms were an emergent property of the phasing of the cell population (49). In short photoperiods, units are highly synchronized, whereas the phases of their firing intervals become dispersed following entrainment to long photoperiods. The patterns of synchronization of electrical activity, moreover, vary regionally. In mice from LD12:12, SCN electrical activity is less phase synchronized dorsally than it is ventrally. However, these regions encode photoperiod differentially: whereas cells of the ventral SCN modulate their phase synchrony, cells of the dorsal SCN additionally modulate their individual waveforms of electrical activity (50).

As electrical activity is a clock-controlled output, it need not faithfully report the phase of the underlying cellular clock. Therefore, it is important to assess whether photoperiod manipulations alter the phase distributions of clock genes. Indeed, as monitored by Per1 mRNA luciferase reporter, cells in the posterior SCN tracked lights on (e.g., morning) over a wide range of photoperiods. In contrast, two populations of cells were distinguished in the anterior SCN: one that tracked lights off and another that became increasingly phase segregated as photoperiod lengthened (51). Using a PER2 reporter, Evans et al. likewise described marked variations in the phase maps of SCN cells as a function of photoperiods lengthened above LD12:12 (52). Over time in culture, these cell populations modulated their phase relationships systematically, rendering the SCN explant a powerful model in which to assess coupling interactions in real time.

Beyond encoding photoperiod, regionally- and temporally-distinguished cell populations model the resetting kinetics of behavior following exposure to advancing and delaying light pulses. The evening and morning peaks of hamster SCN electrical activity revealed in a horizontal slice preparation are differentially shifted by application of glutamate (53). Similarly, following a 6 h phase advance in the photoperiod, differential resetting kinetics of ventral versus dorsal cell populations are apparent in the distribution of peak phases of PER2 luciferase reporting (54).

Thus, phase dispersion of neural clocks appears to be an organizing principle of altering the SCN's waveform. This conclusion, however, does not exclude the possibility that individual neurons are themselves altering their individual waveforms as was the case in the electrical activity rhythms of cells in the dorsal SCN of mice (50). Indeed, with a green fluorescent protein reporter of Per1 activity, the waveform of SCN neurons was persistently modulated by the developmental photoperiod (55). This apparent imprinting was not as large as the overall effect on SCN waveform suggesting that both individual neuron and network interactions contribute to this modulation of SCN waveform.

Although convergent evidence suggests a strong link between waveforms in behavior and in SCN network dynamics, much work remains to understand their relationship and specific mechanisms. Whereas a role of synaptic communication from network organization is clearly established, multiple neurotransmitters may regulate the network in a complex manner. VIP (vasoactive intestinal polypeptide) signaling contributes to neural synchrony but also has the potential to desynchronize the network as a function of timing and dose (56). $GABA_A$ (γ-amino butyric acid) signaling has been demonstrated to desynchronize SCN neurons, at least when VIP signaling is attenuated (52,57). The effects of $GABA_A$, moreover, are sensitive to prior photoperiodically-entrained state of the network (52).

Consequences of waveform manipulation

Besides providing a mechanistic basis for an internal calendar through which to regulate seasonal physiology, photoperiodic entrainment appears to modulate core features of the pacemaker itself. As mentioned above, the fraction of the endogenous cycle in which light induces phase resetting is expanded in winter versus summer conditions (13). But more surprisingly, experiments examining the effects of photoperiod on phase-shifting by brief light pulses indicate that animals under short, winter-like days have a higher amplitude PRC and thus respond to brief light pulses with phase-shifts of greater magnitude than long day counterparts (8,58).

Whereas the PRCs to bright light pulses establish photoperiodic differences in the response to light, there appears additionally to be variations in light sensitivity. Photic sensitivity can be operationalized either in terms of the threshold of light capable of inducing a phase shift, the irradiance sufficient to generate the maximum phase response, or most commonly, the irradiance that produces half of the maximal phase shift response. Each measure is separate from the question of the size or direction of phase shifts. In Syrian hamsters tested late in subjective night, approximately 40X more light (~1.5 log units) was necessary to produce half of the maximum phase shift response following entrainment to summer conditions than after winter conditions (59). The disparate irradiances demonstrated to generate comparable phase advances under short

versus long days likewise yielded comparable phase delays. Moreover, equal photon doses produced significantly larger delays in the short photoperiod condition, as well as markedly greater pERK, PER1, and cFOS immunoreactivity in the suprachiasmatic nuclei. Patterns of immunoreactivity in all 3 proteins were related to the size of the phase shift rather than the intensity of the photic stimulus, suggesting that photoperiod modulation of light sensitivity lies upstream of these events within the signal transduction cascade. An analogous effect of resetting sensitivity generalized to Siberian hamsters (60). In that species, however, the regulation of melatonin was not comparably modulated.

