Neurophysiological Analysis of the Suprachiasmatic Nucleus: A Challenge at Multiple Levels

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Abstract

Understanding the neurophysiology of the circadian timing system requires investigation at multiple levels of organization. Neurons of the suprachiasmatic nucleus (SCN) function as autonomous single-cell oscillators, which warrant studies at the single-cell level. Combining patch-clamp recordings of ion channels with imaging techniques to measure clock gene expression and intracellular calcium has proven extremely valuable to study cellular properties. To achieve and maintain rhythmic activity, SCN neurons require sufficient stimulation (i.e., input) from surrounding cells. At the network level,

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SCN rhythms are robust and can be measured *in vitro*, for example, in brain slices that contain the SCN. These recordings revealed that the collective behavior of the SCN neuronal network is strongly determined by the phase dispersal of the neurons. This phase dispersal is plastic, with high synchronization in short photoperiod, desynchronization in long photoperiod, and antiphase oscillations in aging and/or continuous light. *In vivo* recordings are needed in order to study the SCN as part of a larger network (i.e., interacting with other brain centers) and to study the SCN's response to light. Interestingly, superimposed on the circadian waveform are higher frequency fluctuations that are present *in vivo* but not *in vitro*. These fluctuations are attributed to input from other brain centers and computational analyses suggest that these fluctuations are beneficial to the system. Hence, the SCN's properties arise from several organizational levels, and a combination of approaches is needed in order to fully understand the circadian system.

1. INTRODUCTION

Circadian rhythms are a fundamental property of living organisms. They have evolved early in evolution and are the consequence of an endogenous pacemaker or clock. Clocks evolved in response to the cyclic 24-h changes in the environment due to Earth's rotation around its axis. The evolutionary advantages of having an endogenous clock are clear, as they help the organism anticipate cyclic changes in the environment. In addition, many organisms also have seasonal rhythms in addition to a circadian rhythm. Both circadian and seasonal rhythms are under the control of overlapping biological mechanisms. In mammals, the central clock is located at the base of the brain in a structure called the suprachiasmatic nucleus (SCN), which sits directly above the optic chiasm. The SCN generates circadian rhythms and perceives external light information, thus enabling it to synchronize with both daily and seasonal cyclic changes. In this chapter, we will discuss and review the neurophysiological studies of the SCN.

The generation of biological rhythms is rooted at the molecular level—a negative feedback loop between clock genes and their protein products provides cell-autonomous oscillators. However, although these oscillators are cell-autonomous, they still require input in order to function properly. In the case of the SCN, it seems that most SCN neurons are part of a network and must be excited (i.e., activated) sufficiently to maintain their rhythm-generating capacity (Herzog, Aton, Numano, Sakaki, & Tei, 2004; Welsh, Takahashi, & Kay, 2010). Thus, the rhythm-generating molecular machinery is itself part of a larger network or loop. At the individual cell level, interactions between molecular machinery—as well as input from other cells—are required to obtain a functional single-cell oscillator.

Additional properties of the circadian system arise at the SCN network level and these properties can be measured *in vitro*.

The SCN network interacts with other brain regions. Based on these interactions, which involve both afferent and efferent pathways, the SCN (i) becomes entrained to the external light cycle, (ii) is responsive to the animal's behavioral activity and sleep stages, and (iii) can function both as a circadian pacemaker and as a mediator of seasonal rhythms. To investigate the SCN in the context of a larger network, including the relevant afferent and efferent pathways, several *in vivo* recording methods have been developed.

In this review, we will discuss the analysis of the mammalian circadian system at each of these levels, and we will evaluate the currently available physiological approaches that have been applied at each level. In addition, we will use examples to discuss the insights that have been gained from studies performed at each level.

2. PART I: CLOCK MECHANISMS AT THE CELLULAR LEVEL

Following the initial discovery of clock genes and clock cells in both invertebrates and mammals, the study of the molecular mechanisms within circadian pacemaker cells became the new focus of circadian clock researchers in the 1990s. Within a relatively short time span of 10 years, the core molecular players in the cellular clock were identified, and—more importantly—the dynamics of feedback loops were discovered, explaining the underlying basis of the 24-h rhythm in clock gene expression.

