RUNNING TITLE: SCN CLOCK CIRCUITRY

In synch but not in step: Circadian clock circuits regulating plasticity in daily rhythms

Jennifer A. Evans^{1*} and Michael R. Gorman²

¹Department of Biomedical Sciences, Marquette University, Milwaukee WI USA

²Department of Psychology, University of San Diego, La Jolla, CA USA

*Correspondence:

Jennifer Evans

Marquette University

Department of Biomedical Sciences

561 N 15th St

Schroeder Complex, Room 446

Milwaukee, WI 53233

jennifer.evans@marquette.edu

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Abstract

The suprachiasmatic nucleus (SCN) is a network of neural oscillators that program daily rhythms in mammalian behavior and physiology. Over the last decade much has been learned about how SCN clock neurons coordinate together in time and space to form a cohesive population. Despite this insight, much remains unknown about how SCN neurons communicate with one another to produce emergent properties of the network. Here we review the current understanding of communication among SCN clock cells and highlight a collection of formal assays where changes in SCN interactions provide for plasticity in the waveform of circadian rhythms in behavior. Future studies that pair analytical behavioral assays with modern neuroscience techniques have the potential to provide deeper insight into SCN circuit mechanisms.

Keywords: circadian; suprachiasmatic nucleus; clock network; coupling; behavior; plasticity

1. Introduction

Daily rhythms are an important and pervasive feature of life on this planet. In mammals, a wide variety of behavioral and physiological processes fluctuate regularly each day (e.g., locomotor activity, sleep, cognitive performance, hormone secretion, protein synthesis, cell division). These and other essential processes are programmed by an endogenous timekeeping system that has evolved to promote survival in a rhythmic environment. The primary evidence for the endogenous basis of daily rhythms is that they persist under constant conditions devoid of environmental time cues. Because the period length adopted under constant conditions is not exactly 24 h, these internally programmed rhythms are referred to as circadian (*circa dies*, about a day). Experimental approaches that track daily changes in behavior and physiology have proven vital for defining formal mechanisms of circadian timekeeping and the process of entrainment to the 24 h environment. Following on this work, the biological generator of daily rhythms was localized to a specific site in the anterior hypothalamus, the suprachiasmatic nucleus (SCN). The discovery that this small region of the brain controls the temporal patterning of behavior and physiology is one of the most striking examples of localized function in the field of neuroscience. Recent advances have built upon this pioneering work to define the cellular mechanisms of circadian timekeeping, revealing the existence of a molecular oscillator built of “clock genes”. We now realize that nearly every cell of the body is a daily clock, and the next major challenge in the field is to define the circuits and signals through which these clocks interact with one another.

In this review, we highlight the evidence that the master circadian clock in mammals contains multiple interacting clock cells organized into a network, as first articulated in 1960 (Pittendrigh, 1960). Importantly, this classic model posits that the master circadian clock contains functionally distinct clocks that coordinate with one another to form a functional pacemaker. A large body of research supports the fundamental premise of this model, which is striking given that it was first formulated based purely on behavioral data obtained before the discovery of the SCN’s role as a pacemaker. Although there is compelling evidence that the SCN does indeed contain multiple cellular clocks, little is known about the circuits, signals, and mechanisms by which SCN cells coordinate with one another. Deeper insight into SCN circuitry is imperative for understanding the intercellular interactions that guide rhythmic neural behavior, which is a common theme emerging throughout neuroscience. Here we will provide a brief summary of clock circuits and then review formal assays where plasticity in circadian behavior is thought to reflect interactions among master clock cells. We hope that re-examining these formal analytical tools through a modern lens will highlight ways that these assays can be used to further define SCN circuits. As with past advances in our field, the continued synthesis of formal mechanistic insight with cutting-edge technical advances is expected to deepen our understanding of principles and mechanisms underlying circadian timekeeping.

2. Circadian circuits

2.1 The circadian timekeeping system: Clocks at multiple levels

Multi-clock models can be categorized as hierarchical or complex (Moore-Ede et al., 1976). Hierarchical circadian models postulate that the body contains multiple circadian clock tissues, with “peripheral” clock tissues regulated by a central pacemaker that maintains internal synchrony within the system (Rosenwasser and Adler, 1986). In contrast, complex circadian models stipulate the existence of multiple, interacting clock cells within the central pacemaker itself (Pittendrigh, 1974, Pittendrigh and Daan, 1976b). There is a great deal of experimental support for each model, reviewed briefly below, and in both models a critical question is how multiple clock tissues or cells are able to communicate with one another. It is important to note that communication is not required in a multi-clock system. For example, a group of cellular clocks with identical period lengths would remain synchronized without intercellular signaling. Although it is theoretically possible that cellular clocks could be identical clones running at the same speed, this is biologically implausible. Indeed, a large number of studies indicate that the intrinsic period of different tissue and cellular clocks are not identical (Abe et al., 2002, Balsalobre, 2002, Granados-Fuentes et al., 2004). Even in the face of heterogeneous period lengths, intercellular communication may not be essential given that external input (i.e., zeitgebers, e.g., light) might effectively synchronize a population of non-interacting clock cells. However, under constant conditions devoid of time cues, non-interacting clock cells would be unable to maintain synchrony. Under these circumstances, temporal desynchrony would lead to arrhythmia at the level of the population and multiple, independent periodicities would manifest in the overt rhythms controlled by these clock cells. Since the vast majority of mammalian species do not display multiple periodicities under constant darkness, this implies that the underlying tissue and cellular clocks interact through coupling mechanisms. In the simplest sense, coupling is the ability of one cellular (or tissue) clock to influence the rhythm of another clock through interactions that may be reciprocal or one-directional. In this regard, coupling may influence any rhythmic property (i.e., period, phase, amplitude, precision), although an emphasis is often placed on coupling mechanisms that maintain period synchrony. In addition, intercellular interactions also regulate phase synchrony among different clocks. In some cases, coupling may inhibit phase synchrony to prevent the simultaneous expression of opposing biological processes. In this manner, coupling would optimize performance of the system as a whole by preventing “phase locking” (i.e., absolute phase synchrony). Thus, coupling can have multiple consequences for the expression of rhythmic parameters, which could be mediated by distinct types of signaling mechanisms.

Findings supportive of the hierarchical clock model derive from studies of human and non-human primates, where independent rhythms of behavioral activity and body temperature emerged under constant conditions or after large shifts in the entraining light:dark (LD) cycle (Aschoff, 1965, Moore-Ede et al., 1977, Sulzman et al., 1977, Tapp and Natelson, 1989, Weibel et al., 1997). Based on the concept that a single oscillator cannot simultaneously express multiple, independent periods, these results were interpreted as reflecting the actions of at least two distinct oscillators disconnected from central control. Multiple clock tissues were also indicated by work demonstrating that food and psychoactive stimulants could restore the expression of daily rhythms in SCN-lesioned animals (Honma et al., 1989, Stephan, 1989, Tataroglu et al., 2006). Although, it has been very difficult to localize non-SCN food- and drug-sensitive tissue clocks (Davidson, 2006), recent work highlights an important role for dopamine signaling in the dorsal striatum (Gallardo et al., 2014). To date, the most compelling evidence in support of the hierarchical clock model is the discovery that numerous glands and tissues are able to generate endogenous rhythms *in vitro* (Abe et al., 2002, Balsalobre, 2002, Granados-Fuentes et al., 2004). Endogenous rhythms are also displayed by single somatic cells, which indicates that the basic mechanism for circadian timekeeping operates at the molecular level.

At the cellular level, circadian rhythms are programmed by a molecular oscillator consisting of a family of clock genes that regulate their own expression (Mackey, 2007, Zhang and Kay, 2010, Buhr and Takahashi, 2013). At its core, circadian rhythms are sustained by a ~24 h transcriptional-translational negative feedback loop (Figure 1A) with positive elements that serve as activators and negative elements that serve as repressors. The positive elements are bHLH-PAS transcription factors CLOCK and BMAL, which form a dimer that initiates transcription of *Period* (*Per1*, *Per2*, *Per3*) and *Cryptochrome* (*Cry1*, *Cry2*) genes (Figure 1A-1). The corresponding protein products (e.g. PER1-3, CRY1-2) are negative elements that form protein dimers, which feedback to inhibit their own expression by antagonizing the transcriptional activity of CLOCK-BMAL (Figure 1A-2). The negative elements are degraded over time, thus relieving repression and allowing transcription to recommence the following day. In addition to this primary loop, there are a number of interconnected accessory loops that act to stabilize and amplify circadian oscillations at the cellular level (Figure 1A-3). This molecular oscillator functions in nearly every cell of the body to regulate biological processes in a tissue-specific manner (Panda et al., 2002, Storch et al., 2002, Zhang et al., 2014).