Until recently, photoperiodic modulation of pacemaker properties has tended to focus on acute (i.e., non-parametric) effects of light on the circadian clock despite ample evidence that, even in rodents, daytime light exerts additional, parametric effects of on clock function (61,62). As noted above, acute light pulses can induce transient changes in circadian waveform, typically reducing α, and perhaps reducing PRC amplitude as a result. Therefore, it is unclear whether winter entrainment should be expected to produce any meaningfully enhanced resetting to changes in full photoperiods. To test this prospect, a novel assay was designed in which winter- and summer-entrained hamsters were transferred to 6 identical summer photoperiods phased at successive 4 h intervals. Because changes in waveform introduce different size phase shifts with respect to different phase markers, there can be no unambiguous identification of the Zeitgeber shift. Nevertheless, phase-resetting kinetics across all the groups can be plotted on circular coordinates to provide an objective measure of phase lability. Syrian hamsters under short photoperiods were, overall, able to reset to new time zones twice as quickly on average as long day counterparts (Figure 3).

The same protocol was used to evaluate the speed of resetting to traditional, long photoperiods after bifurcation. Here again, where the total light exposure was the same as in long photoperiods, the circadian system adapted more rapidly to phase-shifts of varying magnitude and direction (Figure 3). Moreover, previously-bifurcated hamsters were shifted to anti-phase times zones (12 h apart) and released into DD (63). As assessed by the free-running locomotor activity rhythm, the master pacemaker was fully shifted into anti-phase time zones in only a matter of days, confirming that the rapid behavioral resetting seen after bifurcation is not a behavioral artifact of masking. While the mechanisms for enhanced rates of resetting after waveform manipulation are unknown, we hypothesize that they will be relatable to the phase and waveform dynamics of oscillators within the SCN.

Summary

- Waveforms of behavioral and physiological rhythms of rodents can exhibit tremendous variation, both transiently and under steady state conditions. While waveform plasticity has obvious ecological utility in the context of photoperiodism, its various manifestations in non-ecological context likely reflect non-adaptive by-products of its mechanistic organization. Although heritability of waveform plasticity has been demonstrated in mammals (19), there has been little attention paid to the genetic basis of this fundamental dimension of circadian organization.
- The SCN, as a result of its multi-oscillator network organization, may encode and modulate pacemaker waveform through changes in phase of SCN neurons, changes in individual SCN neuron function and perhaps via other yet to be discovered mechanisms.

Recent investigations of SCN neural circuitry establish the roles of multiple, interacting neurotransmitter and signaling mechanisms in the regulation of multi-cellular pacemaker network.

- Earlier reports that winter photoperiods increase the amplitude of the phase response curve (8) have now been shown to generalize to shifts in full photoperiods that are of greater relevance to time-zone travellers and to shift-workers. Moreover, novel, but unecological, entrainment conditions such as bifurcation -- itself a novel entrainment condition worthy of consideration for shift-workers (30) -- likewise demonstrate a linkage between waveform and circadian resetting that may afford new approaches to circadian adaptation (63).

Key questions of interest and suggested readings:

- How do taxononically diverse organisms adapt to photoperiodic change? (6)
- How might circadian waveform manipulations be relevant to human shift-workers? (30)
- What are the emergent properties of SCN networks? (12,14)

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Figure Legends

Fig 1. Examples of waveforms in various environmental and physiological rhythmic oscillations. The daily rhythm in light intensity at a temperate latitude over one month differs markedly in summer (A) versus winter (B; data from https://ndawn.ndsu.nodak.edu). Similarly, seasonal changes in daylength are reflected in waveform differences in the melatonin rhythm in Siberian hamsters in summer (C) versus winter conditions (D; data adapted from (64)). Concentrations of luteinizing hormone remain at basal levels except for a rapid, burst-like pattern late in the day of proestrus (E; data adapted from (65)). Daily variation in alertness in humans is bimodal (F; data adapted from http://www.nhtsa.gov/people/injury/drowsy_driving1).

Fig 2
Behavioral consequences of waveform manipulation in hamsters. Representative double-plotted actograms under long days and short days (A). Locomotor activity expands under short photoperiods, and is maintained after release into constant conditions. Individual hamsters respond differently to changes in photoperiod (B). Phase-jumping occurs under changing skeleton photoperiods, wherein as the original scotophase is shortened, activity "jumps" to the longer scotophase (C). Hamsters bifurcate more robustly under dim, not dark, scotophases (D). Bifurcation can be maintained under a variety of manipulations of the light cycle, including unequal scotophase duration, transient removal of a scotophase or photophase, and under skeleton LDLD cycles (E). Data in E are single-plotted, across 24 h. Data are previously unpublished or adapted from (25,30,37,62).