2.1. The molecular feedback loops and beyond

Molecular feedback loops were discovered as the core of circadian rhythm generation; these loops consist of transcription-translation feedback loops (TTFLs) that involve clock genes whose products either inhibit or activate transcription. This mechanism follows a basic oscillator principle in which at least one negative feedback loop and a time delay are required to generate an oscillation. In the case of the circadian clock, intracellular levels of *Per* and *Cry* mRNA increase in the morning, and subsequently PER and CRY proteins are produced throughout the day (Albrecht, 2012). These proteins form dimer complexes that translocate to the nucleus near the end of the day, where they act to suppress clock gene transcription, causing the mRNA levels to decrease again during the night. The PER/CRY dimers are actively degraded during the night, providing an integrative component

of the clock machinery and contributing to a delay of 24 h. In addition, positive regulatory elements—including the *Rev-erbα*, *Rev-erbβ*, and *RORA* genes—both augment and stabilize the molecular oscillator.

Recently, our knowledge of the core clock mechanism was expanded beyond the TTFL to include cytosolic and metabolic oscillators, both of which are believed to interact with the TTFL to maintain proper clock function and rhythmic control of cellular physiology (Rey & Reddy, 2013). As part of the metabolic oscillator, a circadian rhythm in redox cycles was found to be highly conserved through evolution and is a potentially independent cellular clock. Alternatively, redox cycle products may act in concert with post-transcriptional machinery to regulate functions of the cellular clock, for example, as recently demonstrated for neuronal excitability in SCN neurons (Wang et al., 2012). Metabolomics is a promising approach for deciphering the metabolic oscillator and its pathways. This systems wide method for measuring metabolites in cellular physiology provides a direct biochemical insight into clock-driven metabolic pathways.

2.2. Cytosolic oscillators

Within the cytosolic oscillators, Ca^{2+}, cAMP, NAD^+/NADH, AMP-activated protein kinase, and glucose have been found to act as more than merely mediators of molecular clock output; they also interact with TTFLs and contribute to the homeostasis and amplitude of cellular circadian oscillations (Rey & Reddy, 2013). Perhaps, the most thoroughly studied cytosolic oscillation in mammals is the rhythmic oscillations in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). In SCN neurons, $[Ca^{2+}]_i$ is high during the day and low during the night, a pattern that is driven—at least in part—by oscillations in electrical activity (Colwell, 2000; Irwin & Allen, 2007; but see Ikeda et al., 2003). Although Ca^{2+} entry through voltage-gated Ca^{2+} channels is required for maintaining the molecular function of the circadian clock (Lundkvist, Kwak, Davis, Tei, & Block, 2005), it may not be the primary source of endogenous Ca^{2+} rhythmicity. It is more likely that endogenous Ca^{2+} stores controlled by IP3Rs (inositol 1,4,5-trisphosphate receptors) and RyRs (ryanodine receptors), which were previously identified in SCN neurons (Diaz-Munoz et al., 1999), are responsible for the initial rise in $[Ca^{2+}]_i$ (Ikeda et al., 2003); however, the precise mechanism that underlies the circadian regulation of Ca^{2+} homeostasis has remained elusive. The discrepancies in data obtained from Ca^{2+} recordings may be due in part to the different techniques that were used in various studies. For example, the

synthetic ratiometric dye Fura-2 AM has been used successfully to measure $[Ca^{2+}]_i$, and recordings using this dye revealed an activity-dependent rhythmicity in $[Ca^{2+}]_i$ (Colwell, 2000). On the other hand, measurements using genetically encoded probes such as yellow cameleon 2.1 (Ikeda et al., 2003) revealed the presence of rhythmic $[Ca^{2+}]_i$ fluctuations despite blocking action potentials using tetrodotoxin (TTX). Differences in Ca^{2+} affinity, compartment specificity, and dynamics between the two types of probes may provide an explanation for this discrepancy (Whitaker, 2010). Ideally, combining new genetically encoded Ca^{2+} probes such as GCaMP3 (Brancaccio, Maywood, Chesham, Loudon, & Hastings, 2013; Tian et al., 2009) with a voltage-sensitive reporter (Cao et al., 2013) should help resolve this issue. Furthermore, measuring and controlling intracellular Ca^{2+} homeostasis will yield important insights into the link between $[Ca^{2+}]_i$ and the molecular clock (Harrisingh, Wu, Lnenicka, & Nitabach, 2007).