These various tissue clocks are regulated by a master clock within the suprachiasmatic nucleus (SCN), a bilateral structure in the anterior hypothalamus located immediately above the optic chiasm (Welsh et al., 2010, Mohawk et al., 2012). Lesion and tissue-graft experiments have demonstrated that the SCN is both necessary and sufficient for sustained circadian rhythms under most experimental conditions (Weaver, 1998). Consistent with its role as master clock, the SCN as a tissue displays circadian rhythms in many

cellular processes, including metabolism, electrical activity, and gene/protein expression (Klein et al., 1991), which it generates intrinsically without the need for input from the environment or other tissues. For example, the whole SCN exhibits circadian rhythms in spontaneous electrical firing and *Period* transcription *in vitro*, with peak expression during the projected daytime hours. Further, these circadian rhythms are reflected in the cellular activity of single SCN neurons, which typically display relatively short ~5 h periods of electrical activity at midday (Schaap et al., 2003, Brown et al., 2006). In addition to its intrinsic oscillatory capacity, the SCN receives input from the environment (e.g., light) that allows it to synchronize to the 24 h solar day. This photic input is transmitted to the SCN through a direct retinal projection, the retinohypothalamic tract, and several indirect pathways, including the geniculohypothalamic tract from the intergeniculate leaflet of the thalamus (Morin, 1994). The majority of retinal ganglion cells that provide photic input to the SCN expresses the photopigment melanopsin and respond intrinsically to photic stimulation (Berson, 2003, Gooley et al., 2003, Hattar et al., 2003, Morin et al., 2003, Panda et al., 2003, Brown and Robinson, 2004). Light stimuli at night increases electrical firing and clock gene expression in the SCN, which is ultimately conveyed to downstream targets to adjust their phase. Thus, the SCN serves as the interface between the external and internal milieus, synchronizing the various body clocks to the 24 h world and one another (Figure 1B).

Similar to the evolution of hierarchical models, both formal and physiological analyses support complex clock models that emphasize the existence of multiple, interacting oscillators within the master clock itself (Pittendrigh, 1960, Pittendrigh and Daan, 1976b). The complex clock model was first proposed based on plasticity in behavioral phenotypes that manifest under various environmental conditions (Pittendrigh, 1960, 1974, Pittendrigh and Daan, 1976b). In contrast to evidence for the hierarchical model, different overt rhythms changed in similar ways and thus it was concluded that this form of environmentally induced plasticity reflected a global change occurring within the central pacemaker itself. It is now well established that the SCN does indeed contain a network of multiple cellular clocks (Figure 1C). Individual SCN neurons are self-sufficient cellular clocks that continue to express circadian rhythms even when synaptic communication is disrupted (Bouskila and Dudek, 1993, Shibata and Moore, 1993, Welsh et al., 1995, Herzog et al., 1997, Liu et al., 1997, Shirakawa et al., 2001). However, when unable to interact, SCN neurons display different period lengths and desynchronize with one another over time. In contrast, when SCN neurons are able to communicate, period synchrony is sustained by intercellular signaling. The ability to maintain period synchrony at the tissue level independent of input appears to be a property that is not shared by other tissue clocks (Yamazaki et al., 2000, Nagoshi et al., 2004, Welsh et al., 2004). As discussed above, coupling mechanisms within the SCN also regulate phase synchrony, with SCN neurons “preferring” to adopt specific phase relations (Yamaguchi et al., 2003) that are modulated by the environment (Jagota et al., 2000, Ohta et al., 2005, Inagaki et al., 2007).

For both hierarchical and complex models, there remain fundamental questions concerning the properties and functions of the underlying clock cells or tissues. For the hierarchical model, important questions include: 1) how does the SCN communicate with downstream tissue clocks, 2) does the local clock in downstream tissues play an important role in controlling rhythmic output, and 3) do the cells and tissues of peripheral clocks interact with one another and/or transmit cues back to the SCN? Likewise, there are outstanding questions about the circuitry and function of the SCN complex. In order to understand the master clock network, it will be critical to: 1) address whether SCN neurons differentially contribute to the emergent properties of the network and 2) define the mechanisms by which SCN communicate with one another to coordinate their rhythmic behavior.

2.2 The clock complex: SCN circuitry

The majority of SCN cells are local projection neurons that connect to other neurons in the SCN and surrounding hypothalamus (Abrahamson and Moore, 2001, Moore et al., 2002). Nearly all SCN neurons produce γ -aminobutyric acid (GABA), yet they can be categorized into distinct subgroups based on neuropeptide expression (Moore and Speh, 1993, Abrahamson and Moore, 2001). Classic work detailing SCN cytoarchitecture in the rat distinguished two spatially segregated compartments: the dorsomedial shell and the ventrolateral core (Moore and Silver, 1998). Within the SCN shell is a dense population of neurons that express arginine vasopressin (AVP), whereas the SCN core contains several different neuronal subclasses, including those that express vasoactive intestinal polypeptide (VIP). This organization typifies the SCN of most eutherian mammals studied to date, although regional anatomy and chemoarchitecture can vary between species (Cassone et al., 1988, Morin, 2007). The SCN shell and core are convenient constructs for describing SCN compartmentalization; however, there is a growing appreciation that this organizational scheme belies a deeper complexity that awaits discovery. For instance, the SCN produces dozens of signaling factors, and the list of important SCN peptides continues to expand even to this day (van den Pol and Tsujimoto, 1985, Lee et al., 2013, Lee et al., 2015). Thus, the SCN remains a complex structure with regions and cell types whose functions are not readily transparent (Moore et al., 2002, Antle et al., 2003, King et al., 2003, Lee et al., 2003, Antle and Silver, 2005).

In addition to compartmentalization of neuropeptide expression, there are also regional differences in SCN function. For example, SCN neurons display regional differences in phase and inherent period length, which manifest even when considering a single neuropeptide subclass of SCN neuron (Shinohara et al., 1995, Schwartz et al., 2000, Quintero et al., 2003, Yamaguchi et al., 2003, Albus et al., 2005, Noguchi and Watanabe, 2008, Evans et al., 2011, Myung et al., 2012). Further, light does not indiscriminately excite all SCN neurons, instead photic stimuli activate only ~25% of SCN neurons and inhibits a smaller population

(Meijer et al., 1986, Jiao et al., 1999, Aggelopoulos and Meissl, 2000, Nakamura et al., 2004, Brown et al., 2011). Tract tracing studies suggest that there is a denser retinal projection to the SCN core than the SCN shell (Abrahamson and Moore, 2001, Lokshin et al., 2015). These anatomical differences in retinal connectivity map onto functional differences in photic responses, with the SCN core displaying light-induced changes in gene expression that precede those in the SCN shell (Silver et al., 1996, Dardente et al., 2002, Yan and Okamura, 2002, Yan and Silver, 2002, Kuhlman et al., 2003, Nagano et al., 2003, Yan and Silver, 2004). The current working model of photic signaling is that the SCN core contains first order neurons that receive afferent input, which process and transmit this information to neurons in the SCN shell. On the other hand, the SCN shell is thought to contain strongly rhythmic cells that provide outputs to reset the phase of downstream tissues (Nakamura et al., 2001, Dardente et al., 2002, Zhou and Cheng, 2005, Kalsbeek et al., 2010, Evans et al., 2015). However, there are aspects of this model that remain unclear. For instance, although some work supports the idea that subclasses of SCN neurons differ in oscillatory capacity (Jobst and Allen, 2002), recent work indicates that cellular rhythmicity is stochastic, relies on network interactions to be sustained stably, and does not segregate with neuropeptide expression (Webb et al., 2009). Further, both AVP and VIP neurons extend processes to target regions in the hypothalamus, thalamus, and forebrain (Abrahamson and Moore, 2001, Buijs and Kalsbeek, 2001, Kalsbeek and Buijs, 2002), which suggests that both SCN shell and core neurons provide signals to downstream clocks. The functional relevance of signals emanating from SCN neurons within different compartments remain ill defined.

2.3 SCN signaling mechanisms

Intercellular communication within the SCN network may involve multiple mechanisms (van den Pol and Dudek, 1993, Michel and Colwell, 2001, Aton and Herzog, 2005). An important role for synaptic communication in SCN coupling is revealed by work demonstrating that blocking Na^{+2} dependent action potentials with tetrodotoxin (TTX) causes SCN neurons to desynchronize *in vitro* (Yamaguchi et al., 2003). But the persistence of SCN timekeeping in the presence of TTX or low Ca^{+2} suggests that the network can use other coupling mechanisms not dependent on synaptic release of signaling molecules (Schwartz et al., 1987, Schwartz, 1991, Bouskila and Dudek, 1993, Dudek et al., 1993, Shibata and Moore, 1993). In addition, the specific mechanisms employed in coupling may depend on the maturity of the network since the SCN displays circadian rhythms before synaptogenesis (Shibata and Moore, 1987, Reppert, 1992). Although this plurality of potential coupling mechanisms presents an obstacle to understanding SCN circuitry, several factors have been identified that influence the emergent properties of the network (i.e., VIP, GABA, AVP). To date, significant progress has been made in defining the specific contribution of

these coupling factors, although recent work suggests that their precise role in SCN coupling may be influenced by the state of the network itself (Evans et al., 2013, Bedont et al., 2014, Wang et al., 2014).