Fig 3
Manipulations of waveform prior to a phase shift accelerate re-entrainment. Shown here are representative double-plotted actograms of wheel-running activity in Syrian hamsters first exposed to one of three different waveforms (LD, SD, and LDLD) and then exposed to each of 6 phase shifts to a new LD 16:8 schedule in a global assay of phase-resetting. Actograms are organized in columns by waveform group (LD, SD and LDLD). For each actogram, 10 days of the baseline waveform and 10 days following the shift are shown. The size of the phase shift is denoted on the left of each row and reflects the change in lights off (+4 h represents a 4 h advance of lights-off in all groups). For LDLD animals, the reference point for lights-off was derived from the pre-bifurcation scotophase. Radar plots below each column reflect the phase mismatch at progressive 3-day intervals post-shift for that waveform manipulation. Each axis of the radar plot represents a shift of varying magnitude and direction. The white hexagon in the center of each plot represents the "target" phase in the new schedule (for activity onsets, this was the new lights-off). Each colored polygon represents the absolute value of the average 3-day deviation of the mean onset from the new lights-off in hours, with colors progressing from coolest (days 1-3 post-shift) to warmest (days 13-15). Similar results were found when midpoints and offsets were examined. Data adapted from (63).

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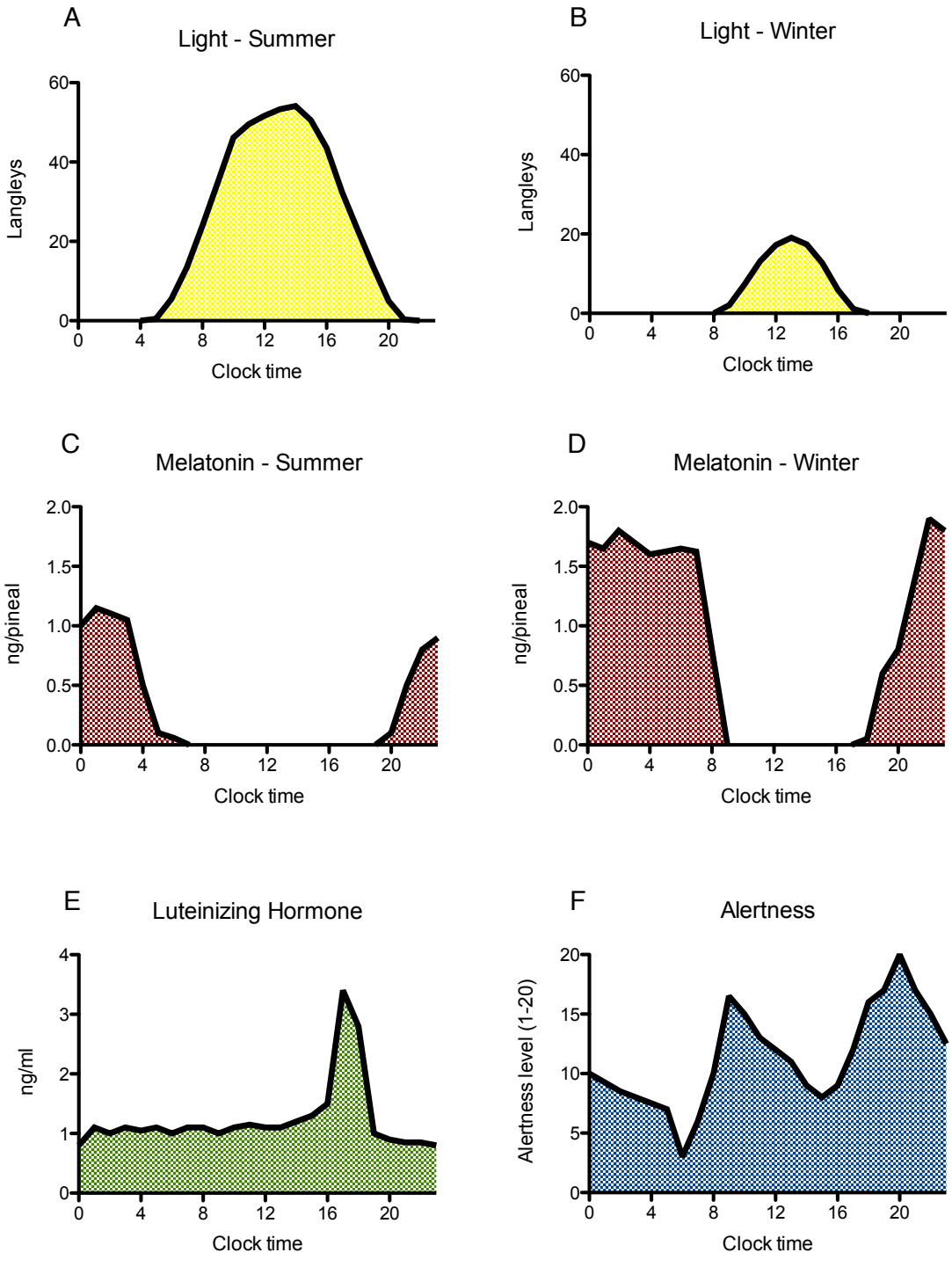
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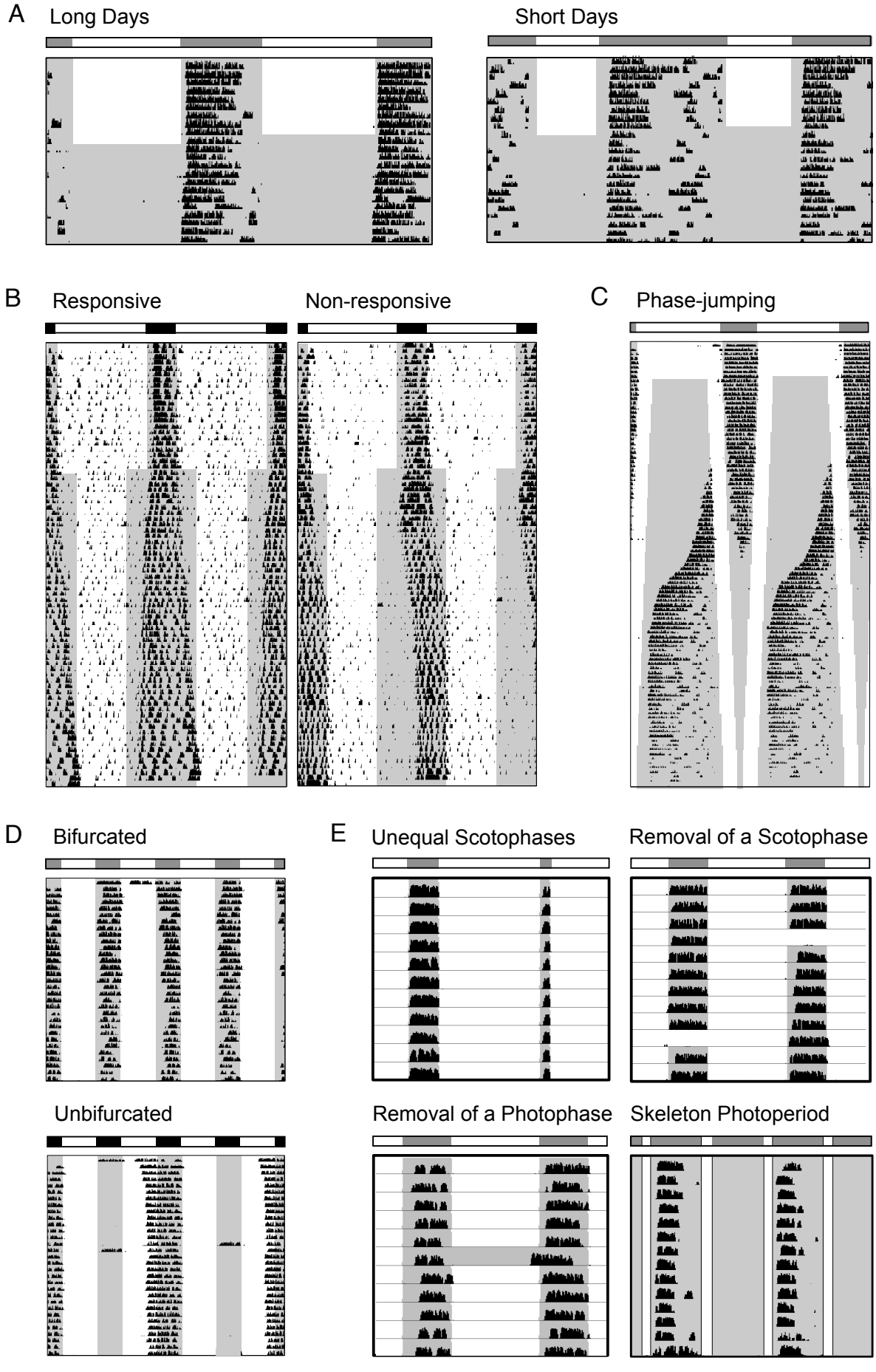
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Removal of a Photophase
Skeleton Photoperiod

Phase shifts of varying magnitude and direction by waveform