In addition to its central role in promoting the molecular clock mechanism, Ca^{2+} is also an intracellular signaling molecule, transducing the cell membrane's excitability state to the nucleus, as well as possibly modulating membrane properties by acting on cyclic nucleotide-gated channels. For example, depolarization via the light-induced release of glutamate from retinohypothamalic nerve terminals in the SCN increases $[Ca^{2+}]_i$, which is required for generating a phase shift in the circadian rhythm at night. This glutamate-induced phase shift in the SCN can be mimicked *in vitro* by applying glutamate agonists and requires the activation of voltage-gated Ca^{2+} channels (Kim et al., 2005).

2.3. Role of neuropeptides

The activity of SCN neurons can be modulated by several pathways, including endogenous pathways, retinal input, synaptic activity originating from other brain areas, and communication between neurons within the SCN. In the majority of synaptic inputs to SCN neurons, neuropeptides play a role as either co-transmitters or modulators. The glutamatergic synapse coreleases pituitary adenylyl cyclase-activating polypeptide (PACAP) to modulate postsynaptic currents and the level of Ca^{2+} influx (Michel, Itri, Han, Gniotczynski, & Colwell, 2006). Another neuropeptide often present in GABAergic synapses of SCN neurons is vasoactive intestinal polypeptide (VIP) (Castel & Morris, 2000), which enhances GABAergic transmission (Itri & Colwell, 2003). VIP also induces Ca^{2+} influx (Irwin & Allen, 2010) and causes a phase shift in both SCN rhythmicity and behavior.

Interestingly, the cyclic expression of clock genes is drastically reduced in SCN neurons of mice that lack either VIP (Loh et al., 2011) or its receptor (Maywood et al., 2006). This finding suggests that VIP promotes SCN communication and synchronization within the SCN, and it suggests that functional VIP signaling is required for the molecular circadian clock to achieve its full dynamic range. This result is in line with findings showing that PER2 rhythmicity is attenuated when action potentials are blocked (Yamaguchi et al., 2003), which could be attributed primarily to impaired VIP signaling during suppressed neuronal activity.

2.4. Circadian modulation of excitability

At the single-cell level, neuronal activity is intrinsically controlled by ion channels in the plasma membrane. In SCN neurons, the interplay between the activities of various ion channels results in daily oscillations in resting membrane potential (V_m) and action potential frequency. The V_m of SCN neurons is approximately 10 mV more depolarized during the day than during the night. This difference in V_m is due to a change in the conductance of TEA-sensitive K^+ channels; this conductance is higher during the night than during the day (Kuhlman & McMahon, 2004). The identity of the channels that modulate V_m has not been determined; however, likely candidates include the family of two-pore K^+ channels, of which *TASK1* and *TASK2* transcripts have been detected in the SCN (Panda et al., 2002). In addition, several ion channel types provide excitatory drive enabling spontaneous activity in SCN neurons. These channels include persistent Na^+ channels (Jackson, Yao, & Bean, 2004), L-type Ca^{2+} channels (Nahm, Farnell, Griffith, & Earnest, 2005; Pennartz, de Jeu, Bos, Schaap, & Geurtsen, 2002), and T-type Ca^{2+} channels (Akasu, Shoji, & Hasuo, 1993; Nahm et al., 2005). The frequency of these spontaneously generated action potentials is modulated by currents through fast-delayed rectifier K^+ channels (Itri, Michel, Vansteensel, Meijer, & Colwell, 2005; Kudo, Loh, Kuljis, Constance, & Colwell, 2011) and transient A-type K^+ channels (Alvado & Allen, 2008; Bouskila & Dudek, 1995; Itri et al., 2010). The circadian regulation of both fast-delayed rectifier K^+ channels and transient A-type K^+ channels is altered in the SCN of aged animals (Farajnia et al., 2012); thus, contributing to the clock phenotype observed in aging and suggesting that these channels play a stabilizing role in clock circuitry (Farajnia, Deboer, Rohling, Meijer, & Michel, 2014).