Vasoactive intestinal polypeptide (VIP) Research conducted by a number of different labs has established a clear role for VIP in SCN coupling (Vosko et al., 2007). Mice deficient in either VIP or its receptor are unable to sustain circadian rhythms in constant darkness (DD), and instead their locomotor rhythms devolve into seemingly random bouts of activity (Harmar et al., 2002, Colwell et al., 2003). The arrhythmic phenotype that manifests in VIP-deficient mice reflects a loss of neuronal synchrony within the SCN and a decrease in the number of SCN neurons able to maintain high amplitude rhythms (Aton et al., 2005, Maywood et al., 2006, Brown et al., 2007, Hughes et al., 2008, Ciarleglio et al., 2009). VIP knockout mice also display a range of other phenotypes, including changes in sleep, metabolism, cardiac function, and reproduction (Bechtold et al., 2008, Sheward et al., 2010, Hannibal et al., 2011, Hu et al., 2011, Schroeder et al., 2011, Fahrenkrug et al., 2012, Loh et al., 2014), which may reflect loss of internal synchrony at the systems level (Loh et al., 2011). Thus, VIP signaling is an important mediator of SCN synchronization whose absence has widespread consequences for behavior and physiology. However, recent work indicates that VIP can also desynchronize the phase of SCN neurons if given at the wrong time or at high doses (An et al., 2013, Ananthasubramaniam et al., 2014), which highlights the need to define precisely the properties and mechanisms of VIP signaling to better understand how it regulates clock function at the cellular, network, and systems levels.

γ -aminobutyric acid (GABA) Unlike VIP, recent work has revealed that GABA influences circadian coupling by desynchronizing SCN neurons (Evans et al., 2013, Freeman et al., 2013, DeWoskin et al., 2015, Myung et al., 2015). Normally, this effect of GABA_A signaling is hard to detect because VIP signaling is a potent synchronizing agent. But in the absence of VIP (e.g., when SCN slices are cultured from VIP knockout mice or cultured with VIP antagonists), SCN neurons desynchronize due to GABA_A signaling within the network (Evans et al., 2013, Freeman et al., 2013). However, the functional role of GABA_A signaling can be influenced by environmentally induced changes in the state of the network (Evans et al., 2013, Farajnia et al., 2014, Myung et al., 2015). For example, although GABA_A signaling acts to inhibit phase synchronization when SCN neurons are tightly synchronized under standard laboratory lighting conditions, it serves to facilitate network re-synchronization when SCN neurons are desynchronized by light *in vivo* (Evans et al., 2013). Interestingly, the change in the functional role of GABA_A signaling under these lighting conditions is associated with an increase in the number of SCN neurons that display GABA-induced excitatory responses (Farajnia et al., 2014, Myung et al., 2015). Similarly, GABA_A signaling can synchronize dissociated SCN neurons *in vitro* (Liu and Reppert, 2000) and is involved in the transfer of resetting information from one SCN compartment to another (Albus et al., 2005, Han et al., 2012). Clearly,

further work is required to define the conditions and mechanisms that determine the functional role of GABA_A signaling in SCN coupling.

Other mechanisms In the absence of VIP signaling, SCN neurons can be synchronized by AVP or GRP (Brown et al., 2005, Maywood et al., 2006, Maywood et al., 2011). Although AVP is traditionally viewed as an output signal, AVP neurons provide signals that modulate network function to regulate the rate of recovery from simulated jetlag (Yamaguchi et al., 2013, Mieda et al., 2015). On the other hand, GRP is mostly known for its role in processing photic signals (McArthur et al., 2000, Dardente et al., 2002, Antle and Silver, 2005, Gamble et al., 2007), and its potential role in SCN coupling remains unclear. In addition to synaptic communication, electrotonic signaling through low-resistance gap junctions may also influence SCN synchrony by allowing the transmission of charged ions and other small molecules (e.g., cAMP) among cells in close apposition (Bennett et al., 1991, Rash et al., 2000, Rash et al., 2001, Connors and Long, 2004). Gap junctions can be found in SCN neurons and glial cells, with the diffusion of labeled molecules (i.e., dye coupling) occurring mostly between cells within a SCN region (Welsh and Reppert, 1996, Jiang et al., 1997, Colwell, 2000, Jobst et al., 2004, Long et al., 2005). When gap junctions are targeted pharmacologically, SCN electrical rhythms become broader, arrhythmic, or bimodal (Prosser et al., 1994, Shinohara et al., 2000a, Shinohara et al., 2000b, Shirakawa et al., 2001). Bimodal rhythms also emerge in the presence of the glial metabolism antagonist, fluorocitrate (Prosser et al., 1994). These changes in the waveform of SCN electrical rhythms are thought to be due to altered communication among SCN neuronal subpopulations (Wang et al., 2014), although it remains unclear how this influences the functional properties of the network. Given the evidence for multiple SCN coupling mechanisms, it will be important to obtain detailed insight into how SCN neurons integrate the various signals produced by the network and the functional consequences of cross-modal interactions revealed by previous work (Colwell, 2000, Shinohara et al., 2000b, Itri et al., 2004, Wang et al., 2014).

3. Formal assays for investigating the emergent properties of the complex clock

The complex clock model was first inspired by changes in the waveform of circadian rhythms that manifest under certain environmental lighting conditions, such as seasonal changes in day length and constant light (Pittendrigh, 1974, Pittendrigh and Daan, 1976b). Other analytical paradigms discovered since also imply the existence of multiple, interacting oscillators within the SCN (e.g., simulated jetlag, non-24 h cycles, 24-h light:dark:light:dark cycles). By investigating circadian plasticity at the behavioral level, these formal assays are used to infer the structure of the complex clock and the properties of its constituent oscillators. A renewed focus on defining the neurobiological bases of circadian plasticity at the behavioral level may provide critical insight into SCN circuitry and may be used to test putative SCN

coupling factors. However, the formal concepts embodied in the complex clock model should be updated and elaborated in order to provide refutable hypotheses for testing the role of different coupling mechanisms. Here we will use terminology originally adopted under the dual oscillator model for its utility, but this is intended to represent at least two populations of clock cells (Winfree, 1967, Pavlidis, 1973, Enright, 1980b, Strogatz, 2003, Izhikevich, 2007). In addition to providing insight into the nature and mechanisms of SCN coupling, the formal assays highlighted in this review may provide means to further distinguish SCN neuronal subpopulations. Moreover, further investigation into the bases of flexibility in circadian waveform may lead to novel approaches to mitigate the harm of circadian disruption in humans (Harrison and Gorman, 2012).

3.1. Changes in circadian waveform after photoperiodic changes in day length

Photoperiod influences myriad physiological and behavioral rhythms, including both reproductive and non-reproductive processes (Illnerova, 1991, Goldman, 2001, Gorman et al., 2001a). In fact, there is a suite of diurnally- and nocturnally-phased events whose duration mirrors the length of the light and night portion of the LD cycle, respectively. The fact that numerous rhythms change in concert is taken as evidence that the central pacemaker itself is sensitive to photoperiod. For instance, the duration of melatonin secretion from the pineal gland is proportional to the length of the night, with a longer duration of release under winter-like short days compared to summer-like long days. Likewise, the duration of locomotor activity (α) compresses under long days and expands under short days (Figure 2A). Melatonin is an important driver of photoperiodic changes in physiological function in some species, but photoperiodic changes in α do not require melatonin (Hastings et al., 1987, Refinetti, 2002). This also is taken as evidence that the SCN itself is altered by changing day lengths. More direct measures of SCN function indicate that it does indeed encode photoperiod. For example, photoperiod modulates the length of the photosensitive phase of the circadian cycle, as determined by light-induced phase shifts and changes in SCN gene expression (Pohl, 1983, Pittendrigh et al., 1984, Pohl, 1984, Binkley and Mosher, 1986, Humlova and Illnerova, 1992, Travnickova et al., 1996, Vuillez et al., 1996, Illnerova and Sumova, 1997, Sumova and Illnerova, 1998, vanderLeest et al., 2009). Proportional changes in SCN rhythms of diurnally expressed markers are also observed, with long days increasing the duration of SCN electrical firing, endogenous *c-fos* expression, and clock gene/protein production (Sumova and Illnerova, 1998, Jac et al., 2000, Jagota et al., 2000, Messenger et al., 2000, Mrugala et al., 2000, Sumova et al., 2003, de la Iglesia et al., 2004b, Johnston et al., 2005, VanderLeest et al., 2007, Naito et al., 2008, Yan and Silver, 2008, Brown and Piggins, 2009). The process of photoperiodic modulation of overt rhythms and SCN function can also be observed after release from long days to constant darkness (DD), which eliminates the masking influence of light (Figure 2A).

Following transfer to DD, circadian rhythms are said to “free-run” and reflect inherent period length (τ). At the same time, α increases in a systematic fashion after release into DD, with α expansion occurring as activity onset advances and activity offset delays each cycle. Advances in the onset of behavioral activity and melatonin secretion are highly correlated, as are the delays in their offsets, suggesting that a common mechanism underlies increases in both rhythms (Elliott and Tamarkin, 1994).

At the formal level of analysis, photoperiodic changes in circadian waveform are thought to reflect adjustments in the phase relationships between two distinct populations of clocks (Figure 2B). Based on the differential control of activity onset and offset, these two clock populations have been labeled Evening (E) and Morning (M) oscillators. Since activity onset advances whereas activity offset delays under DD and short day lengths, it is theorized that E oscillators have $\tau < 24$ h and M oscillators have $\tau > 24$ h. According to this model, the inherent differences in τ alter the phase angle between E and M oscillators (ϕ_{EM}), which leads to increases in the duration of subjective night (Pittendrigh, 1974, Illnerova, 1991, Elliott and Tamarkin, 1994, Gorman et al., 1997). It is further predicted that during entrainment, the E oscillator with $\tau < 24$ h is decelerated daily by light at dusk and the M oscillator with $\tau > 24$ h is accelerated daily by light at dawn. When day length changes, these resetting-actions of light would influence ϕ_{EM} (Figure 2B), which is expected to alter the waveform of output signals from the SCN and the overt rhythms it programs (e.g., α , melatonin duration). Due to the reduced influence of photic cues under short days and DD, the changes that manifest are thought to due to inherent τ differences between E and M and/or ϕ_{EM} -dependent coupling.

Typically, the duration of subjective night stabilizes under DD, which would not be expected if its expansion were determined solely by inherent differences in τ . Instead, the rhythms of independent free-running oscillators would periodically diverge and converge to produce a “beating” pattern when monitored over many cycles. In contrast to this prediction, the maximal degree of subjective night expansion is relatively fixed under most conditions (Hastings et al., 1987, Elliott and Tamarkin, 1994, Gorman et al., 1997). In fact, DD-induced increases in α and melatonin secretion are typically proportional to the length of the scotophase under the previous LD cycle (Illnerova, 1991, Elliott and Tamarkin, 1994), with little to no further expansion occurring after release from very short days (Figure 2A). Thus, the systematic yet constrained pattern implies that coupling synchronizes the period of E and M to regulate ϕ_{EM} and prevent arrhythmia or “beating” from manifesting (Pittendrigh and Daan, 1976b, Illnerova, 1991, Elliott and Tamarkin, 1994).

Since its conception, this complex clock model has captured the interest of chronobiologists and inspired a search for the location of E and M oscillators. Based on this model, E and M oscillators are predicted to display 1) adjustable phase relationships dependent on photoperiod, 2) differential control over activity onset versus offset, and 3) inherent period differences with $\tau_E < 24$ h $<$ τ_M . Several studies

investigating SCN electrical firing rhythms and clock gene/protein expression have identified subgroups of SCN neurons whose phase relationships are modulated by photoperiod, with these neuronal subgroups organized along a rostral-caudal axis and/or dorsal-ventral axis (Jagota et al., 2000, Hazlerigg et al., 2005, Inagaki et al., 2007, Naito et al., 2008, Yan and Silver, 2008, Brown and Piggins, 2009, Evans et al., 2013, Myung et al., 2015). Furthermore, studies have identified specific subgroups of SCN neurons within the rostral and caudal SCN that differentially control activity onset and offset (Inagaki et al., 2007). Lastly, period differences have been reported for SCN neurons located within discrete regions (Shinohara et al., 1995, Noguchi et al., 2004, Noguchi and Watanabe, 2008, Myung et al., 2012). Collectively, this work suggests that long photoperiods modulate the phase relationships between SCN neurons, but questions remain about this process and the underlying coupling mechanisms.

Important questions concerning the nature of this coupling process need to be addressed by additional studies. For example, the basic premise of $\tau_E < 24 \text{ h} < \tau_M$ of the original E-M model does not take into account the fact that α expansion under DD reflects the actions of a coupled system. Thus, DD-induced α expansion could be driven by inherent period differences OR by phase shifts produced by intercellular signaling factors produced by the network. Although it has been extremely difficult to distinguish these two possibilities, this is a non-trivial issue that hinders insight into how SCN subgroups are interacting during photoperiodic changes in circadian waveform. For example, if α expansion is driven by inherent period differences, then coupling interactions need not engage until a specific ϕ_{EM} is achieved (Figure 2C). In this case, coupling reflects an ϕ_{EM} -dependent interaction that resets E and M oscillators so that they synchronize with a similar period when in a specific relationship. In effect, this would limit further changes in ϕ_{EM} and prevent phase locking from manifesting (Figure 2C). Alternatively, α expansion may not reflect inherent period differences but instead could be driven by intercellular interactions that cause E and M to reset one another (Figure 2D). In this model, phase locking is prevented by ϕ_{EM} -dependent changes in coupling strength (Figure 2D). Thus, both models involve a recursive mutual resetting process that is ϕ_{EM} -dependent (Daan and Berde, 1978, Oda and Friesen, 2002). Although pitted against one another here, it is worth noting that these two models are not mutually exclusive (Figure 2E) and may be accounted for by a single coupling process that involves sinusoidal rhythms in resetting (Daan and Berde, 1978). Recent work has validated this general concept by demonstrating that SCN neurons can interact through ϕ -dependent resetting (Evans et al., 2013), and an important issue for future work is testing whether this type of “coupling response rhythm” varies with SCN subclass and/or environmental conditions.

The conceptual models described above are designed to illustrate possible ways of envisioning the formal process of coupling, which is an important step towards defining its neurobiological basis. Although it remains untested whether the strength or nature of SCN coupling is systematically influenced by neuronal

phase relationships, formal analyses may begin to examine this issue by investigating the rate of α expansion following release from different pretreatment conditions. Further, work suggests that distinct SCN factors promote period synchrony (VIP) and desynchrony (GABA), and future studies should test whether these factors influence photoperiodic plasticity in circadian waveform. It is clear that VIP signaling plays an important role in stabilizing circadian waveform in the absence of environmental time cues (Harmar et al., 2002, Colwell et al., 2003), but additional work should test whether the DD phenotype of VIP-deficient mice is caused directly by loss of the VIP-related synchronizing cue or indirectly by a GABA-related desynchronizing signal (Freeman et al., 2013). Moreover, VIP knockout mice fail to maintain photoperiodic changes in α and SCN electrical activity upon release into DD (Lucassen et al., 2012), but the strength of rhythms displayed by VIP mice is enhanced by short day entrainment. In addition to short day lengths, rhythms of VIP-deficient mice are improved by constant light and use of a running-wheel (Power et al., 2010, Hughes et al., 2015). Although the neurobiological basis remains unclear, it should be investigated whether these environmental conditions restore rhythmicity in VIP-deficient mice by changing the strength of other SCN coupling factors. When paired with manipulation of specific signaling mechanisms, formal analyses of photoperiodic plasticity in circadian waveform has the potential to shed new light on the process and mechanisms underlying SCN coupling.

3.2. Changes in circadian waveform under constant light

Support for the complex clock model also derived from the observation that locomotor activity rhythms could dissociate into multiple components under constant light (LL) conditions (Pittendrigh and Daan, 1976b). In nocturnal rodents, LL lengthens τ , compresses α and decreases locomotor activity levels (Aschoff, 1960). In addition, chronic exposure to LL can induce a temporal reorganization known as “splitting” (Figure 3A), which is characterized by two bouts of rest and locomotor activity per circadian cycle (Morin and Cummings, 1982, Turek et al., 1982, Cheung and McCormack, 1983, Lees et al., 1983, Boulos and Morin, 1985, Meijer et al., 1990, Puchalski and Lynch, 1991b, Pickard et al., 1993, Lax et al., 1998). While the LL-induced split is emerging, the two activity components may free-run with different circadian periods, one shorter and the other longer than 24 h. However, when the two activity bouts reach antiphase, the split rhythm stabilizes such that two distinct activity components are maintained. After transfer from LL to DD, the two activity bouts rapidly rejoin, with unsplit activity rhythms re-emerging after a brief interval (Earnest and Turek, 1982). The presence of activity bouts with distinct period lengths during induction and resolution of the LL-induced split was taken as evidence that this reflects the actions of multiple clocks cycling in antiphase. That these clocks were located within the master clock itself was further based on observations that overt rhythms besides locomotor activity are likewise split under LL

(Shibuya et al., 1980, Pickard et al., 1984, Swann and Turek, 1985) and that SCN electrical rhythms were bimodal in LL-split animals (Mason, 1991, Zlomanczuk et al., 1991). It is worth noting that the incidence of splitting under LL is influenced by many different factors, including LL intensity, species, sex, age and wheel running. In some animals chronic LL will cause the complete loss of circadian rhythmicity (Figure 3A), with ultradian rhythms thought to reflect the independent programs of many desynchronized SCN neurons (Honma and Hiroshige, 1978, Mason, 1991, Lax et al., 1998). Lastly, some inbred strains of mice display spontaneous splitting under conditions of DD rather than LL, although this phenotype appears to arise due to reorganization of non-SCN clocks (Abe et al., 1999, Abe et al., 2001).