The molecular control of neuronal excitation has been investigated in mice that lack specific clock genes, including cryptochrome-1/2

double-knockout mice (Albus et al., 2002). Nevertheless, surprisingly little is known regarding the mechanisms by which the molecular clock affects the activity of ion channels (Colwell, 2011). It is possible that the expression and turnover of ion channels with a half-life of 2–12 h could translate to rhythmicity in membrane currents. Another potential factor could be the circadian control of ion channel trafficking (i.e., the insertion and removal of channels in the plasma membrane). However, posttranslational mechanisms that include kinases and phosphatases account for the majority of ion channel modulation in most neurons. Second messengers that can regulate ion channel activity include cGMP, cAMP, and Ca^{2+}, all of which exhibit circadian oscillations in clock neurons as described above (Colwell, 2000; Ko, Ko, & Dryer, 2001; O'Neill, Maywood, Chesham, Takahashi, & Hastings, 2008). A more thorough analysis of the circadian modulation of ion channels will require combining high-level electrophysiology with simultaneous recordings of second-messenger molecules and pathways. The role of clock genes and clock-controlled genes in modulating neuronal excitability should be investigated using various knockout mice, knockdown models, or siRNA approaches in combination with the aforementioned methods.

2.5. The road back: Neuronal activity impacts clock gene expression

The expression of clock genes can be drastically reduced by blocking action potentials using TTX (Webb, Angelo, Huettner, & Herzog, 2009; Yamaguchi et al., 2003) or by eliminating neuromodulator activity, for example in VIP receptor-deficient mice (Maywood et al., 2006). Manipulating the level of excitability in the clock neurons of *Drosophila* provided compelling evidence that a functional electrical rhythm is required for the rhythmic expression of *PER* and *timeless* (Nitabach, Blau, & Holmes, 2002). However, restricting silencing of clock neurons to adult stages disrupted circadian rhythms in behavior without affecting the molecular clock in *Drosophila* (Depetris-Chauvin et al., 2011). Once again, Ca^{2+} seems to play an important role in transducing neuronal activity to the nucleus (Lundkvist et al., 2005); moreover, it is possible that RyR-controlled intracellular Ca^{2+} stores—in concert with Ca^{2+}-activated BK channels—are part of this signaling pathway (Li et al., 2014). In mammals, the laboratory methods used to manipulate neuronal activity are still quite crude, and the TTX sensitivity of gene expression seems to be restricted to the neonatal SCN (Baba, Ono, Honma, & Honma, 2008) and may depend upon its effect on synaptic vesicle release as a consequence of blocking action potentials

(Deery et al., 2009). Optogenetic tools have been developed for use in the SCN (Sidor & McClung, 2014) and would provide better experimental control over the level and frequency of neuronal activity and the subsequent regulation of gene expression.

3. PART II: THE SCN AS A MULTI-OSCILLATOR

The SCN is a heterogeneous structure containing many cell-autonomous oscillators (Antle & Silver, 2005; Welsh et al., 2010). Functional coupling between these neurons is essential for obtaining a reliable timing signal. It is perhaps extraordinary that this heterogeneous network can generate rhythms with such high precision, with day-to-day deviations on the order of only a few minutes. This high precision is achieved only at the level of the neuronal network and is not present at the level of individual cells (Herzog et al., 2004; Honma, Nakamura, Shirakawa, & Honma, 2004), thus underscoring the importance of performing studies at the network level. Recordings from SCN-containing brain slices have contributed significantly to our understanding of the mechanisms that underlie SCN network organization. As illustrated below, the network organization and phase synchrony of SCN neurons determine the overall waveform of the SCN's electrical activity rhythm. These features also provide considerable plasticity (for example, in response to a change in day length) and are essential for day-to-day synchronization, as well as for the synchronization to a shift in the light cycle. Thus, plasticity within the neuronal network underlies specific functionally relevant physiological adaptations (for review, see Frenkel & Ceriani, 2011; Honma et al., 2012; Meijer, Michel, Vanderleest, & Rohling, 2010; Muraro, Pirez, & Ceriani, 2013; Welsh et al., 2010).

3.1. Using acute slice preparations to study phase resetting

Circadian rhythms in gene expression (Nakamura, Yamazaki, Takasu, Mishima, & Block, 2005; Yamazaki et al., 2000), neurotransmitter content and/or release (Shinohara, Honma, Katsuno, Abe, & Honma, 1998), electrical activity (Gillette et al., 1995), and $[Ca^{2+}]_i$ persist in *in vitro* preparations, including dissociated cells (Welsh, Logothetis, Meister, & Reppert, 1995), organotypic slice cultures (Herzog, Geusz, Khalsa, Straume, & Block, 1997; Nakamura, Honma, Shirakawa, & Honma, 2001), and acute hypothalamic slices (Gillette, 1986; Gillette et al., 1995; Kim & Dudek, 1991; Schaap et al., 2003).

The first recordings from the SCN were performed in acute brain slices by three independent research groups. All three groups found that when the SCN was cultured in isolation, it continued to oscillate with a period of approximately 24 h (Green & Gillette, 1982; Groos & Hendriks, 1982; Shibata, Oomura, Kita, & Hattori, 1982). Although these studies were originally designed to demonstrate the SCN's ability to generate endogenous circadian rhythms, this preparation provides a valuable opportunity to study the properties of the SCN network in much more detail than currently available *in vivo* methods.

Acute brain slices differ fundamentally from cultured slices. In acute brain slices, the recordings are performed within an hour of preparation; moreover, during preparation, the brain is submerged in ice-cold solution to minimize its energy requirements, thereby inhibiting physiological processes. This procedure does not interfere with the phase of the pacemaker; moreover, irrespective of the time of day in which the slices are prepared, the peak in electrical activity occurs at mid-day (Fig. 1; vanderLeest et al., 2009).

The important feature of recording from acute brain slices—also referred to as *ex vivo* recording—is that the consequences of prior changes in environmental conditions can be studied in the slice. After exposing an animal to a shift in the light–dark cycle, one can study the effect of this shift on the rhythmic pattern of the SCN network, and one can investigate the potential changes in regional organization in response to the shift. For example, Albus and colleagues observed that the ventral part of the SCN has an immediate phase shift in response to a shift in the light cycle (Albus, Vansteensel, Michel, Block, & Meijer, 2005). This finding may not be surprising, given that the light-receiving SCN neurons are located in the ventral SCN. This finding is also consistent with studies that used gene expression profiles as an output parameter (Nagano et al., 2003; Nakamura et al., 2005; Yan & Silver, 2004). When the slices are prepared 3 days after the light cycle is shifted, the SCN's phase dichotomy remains, and—more surprisingly—a common finding is that at day 3, the shifted population now shows an overshoot in the shift (i.e., the shift in the ventral SCN exceeds the shift in the external light cycle). However, when the slices are prepared 6 days after the light cycle was shifted, the SCN appears to have shifted completely. These findings are consistent with the time course of the behavioral response to a shift in the light cycle, and they indicate that at least some of the SCN properties measured *ex vivo* reflect actual changes that occurred *in vivo*.

The interpretation of these findings is that the shifted neurons are functionally dominant over the nonshifted neurons and therefore "pull" the

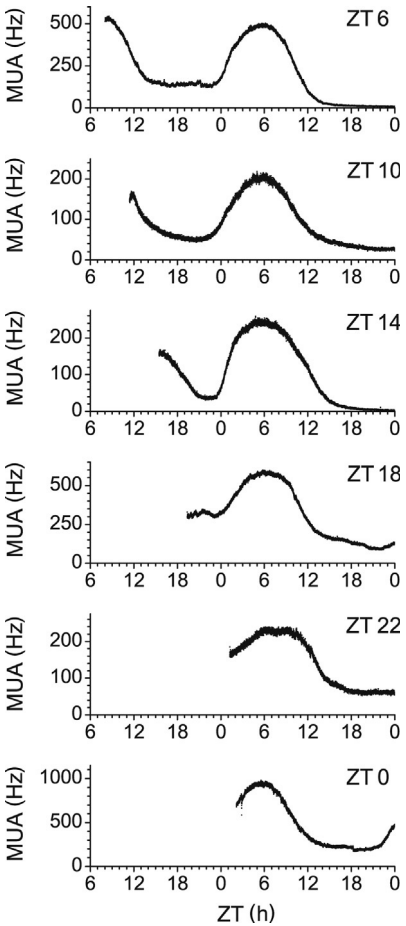

Figure 1 Stability of peak phase of electrical activity rhythm in the SCN slice preparation. Multiunit activity (MUA) rhythms measured in SCN slices prepared at ZT times (top to bottom): ZT 6, ZT 10, ZT 14, ZT 18, ZT 22, and ZT 0. ZT, Zeitgeber time with ZT 0 defined as the time of lights off. *Reproduced with permission from vanderLeest, Vansteensel, Duindam, Michel, and Meijer (2009).*