The neurobiological basis of LL-induced split rhythms has been localized to antiphase oscillations of the left and right lobes of the SCN (de la Iglesia et al., 2000, de la Iglesia et al., 2003, Ohta et al., 2005, Yan et al., 2005, Butler et al., 2012), which are connected by contralateral projections extending across the midline (Moore and Leak, 2001). Consistent with the idea that each split bout is generated by a separate lobe, unilateral SCN lesions can cause the emergence of an unsplit rhythm (Pickard and Turek, 1983), although lesions of non-SCN tissue can produce similar effects (Harrington et al., 1990). However, temporal dissociation of left and right SCN may not be the exclusive means by which splitting arises because animals sustaining unilateral SCN lesions can display LL-induced split rhythms (Davis and Gorski, 1984). Further Siberian hamsters split by LL do not display antiphase electrical rhythms in the left and right SCN, which suggests rearrangement of SCN neurons within each lobe (Zlomanczuk et al., 1991). Consistent with this, LL-split hamsters display antiphase rhythms in the shell and core of each SCN lobe as well as antiphase rhythms between lobes (Yan et al., 2005, Butler et al., 2012); however, it remains unknown if this pattern generalizes to other nocturnal rodent species.

Although photoperiodic and LL-induced modulations of circadian waveform both provide support for the concept of the SCN being a complex comprised of multiple interacting clocks, some studies suggest that photoperiodic and LL-induced changes in circadian waveform are mediated by distinct neurobiological mechanisms. For instance, E and M oscillators are modeled as differentially controlling activity onset and offset, but the left and right SCN provide redundant programming (Davis and Gorski, 1984, Davis and Viswanathan, 1996). Also, photoperiod does not alter the phase relationship between left and right SCN (de la Iglesia et al., 2004b) and unilateral SCN lesions do not compromise photoperiodic changes in gonadal function (Hastings et al., 1987). The relationship between photoperiodism and LL-induced splitting remains difficult to test because these two paradigms involve incompatible experimental conditions and bright LL masks many overt rhythms in nocturnal rodents that are highly informative (e.g., suppression of melatonin secretion). Thus, it remains possible that these two forms of plasticity are fundamentally distinct in terms of their underlying coupling mechanisms.

Despite progress in identifying the neuroanatomical substrate(s) for LL-induced rhythms, the process by which the SCN reorganizes under LL remains unclear. Under the original complex clock model, the two split bouts under LL were originally labeled E and M because in some records the split activity bouts appeared to derive from the evening and morning portions of the unsplit activity rhythm (Earnest and Turek, 1982, Morin and Cummings, 1982, Swann and Turek, 1982, Lees et al., 1983). However, this is not always the case and in some records the origin of the split bouts is unclear (e.g., [Figure 3A](#)). Although the neurobiological evidence discussed above indicates that subsets of E and M oscillators likely control each split bout, it was originally hypothesized that LL lengthened τ_E and shortened τ_M to cause reductions in ϕ_{EM} and α compression (Pittendrigh, 1974, Pittendrigh and Daan, 1976b). Alternatively, it has been proposed that LL-induced splitting could reflect light-induced changes in the strength and/or nature of the coupling process (Daan and Berde, 1978, Kawato and Suzuki, 1980, Oda and Friesen, 2002). It remains unclear precisely what causes the activity rhythm to split into two synchronized bouts that cycle in antiphase, but may involve changes in coupling that involve either attractive or repulsive interactions (Daan and Berde, 1978, Oda and Friesen, 2002) and may be modeled using conceptual processes like those postulated to regulate photoperiodic modulation of circadian waveform.

An important issue for future work will be to understand precisely how LL influences coupling between SCN neurons to fragment circadian waveform into split and arrhythmic patterns. Little is known about how SCN signaling changes during LL-induced splitting and arrhythmia, which may reflect the fact that these forms of plasticity develop over a long period time with little experimental control. Previous research has demonstrated that VIP in rats is downregulated by acute light exposure and 3-wk exposure to LL (Albers et al., 1987, Shinohara et al., 1993, Isobe and Nishino, 1998, Shinohara et al., 1999). However, it is unknown if VIP levels rebound during long-term LL exposure like that necessary to induce splitting and arrhythmia. Further, the depressive effects of light on VIP may not generalize to other rodent species like the mouse (Dardente et al., 2004). It may be possible to test whether downregulation of VIP under LL provides the impetus for loss of synchronization that occurs during LL-induced arrhythmia, but this mechanism fails to fully account for the observation that split bouts are able to synchronize once aligned into an anti-phase configuration. Another possibility is that the emergence of LL-induced splitting reflects coupling mechanisms that resist light-driven α compression through a repulsive type of interaction. Further work is needed to understand how LL may influence SCN coupling. One way in which this question can be addressed is by systemically evaluating the effects of chronic light on putative SCN coupling factors and testing whether down/up regulation is causally related to LL-induced plasticity in circadian waveform. Given the species differences in the incidence and thresholds for LL-induced splitting, future studies may also benefit from a comparative approach using a variety of models – both nocturnal and diurnal.

3.3. Changes in circadian waveform after light-induced resetting

The SCN receives light cues that allow it to synchronize to local time (Meijer and Schwartz, 2003). Photoc entrainment is mediated by a circadian rhythm in light sensitivity described in the photic phase response curve (PRC), which illustrates how light applied at different phases of the circadian cycle shifts the phase of overt rhythms (Johnson, 1999). Specifically, light pulses early in subjective night produce phase delays, light pulses late in subjective night produce phase advances, and light pulses during the subjective day produce negligible effects. In nocturnal rodents, light pulses also produce acute changes in circadian waveform that depend on the direction of phase resetting (Figure 4A). Following late night light pulses, activity/melatonin offset advances readily but activity/melatonin onset requires several cycles to shift completely (Boulos and Rusak, 1982, Honma et al., 1985, Illnerova and Vanecek, 1987, Meijer and De Vries, 1995). The different resetting kinetics of distinct phase markers causes the emergence of “transient” cycles where subjective night is compressed, which is resolved as activity onset shifts gradually over subsequent days (Figure 4A). In extreme cases, transient cycles may be characterized by the complete loss of nocturnal events, such as melatonin secretion (Illnerova and Vanecek, 1987). 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 (Figure 4A). Direction-dependent transients in circadian waveform also emerge following shifts of the LD cycle that simulate travel across time zones (Figure 4B), although shifts in activity onset and offset are oftentimes masked by light under these conditions.

Differential photic resetting also occurs within the SCN itself, with distinct resetting patterns evident between different clock genes and SCN regions. In hamsters, the evening and morning peaks of SCN electrical activity specifically detected in a horizontal slice preparation are differentially shifted by application of glutamate *in vitro* (Jagota et al., 2000). Furthermore, discrepancies in resetting are evident among different SCN clock gene/protein rhythms (Sumova and Illnerova, 1998, Zylka et al., 1998, Reddy et al., 2002, Yan and Silver, 2002, 2004). In addition, SCN regional differences in the rate of re-entrainment have been reported in both the advance and delay direction (Nagano et al., 2003, Albus et al., 2005, Nakamura et al., 2005, Davidson et al., 2009, Nagano et al., 2009, Rohling et al., 2011, Sellix et al., 2012). Results of bioluminescence imaging with real-time reporters of clock protein indicate that the SCN core shifts faster than the SCN shell after a 6 h advance of the LD cycle (Sellix et al., 2012), but the spatiotemporal kinetics of re-entrainment in the delay direction have yet to be examined with this approach.

At the formal level of analysis, light-induced changes in circadian waveform have been interpreted within the context of the complex clock model. According to this model, direction-dependent resetting kinetics reflect differences in the light sensitivity of E and M and their mutual coupling strength (Boulos and Rusak, 1982, Honma et al., 1985, Illnerova, 1991, Meijer and De Vries, 1995). In the case of

differential light sensitivity, the immediate phase advance of activity offset suggests that M is highly sensitive to light provided during late subjective night, while the sluggish response of activity onset indicates E is less sensitive to light at this phase. That the steady state phase shift of onset often equals that for offset has been interpreted as evidence for coupling between E and M (Illnerova, 1991, Meijer and De Vries, 1995), but this process remains ill defined. Subsequent decompression of α during transient cycles could be explained by coupling that promotes period synchrony (Figure 2D) or desynchrony (Figure 2C) in an ϕ_{EM} -dependent manner. Conversely, neither process can account for the lack of delaying transients, unless 1) E and M are equally responsive to light presented during early subjective night or 2) there is an asymmetry in the mutual coupling between E and M (Illnerova, 1991).

Given the translational appeal in better understanding this process, there are a number of things that could be addressed in future work. First, a detailed understanding of how putative E and M oscillators respond to photic stimuli could be used to test whether they are characterized by distinct PRCs. Formal analyses may also provide insight by testing whether nonphotic resetting is similarly marked by transients. The results of such a study may provide insight into whether direction-specific resetting reflects a general feature produced by fundamental differences in the ability of underlying oscillators to reset one another. Conversely, if this phenomenon reflects that E and M have different photic PRCs, then there is little reason to suspect that nonphotic phase shifts will involve transients or that those that might emerge will bear any similarity to light-induced advancing transients. Furthermore, future studies can be designed to compare and contrast the paradigms presented in the proceeding two sections (i.e., changes in circadian waveform after release into DD and after discrete light pulses) in order to resolve whether they reflect identical or disparate processes. Lastly, studies should investigate whether specific SCN coupling factors influence the kinetics of photic re-entrainment. Although transients are not commonly quantified, it is of interest that the rate of re-entrainment is accelerated by either a gain in VIP signaling (Shen et al., 2000, An et al., 2013) or a loss of AVP signaling (Yamaguchi et al., 2013, Mieda et al., 2015). A re-examination of light-induced transients in the context of altered SCN signaling may provide insight, as would studies that couple these manipulations with approaches that quantify the associated changes in SCN spatiotemporal organization.

3.4. Photoperiodic non-responsiveness

Within many rodent species, some animals fail to adopt the typical short day phenotype of reproductive quiescence, and are commonly referred to as short day nonresponders (NR) (Nelson, 1987). In Siberian hamsters, insensitivity to short day lengths has a circadian basis (Puchalski and Lynch, 1988, 1991a, b, 1994, Freeman and Goldman, 1997, Gorman et al., 1997, Gorman and Zucker, 1997, Prendergast and Freeman, 1999, Gorman and Elliott, 2004). NR Siberians express both a short α (Figure 4C) and a short

melatonin signal under short day lengths, with each rhythm phase locked to dawn in the large majority of animals (Puchalski and Lynch, 1986, Margraf et al., 1991, Margraf and Lynch, 1993, Gorman et al., 1997, Gorman and Zucker, 1997, Prendergast and Freeman, 1999, Gorman and Elliott, 2004). A complementary pattern can be seen in the rhythm of spontaneous electrical activity within the SCN of NR Siberian hamsters (Margraf et al., 1991). NR Siberian hamsters display short day responses if long melatonin infusions are provided; indicating that peripheral sensitivity is intact but that the appropriate signal is absent in these animals (Margraf and Lynch, 1993). Further, Siberian hamsters from artificially selected NR lines, if raised in DD or a short day photoperiod, can exhibit photoperiodic responsiveness under short day lengths (Stanfield and Horton, 1996, Freeman and Goldman, 1997, Goldman and Goldman, 2003). Lastly, pre-treatment to very long day lengths can cause unselected animals to display a state of short day nonresponsiveness qualitatively similar to that produced by artificial selection (Freeman and Goldman, 1997, Gorman et al., 1997, Gorman and Zucker, 1997, Prendergast and Freeman, 1999, Goldman et al., 2000). Collectively, these data suggest that the NR phenotype in Siberian hamsters is caused by a fundamental change in the function of the SCN that limits plasticity in circadian waveform.

The nature of the change in the SCN that causes short day nonresponsiveness remains unclear. It has been proposed that short day nonresponsiveness may reflect a change in τ and/or altered coupling (Puchalski and Lynch, 1991b, 1994, Gorman and Zucker, 1997). As described above, it is theorized that photoperiodic expansion of α reflects inherent differences in the period of E and M, with $\tau_E < 24 \text{ h} < \tau_M$. One possible explanation of photoperiodic non-responsiveness contends that lengthening of τ_E causes it to be $> 24 \text{ h}$, which lengthens overall τ and inhibits the ability to expand α under short days. Consistent with this hypothesis, it has been reported that animals from artificially selected NR lines will display gonadal regression under LD cycles $> 24 \text{ h}$ (Puchalski and Lynch, 1994). However, a lengthened τ is not always observed in NR Siberian hamsters, and it has been suggested also that a change in the coupling between E and M oscillators may constrain increases in ϕ_{EM} in these animals. Originally, it was postulated that NR animals have stronger coupling; however, it remains difficult to specify changes in coupling strength given the possibility of both synchronizing and desynchronizing signaling mechanisms. Given the utility of exploring this phenotype further, future studies may benefit from recent advances that could allow for genetic manipulations in this species (Hsu et al., 2014, Sander and Joung, 2014).

3.5. Changes in circadian waveform under skeleton photoperiods and the phenomena of “phase jumps”

Under skeleton photoperiods, the full photophase is replaced with two short light pulses simulating light transitions at dusk and dawn (Figure 4D). These conditions have ecological relevance for nocturnal rodents that primarily receive light exposure as they emerge from and return to darkened burrows. Skeleton

photoperiods also serve as useful analytic tools for studying seasonal changes in pacemaker function, since long and short day lengths are simulated with equivalent light exposure. For the most part, entrainment under skeleton photoperiods resembles that elicited by full photoperiods, except when the former simulates very long day lengths (Pittendrigh and Daan, 1976a). A “phase jump” occurs under these conditions (Figure 4D), where activity onset crosses one of the entraining light pulses and α realigns within the longer of the two available scotophases (Pittendrigh and Daan, 1976a, Rosenwasser et al., 1983, Stephan, 1983, Sharma et al., 1997, Evans et al., 2005). In contrast, phase jumps rarely occur under comparable full photoperiods, suggesting that the continued light exposure contributes to stable entrainment under very long day lengths (Pittendrigh and Daan, 1976a, b).

Previous models have largely accounted for phase jumps through an asymmetry in the phase delay and advance regions of the photic PRC (Pittendrigh and Daan, 1976a, Stephan, 1983, Sharma et al., 1997). These early models, however, do not take into account photoperiod-induced changes in the amplitude of the photic PRC, where reduced phase shift magnitude correlates with decreases in α (Pohl, 1983, Pittendrigh et al., 1984, Pohl, 1984, Shimomura and Menaker, 1994). Therefore, during entrainment to very long day lengths, like those simulated under skeleton photoperiods, light-induced phase shifts are markedly attenuated and less clearly able to generate phase jumps. Mutual coupling mechanisms may account for the emergence of phase jumps under skeleton photoperiods (Pittendrigh and Daan, 1976b, Evans et al., 2005). As α is compressed, a phase jump could emerge as ϕ_{EM} decreases through either a loss of period synchrony or by more direct repulsive interaction. After the initiation of the phase jump, ϕ_{EM} would increase, which would allow for synchrony and complete realignment into the alternative scotophase. As the alternative scotophase is typically longer than its counterpart, E and M can then adopt the ϕ_{EM} that is more conducive to stable entrainment. Very long day lengths incorporating full photophases may inhibit phase jumps by countering the oscillator interactions that instigate the jump. These photic effects may impose long-term changes in circadian function like that described above (e.g., NR phenotype in Siberian hamsters) by increasing τ_E and/or by changing ϕ_{EM} -dependent mutual coupling mechanisms. Further study of phase jumping may thus serve to characterize changes induced by exposure to simulated long day lengths and examine conceptual models of ϕ_{EM} -dependent interactions.

3.6. Forced desynchrony under non 24-h LD cycles

As discussed in the preceding sections, adaptive phase relationships of oscillators may be the product of coupling processes. But if pacemakers are subjected to non-ecological conditions (e.g., LD cycles markedly longer or shorter than 24 h), coupling may not be sufficient to maintain a coherent rhythm. Indeed, there exists a range of environmental frequencies with which the circadian pacemaker can resonate. The

range of non-24-h LD cycles (i.e., T cycles, where T = period of the external cycle) to which a given species of mammals can synchronize can be predicted by τ and the photic PRC typical of that species. Further, a collection of oscillators marked by heterogeneous τ would be predicted to differentially entrain to non-24-h T cycles (Shinbrot and Scarbrough, 1999). For example, oscillators with τ close to T would be expected to entrain, whereas oscillators with τ markedly deviating from T would be expected to free-run. Long-term records of such rhythms would be predicted to have at least two dominant rhythms that beat in and out of phase with one another. Support for these predictions can be found in the activity rhythms of rats held under short T cycles (T21-T22), which is near the lower limit of entrainment for this species (Vilaplana et al., 1997a, Campuzano et al., 1998, Cambras et al., 2000, Cambras et al., 2004). In these records, a non-entrained activity component with $\tau > 24$ h appears to be superimposed onto another activity rhythm entrained to the lights-off transition of the T cycle. When the two activity bouts cross, they exhibit relative coordination (Schwartz et al., 2009). When T approaches 24 h, this pattern is altered in that the entrained component becomes more robust and the non-entrained activity band diminishes. Measures of the free-running rhythms and anticipatory activity preceding the lights-off transition indicate that these patterns of activity do not result solely from the masking effects of light. Instead, these “beating” patterns are thought to reflect that the master clock is partially entrained, which has been supported by investigations revealing that the SCN shell and core are desynchronized under these conditions (de la Iglesia et al., 2004a). Importantly, differential gene expression in these SCN regions appears to persist temporarily after release into DD, arguing against a pure masking effect by light and darkness. To date, the forced desynchrony paradigm has been used to provide new insight into the function of outputs specifically produced by SCN shell and core compartments (Lee et al., 2009, Schwartz et al., 2009, Smarr et al., 2012, Wotus et al., 2013), as well as the propagation of resetting signals within the SCN network (Schwartz et al., 2010). It remains unclear if analogous behavior is observed in other species, but if the rat is unique in its expression of this behavior, then this may provide an interesting comparative approach for studying circuit properties that enable this form of plasticity. In addition, investigating the process of desynchronization and resynchronization using the forced desynchrony model may provide novel insight into SCN coupling mechanisms.