nonshifted neurons to the new phase. Why are these neurons dominant, given that they are fewer in number than the nonshifted neurons? We recently found that the shifted population is highly synchronized in phase (Rohling, vanderLeest, Michel, Vansteensel, & Meijer, 2011). When neurons are highly synchronized, they exert a much stronger effect on other populations. Additionally, the flow of information from the ventral (i.e., shifted) neurons to the dorsal (i.e., nonshifted) neurons is stronger than

the flow of information from the dorsal SCN to the ventral SCN. Evidence for this asymmetrical flow of information was obtained from tracing studies (Han et al., 2012; Leak, Card, & Moore, 1999; Moore, Speh, & Leak, 2002; Romijn, Sluiter, Pool, Wortel, & Buijs, 1997). Finally, ventral GABAergic neurons exert both inhibitory and excitatory effects on the dorsal SCN, whereas the GABAergic input from the dorsal SCN to the ventral SCN is predominantly inhibitory (Albus et al., 2005). The surprising excitatory action of GABA in the dorsal SCN is explained by the activity of the NKCC1 chloride cotransporter (Belenky et al., 2010; Belenky, Yarom, & Pickard, 2008; Choi et al., 2008). Changing the activity of NKCC1 can cause GABAergic input to switch from its classic inhibitory role to exerting an excitatory effect (Kim et al., 2011). We expect that excitatory action (existing in dorsal direction) may have larger phase-shifting capacity than inhibitory action (in ventral direction). Indeed, light's strong phase-shifting effects on the SCN are mediated by excitatory glutamatergic fibers, whereas dark pulse mediated inhibition (Ellis, McKlveen, & Turek, 1982; Meijer, Daan, Overkamp, & Hermann, 1990) or direct hyperpolarization of clock cells (Block, Khalsa, McMahon, Michel, & Geusz, 1993) often have smaller phase-shifting capacity. Nevertheless, the extent to which GABA contributes to the interaction between the dorsal SCN and ventral SCN remains unclear (Albus et al., 2005; Han et al., 2012), as does the role of other transmitters, e.g., AVP, in this process (Maywood, Chesham, O'Brien, & Hastings, 2011).

3.2. Using acute slices to study photoperiod-encoding mechanisms

The effects of shifting the photoperiod are clearly preserved in acute slices. This finding was first reported by Schwartz and colleagues (Mrugala, Zlomanczuk, Jagota, & Schwartz, 2000), who exposed hamsters to a short (LD 8:16), equinoctial (LD 12:12), or long (LD 16:8) photoperiod. They found that the SCN's pattern of electrical activity is strongly dependent on photoperiod. When entrained to a short photoperiod, the ensemble activity pattern is compressed; when entrained to a long photoperiod, the pattern is decompressed. Their finding that the photoperiod's influence on the SCN is preserved in *in vitro* slices was important, as it enabled researchers to perform a detailed analysis of the changes in SCN electrical activity that occur at the subpopulation and single-unit levels, studies that are not currently possible at the *in vivo* level.

Single-unit recordings revealed that the distribution of single-cell activity patterns differs considerably between long and short photoperiods. The electrical activity of individual SCN cells has a 24-h rhythm, and this pattern is not sinusoidal; instead, the cells are active for up to 5–6 h each cycle (Schaap et al., 2003). Thus, the activity pattern of the entire SCN population does not mirror the activity pattern of the individual cells (Fig. 2A). When the animal is exposed to a short photoperiod, the phase relationship between the SCN neurons is more tight, leading to a narrower activity peak at the population level (Fig. 2B). This narrow peak reflects the short duration of the photoperiod. In contrast, when the animal is exposed to a long photoperiod (Fig. 2C), the cells become more desynchronized (i.e., more out of phase), resulting in a broader activity peak at the population level (Brown & Piggins, 2009; Naito, Watanabe, Tei, Yoshimura, & Ebihara, 2008; VanderLeest et al., 2007). Long-term recordings of individual neurons require extracellular recording, as intracellular recordings can distort the