3.7. Bifurcated rhythms under 24-h LDLD cycles

Exposure to 24-h light:dark:light:dark (LDLD) cycles generates bifurcated rhythms, where each activity bout is entrained to one of the daily dark periods (Figure 4E). A variety of protocols using 24-h LDLD cycles rapidly induces bifurcated rhythms in hamsters and mice (Mrosovsky and Janik, 1993, Gorman, 2001, Gorman and Lee, 2001, Gorman and Elliott, 2003, Gorman et al., 2003, Gorman and Elliott,

2004, Evans et al., 2005). The first procedure for generating bifurcated rhythms under LDLD cycles utilized scheduled exposure to novel wheel running (NWR) during the subjective daytime (Mrosovsky and Janik, 1993, Gorman and Lee, 2001, Evans and Gorman, 2002). This protocol is also referred to as behavioral decoupling to acknowledge the role of scheduled wheel running and to distinguish it from LL-induced splitting (Mrosovsky and Janik, 1993). Comparable split rhythms also emerge without NWR when animals are exposed to a LDLD cycle with two, short scotophases of equal duration (Gorman, 2001). Both activity bouts under LDLD correspond to physiological indices of subjective night (e.g., melatonin secretion and light-induced gene expression) and subjective night markers are absent during the intervening bouts of behavioral quiescence (Gorman et al., 2001b, Edelstein et al., 2003, Raiewski et al., 2012). Moreover, LDLD-induced bifurcated rhythms rejoin after release into DD through a series of transients, suggesting that each LDLD activity bout is programmed by a separate group of oscillators. Of interest, 24-h LD cycles with more than two scotophases induce multi-modal rhythms in rats (Vilaplana et al., 1997b) and hamsters (Figure 4F). Several factors are postulated to contribute to the emergence of bifurcated rhythms under LDLD (Gorman et al., 2003, Evans et al., 2005). First, robust novelty-induced wheel running phase is thought to shift a subset of oscillators into the daytime scotophase through nonphotic resetting (Mrosovsky and Janik, 1993, Gorman and Lee, 2001, Evans and Gorman, 2002). Second, the short nighttime scotophase under LDLD challenges entrainment and may instigate a phase jump of a subset of oscillators into the daytime scotophase (Pittendrigh and Daan, 1976a, Stephan, 1983, Evans et al., 2005). As postulated to occur under skeleton photoperiods, a phase jump may be instigated by a change in the interactions between constituent oscillators that cause component oscillators to repel one another. However, under LDLD, the alternative scotophase is not of sufficient length to complete the phase jump, which promotes a stably bifurcated state.

Formal and physiological data indicate that exposure to LDLD produces bifurcated rhythms that are distinct from LL-induced split rhythms. First, LDLD-induced bifurcated activity bouts rejoin upon transfer to LL, which will independently induce splitting within several weeks (Gorman, 2001). Further, there is no indication of a left-right asymmetry in the SCN during LDLD-induced bifurcation, rather light-induced *c-fos* and *Period* gene expression is observed throughout both lobes of the SCN (Gorman et al., 2001b, Edelstein et al., 2003, Watanabe et al., 2007, Yan et al., 2010). Thus, there is reason to believe that LDLD-induced bifurcation arises from the dissociation of oscillators operating within each lobe of the SCN, although their precise location remains undetermined.

4. Advancing the current working model of the SCN complex

4.1. Multiple clock populations within the SCN network

The classic multi-oscillator model first described by Colin Pittendrigh has received wide acclaim since its formal introduction. As described in the preceding sections, there is a large body of neurobiological evidence supporting the theory that the SCN contains multiple independent and heterogeneous oscillators. Analysis of SCN rhythmicity after exposure to changing environmental conditions indicates that the SCN network can be temporally reorganized in a variety of ways, with complex patterns sometimes emerging within the same paradigm. For instance, LL-induced splitting is associated with antiphase oscillations of the left and right SCN lobe, but also antiphase oscillations of shell and core regions within each lobe (Mason, 1991, de la Iglesia et al., 2000, de la Iglesia et al., 2003, Ohta et al., 2005, Yan et al., 2005, Butler et al., 2012). Further, there is evidence that photoperiodic modulation of circadian waveform corresponds with changes in the phase relationships of SCN neurons within the rostral and caudal poles of the network, but also those within the shell and core compartments (Jagota et al., 2000, Hazlerigg et al., 2005, Inagaki et al., 2007, Naito et al., 2008, Yan and Silver, 2008, Brown and Piggins, 2009, Evans et al., 2013, Myung et al., 2015). Lastly, forced desynchrony corresponds to free-running rhythms in the shell and core compartments of the rat, which free-run rather than stably reorganize as in other behavioral paradigms (de la Iglesia et al., 2004a). The plurality of ways in which the SCN network can be rearranged suggests that multiple subpopulations exist. Intra- and inter-SCN coupling mechanisms are likely differentially sensitive to factors operating under these distinct behavioral paradigms, which warrants further study. Real-time imaging of molecular rhythmicity has proved to be an important technique for revealing subgroups of SCN neurons; however, it should be noted that *ex vivo* preparations usually reduce the complexity of the SCN network and therefore may not capture all aspects of its circuitry. Further technological advances that allow for real-time visualization of SCN function while the network is fully intact and integrated into the larger system is expected to provide important insight into network function (Hamel et al., 2015).

The formal assays highlighted in this review may be exploited further to distinguish SCN neuronal subpopulations and define the processes by which they interact. If the objective is to localize functionally distinct oscillators within the SCN in order to study their connections, intrinsic differences, and interactions, the best paradigms with which to pursue this goal may be those involving photoperiodism, LDLD-induced bifurcation, and forced desynchrony, since these paradigms appear to disassociate functionally and anatomically distinct oscillators operating within each lobe of the SCN. Convergent analyses using these paradigms may elucidate distinct subgroups of oscillators that regulate overt periodicity and circadian waveform through their interactions. Furthermore, comparative studies are needed to investigate whether the coupling processes modulating circadian waveform are distinct for the different behavioral assays discussed in this review. Previous work using the Siberian hamster demonstrates a clear relationship between plasticity in circadian waveform across three specific behavioral assays (i.e., photoperiodic

responsiveness, LDLD-induced bifurcation, and arrhythmia under constant dim lighting conditions), which suggests that these are regulated by a common underlying coupling mechanism (Evans et al., 2012a). In contrast, circadian plasticity in these three assays was not related to changes in circadian waveform that emerged immediately after release from entrained to constant conditions or during light-induced resetting transients. This pattern of results suggests that these latter behavioral assays here may reflect the actions of coupling mechanisms that are distinct. Given the known species differences in the behavioral response to the behavioral assays discussed in this review, a comparative approach investigating SCN circuitry in non-murine species may be useful. Future studies may benefit from recently developed methods (Hsu et al., 2014, Sander and Joung, 2014) that allow for genetic manipulations in non-murine animal models that display interesting behavioral phenotypes indicative of unique coupling of SCN oscillators (e.g., the hamster). Research incorporating both nocturnal and diurnal animal models is likewise of interest.

4.2 Influence of light on SCN coupling

Under each of the paradigms presented above, photic stimulation was a critical agent influencing circadian waveform. Under unconventional photic conditions, bi-stability (LL, LDLD) or desynchronization (LL, non 24-h T cycles) can emerge depending on the intensity and/or duration of illumination (Pickard et al., 1993, Gorman et al., 2003, Cambras et al., 2004, Gorman and Elliott, 2004, Gorman et al., 2005, Rosenthal et al., 2005). While bright light is necessary for splitting under LL, dim nighttime illumination appears to be a pivotal factor influencing circadian plasticity under short day photoperiods, skeleton photoperiods, simulated jetlag, LDLD, and non 24-h T cycles (Gorman and Elliott, 2004, Evans et al., 2005, Gorman et al., 2005, Evans et al., 2009). Many questions remain about how light produces these effects. For example, does bright and dim light influence circadian plasticity by directly influencing mutual coupling mechanisms or does it alter the inherent period of SCN neurons? Do different subtypes of SCN neurons exhibit differences in their sensitivity and/or nature of responses to light? How is photic information being processed and propagated through the SCN network (Antle et al., 2003)?

While the present discussion focuses on changes in circadian waveform, the analytical paradigms presented in the preceding sections often produce changes in τ , commonly referred to as circadian aftereffects. For example, exposure to constant light both lengthens overt τ and changes circadian waveform. Does light influence α and τ via a common mechanism or are these effects produced through distinct means? Simulations using mathematical models with separate parameters modulating the collective frequency and relative phase of coupled nonlinear oscillators suggest that either parameter can be used to synchronize a population of high frequency nonlinear oscillators (Shinbrot and Scarbrough, 1999). Behavioral and physiological studies addressing circadian responses to light may attempt to disentangle

photic effects on τ and circadian waveform in order to further address the role of light in modulating circadian plasticity under the above paradigms.

4.3. Alternatives to the complex clock model

Over the years, alternative models to the complex clock have been proposed to account for specific behavioral phenotypes. Prominent among these alternative models are those that posit the circadian system contains a “gate” that regulates the expression of overt rhythmicity, which can be modulated by external conditions to affect circadian waveform. This type of model has been largely invoked to account for photoperiodic changes in circadian waveform, where short day photoperiods may lower the threshold of the gate and thereby produce the expansion of subjective night through seemingly independent periodicities of its onset and offset. Accordingly, short day NR animals could have more rigid thresholds; however, this model is less able to account for the phase angle of entrainment displayed by the majority of NR animals (i.e., locked to lights-on or lights-off). Mathematical models where overt rhythmicity is controlled by the actions of multiple oscillators with variable τ and gated by a threshold have been used to simulate a wide variety of circadian behaviors (Enright, 1980b, a, Shinbrot and Scarbrough, 1999). Under this premise, a population of oscillators with variable τ could form a highly precise, functional pacemaker through the collective actions of multiple, imprecise short-term oscillators. The emergent “neuronal” rhythm may be used to regulate output from the system through the electrical firing pattern of the SCN neurons themselves (Shinbrot and Scarbrough, 1999), or through the actions of a “discriminator” node that need not be inherently rhythmic (Enright, 1980b). In this latter model, direct interactions between individual oscillators are not necessary, if the discriminator can influence the period and/or phase of all the oscillators within the population (Enright, 1980a). This discriminator model may bear on the organization of the central pacemaker, being similar to one of the functions proposed for the calbindin-immunoreactive sub-nucleus within the hamster SCN (Antle et al., 2003). Thus, it stands to reason that alternative models should be considered as potentially relevant elements of SCN circuits, regardless of the importance and popularity of the complex SCN model.

5. Conclusions

The behavioral and physiological analyses highlighted in this review indicate that the central pacemaker is composed of a population of self-sufficient clocks that couple together to form a plastic network. The next step is to advance our understanding of the coupling mechanisms that govern pacemaker function and the neural structure that supports this complex. This review presents models of SCN coupling

that can be subjected to empirical tests. In particular, these coupling models posit that SCN neurons assume specific relationships through inherent differences in τ and/or ϕ_{EM} -dependent interactions. While previous experimental work has focused largely on putative coupling that synchronizes SCN neurons, the forms of circadian plasticity highlighted in this review can be conceptualized as either attractive (Figure 2D) or repulsive coupling mechanisms (Figure 2C). Whether “attractive” and “repulsive” coupling processes reflect distinct signaling mechanisms remains to be determined through empirical study, but it is of interest that similar processes have been employed in mathematical models of coupled, nonlinear oscillators (Shinbrot and Scarbrough, 1999). It will be important to gain a better understanding of the source and temporal patterning of coupling signals such as these, which can be tested effectively by synthesizing formal, physiological, and molecular analyses.

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Figure Legends

Figure 1. Circadian clocks at multiple levels. A) Simplified model of circadian timekeeping at the molecular level depicting clock gene transcriptional-translational feedback loops. 1. The transcription factors CLOCK and BMAL1 bind to E-box elements (black box) within the promoter sequences of a variety of clock genes. Note that NPAS2 is a paralog of CLOCK that functions in some tissues to activate transcription. 2. The protein products of *Period* (*Per*) and *Cryptochrome* (*Cry*) genes form repressors that inhibit their own transcription. Note there are three paralogs of *Per* (*Per1*, *Per2*, *Per3*) and two paralogs of *Cry* (*Cry1*, *Cry2*). 3. Additional feedback loops involve additional clock genes that interact with the elements of the core loop to amplify and stabilize molecular clock function. For example, the protein products of *Rev-erb* and *Ror* genes compete for binding at ROR elements (white box), whereas the protein products of *Dbp* and *E4bp4* compete for binding at D-box elements (gray box). B) Simplified model of circadian timekeeping at the systems level. The master clock within the SCN (red circle) receives light input that synchronizes it to the 24 h day. The SCN then provides outputs to downstream clocks in the brain and body to coordinate their function. C) Left: The SCN contains ~10,000 neurons in each lobe that express the clock protein PER2. Middle: SCN neurons can be classified based on neuropeptide expression, with the major groups being those that express Arginine Vasopressin (AVP) and Vasoactive Intestinal Polypeptide (VIP). Right: Simplified model of circadian timekeeping within the SCN network. Briefly, VIP (depicted in red) and other neurons in the SCN core (depicted in blue) receive and process photic inputs, which they then transmit to neurons in the SCN shell (green) that provide outputs to downstream tissues. See text for additional details.

Figure 2. Photoperiodic modulation of circadian waveform. A) Representative double-plotted actograms illustrating changes in locomotor activity rhythms of Syrian hamsters held under long day and short day photoperiods before release into constant darkness. White and black bars above each actogram illustrate initial housing conditions, with the internal shading indicating the change in lighting conditions. Data replotted from (Evans et al., 2007). B) Schematic representation of the complex clock model of Pittendrigh and Daan. 1. Under long day photoperiods, Evening (E) and Morning (M) oscillators define the times of activity onset and offset, respectively. E is phase delayed by light at dusk whereas M is phase advanced by light at dawn (lighting bolts). The phase angle of E and M (ϕ_{EM} , internal red angle) determines the duration of nighttime locomotor activity (α , semi-circular brown bar). Importantly, α is inversely related to the duration of electrical and molecular activity of SCN neurons (blue spikes). 2. When transferred to short day photoperiods, E and M oscillators adopt different period lengths, < 24 h and > 24 h, respectively (solid arrows). Over time, this causes changes in ϕ_{EM} , increases α , and decreases the phase distribution of SCN neurons. 3. Stability in circadian waveform is established when E and M are re-entrained by light. 4.

Maximal expansion under constant darkness resembles that achieved under very short day photoperiods.

C) Conceptual model for a repulsive coupling mechanism. Top: Changes in ϕ_{EM} are caused by inherent differences in period ($E < 24$ h, $M > 24$ h) that cause E and M shift closer to one another over consecutive days (solid arrows). Bottom: Changes in ϕ_{EM} are ultimately limited by a repulsive coupling interaction between E and M (red shading) that causes each oscillator to shift in the direction opposite of their free-run (dashed arrows). The strength of this repulsive coupling interaction is expected to depend on ϕ_{EM} , as illustrated by changes in shading. D) Conceptual model for attractive coupling mechanisms. Top: Changes in ϕ_{EM} is caused by an attractive coupling interaction (green shading) between E and M that causes them to shift close to one another (dashed arrows). The strength of coupling is expected to depend on ϕ_{EM} , as illustrated by changes in shading. E) Conceptual model for combined attractive and repulsive coupling mechanisms. In the combined model, both types of coupling interactions influence the steady state of the network. See text for more details.

Figure 3. Modulation of circadian waveform under constant light (LL) conditions. A) Representative double-plotted actograms illustrating changes in circadian waveform of Syrian hamsters (left) and mice (right) during exposure to LL. White bars above each actogram illustrate lighting conditions. Hamster and mouse data are replotted from (Gorman, 2001) and (Evans et al., 2012b), respectively.

Figure 4. Plasticity in circadian waveform during light-induced resetting (A), simulated jetlag (B), photoperiodic non-responsiveness (C), skeleton photoperiods (D), LDLD cycles (E), and multi-model lighting conditions (F). Data in panel A are from (Evans et al., 2007), panel B are from (Evans et al., 2009), panel C are from (Gorman and Elliott, 2004), panels D-E are from (Evans et al., 2005), and panel F are from (Evans & Gorman, unpublished observations).