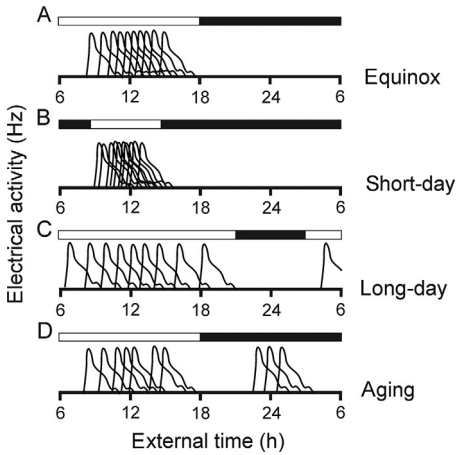

Figure 2 Influence of day length and age on phase distribution of electrical activity patterns of single SCN neurons (schematic). (A) In light regimes with 12-h light and 12-h darkness, SCN neuron activity pattern cluster around mid-day. (B) In short days (8-h light–16-h dark), phase differences between SCN neurons are small, resulting in a narrow peak at the ensemble level in the middle of the day. (C) In long days (16-h dark–8-h light), these phase differences are large, resulting in a broad peak at the ensemble level. An increase in phase dispersal causes a broader peak and reduces the rhythm's amplitude. (D) In aging (LD 12:12), the phase distribution changes in a characteristic way, and a subpopulation of neurons appears to become active in antiphase to the main population, both leading to a decrease in the rhythm's amplitude. Time on *x*-axis is expressed as "external time" (ExT) to be able to compare between different photoperiods. ExT 12 h is defined as the middle of the day and ExT 24 h defining the middle of the night.

circadian activity rhythm (Schaap et al., 1999). To overcome this limitation, Brown and colleagues developed a method to increase the effectiveness of single-cell recordings using a stationary glass suction electrode (Brown, Banks, & Piggins, 2006). Despite small difference in their recording techniques, the aforementioned studies yielded similar results, and all studies suggest that desynchronization is increased under long photoperiods.

3.3. Using acute slices to study synchronization within the SCN

Long-term exposure to continuous light causes a loss of rhythmicity and can split the unimodal circadian behavioral activity band into two activity bands that are separated by approximately 12 h (Pittendrigh & Daan, 1976). This antiphase/phase relationship is a state that is often observed in situations in which synchronization is suboptimal. The two behavioral activity bands are stably coupled in antiphase, suggesting that they arise from cell populations that interact. An *ex vivo* analysis of slices from hamsters with split activity rhythms revealed an antiphase relationship between the left and right SCN (Jagota, de la Iglesia, & Schwartz, 2000). The two sides of the SCN are connected and are therefore functionally coupled. Coupling between the left and right SCN has been studied by applying electrical stimulation to one side while recording the electrical responses in the other side. Patch-clamp recordings of the responsive neurons revealed an excitatory response with sufficient magnitude to elicit action potentials. Approximately 30% of SCN neurons respond to contralateral stimulation. Because the responses are often—but not always—blocked by the AMPA receptor antagonist CNQX, the connection between the left and right SCN is at least partially glutamatergic (Michel et al., 2013).

Acute slices prepared from arrhythmic animals that were exposed to continuous light revealed a strong—and in some cases, complete—desynchronization of single-cell activity patterns (Ohta, Yamazaki, & McMahon, 2005). While the individual cells of the SCN are robustly rhythmic in Per1::GFP expression, the near-complete asynchrony among these cells leads to an absence of rhythmicity at the population level (Coomans et al., 2013; Ohta et al., 2005).

Aged animals exhibit evidence of a decrease in synchrony within their SCN (Farajnia et al., 2012). For example, slices prepared from 2-year-old mice have a wide phase distribution among their neurons. In the SCN neurons of aged animals, a characteristic bimodal distribution emerges (Fig. 2D). The two antiphase populations have dissimilar magnitudes—the majority of

neurons are active during the day, whereas a smaller yet distinctive group of neurons is active during the night. Note that this antiphase/phase relationship is typical of a decrease in interneuronal coupling. This decrease in coupling among neurons within an aged network is consistent with a decrease in neurotransmitter function within the SCN, including GABA (Farajnia et al., 2012; Palomba et al., 2008) and VIP (Duncan, Herron, & Hill, 2001; Zhou, Hofman, & Swaab, 1995), both of which are believed to play a major role in coupling within the SCN (Albus et al., 2005; Colwell et al., 2003; Liu & Reppert, 2000; Maywood et al., 2006).

3.4. Rescue of single-cell deficiencies by the neuronal network

The presence of networks gives rise to robustness in the system. For example, genetic perturbations have less effect on the SCN network than on single cells (Liu et al., 2007). In the aged SCN, relatively isolated cells have a more severely distorted pattern of circadian activity (Farajnia et al., 2012). This finding was evident from cells that were recorded near the surface of the slice, where the network is highly disrupted and only a few connections are preserved. On the other hand, when cells were recorded from the middle of the slice (where the network is relatively intact), the cells showed a robust rhythm, despite a decrease in overall amplitude. These results suggest that the presence of the network can compensate for the loss of robust single-cell oscillators. In theory, this compensation can be achieved in one of the following ways: (i) a small group of cells has not deteriorated and can "boost" the remaining cells in the population, or (ii) a large group of weak oscillators will together, as a result of their communication (e.g., resonance) and acquire a higher amplitude rhythm. Indeed, individually weak oscillators can produce robust rhythms when they are combined to form a larger network (Ko et al., 2010).

The enhanced stability of the network was demonstrated elegantly by Buhr, Yoo, and Takahashi (2010), who showed that heat-shock pulses cause a larger phase shift in SCN slices that are severed (i.e., the dorsal SCN is separated from the ventral SCN) compared to intact slices (Herzog & Huckfeldt, 2003; Ruby, Burns, & Heller, 1999).

4. PART III: *IN VIVO* ELECTROPHYSIOLOGY RECORDINGS FROM THE SCN IN ANESTHETIZED AND FREELY MOVING ANIMALS

The properties of a circadian system are critically dependent upon interactions between the SCN and other structures. A clear example of this

dependence is the phenomenon that photic entrainment—one of the circadian system's primary attributes—is entirely dependent upon the flow of information from intrinsically photosensitive retinal ganglion cells (ipRGCs) to the SCN. Thus, *in vivo* recording is currently the best system for studying the SCN as a component of a larger network with intact connections (i.e., inputs and outputs) with other brain nuclei.

In vivo recordings from the SCN were first performed in freely moving rats (Inouye & Kawamura, 1979). The SCN showed clear circadian patterns in electrical activity—specifically, the frequency of electrical impulses was high during the day and low during the night. This rhythm was preserved when an incision was made around the SCN, suggesting that the rhythm was not dependent upon input from other brain areas (although other rhythms such as humoral input and/or temperature could not be excluded). When the SCN was surgically isolated, the animal lost its behavioral activity rhythm. This study provided the first compelling evidence that the SCN is both an autonomous oscillator (i.e., a structure that can generate its own circadian rhythm) and a pacemaker (i.e., a structure that can impose its rhythm upon other structures), and it provided strong support of conclusions drawn from lesion studies performed in 1972 (Moore & Eichler, 1972; Stephan & Zucker, 1972). A remaining question is whether humoral and/or electrical factors are required to transmit information from the SCN; in this respect, several factors have been suggested to play a role in the transmission of information to non-SCN areas; these factors include prokineticin-2 (Cheng et al., 2002), TGF-alpha (Kramer et al., 2001), cardiotrophin-like cytokine (Kraves & Weitz, 2006), and glutamate (Sun, Whitefield, Rusak, & Semba, 2001). Chronic infusion of TTX causes arrhythmic activity patterns (Schwartz, Gross, & Morton, 1987); however, because the release of humoral factors can also depend upon electrical activity, electrical activity clearly plays a key role. Indeed, the results obtained from introducing an incision around the SCN support the hypothesis that electrical output is important, as humoral factors would diffuse readily across the incision. On the other hand, implanting an encapsulated SCN can restore rhythms in animals that were previously rendered arrhythmic by an SCN lesion (Silver, LeSauter, Tresco, & Lehman, 1996). This issue certainly warrants further study in order to determine the role of electrical input and/or humoral factors in SCN output.

Simultaneous recordings of SCN electrical activity and behavioral activity indicate that the transition from rest to activity—and vice versa—occurs when the SCN has reached half-maximum levels in electrical activity

(Houben, Deboer, van Oosterhout, & Meijer, 2009). The transition can be described by a probability function with a maximum probability for a transition at the 50% level (Houben et al., 2009). Within the active period, the level of electrical activity contributes to the activity pattern. In nocturnal animals the most intense levels of behavioral activity are observed during the trough of the electrical activity rhythm, and acute suppression of SCN electrical activity by TTX acutely induces behavioral activity. These results indicate that the SCN's electrical activity level determines the behavioral activity pattern both at circadian level and at the ultradian time scale (Houben, Coomans, & Meijer, 2014; Hu, Scheer, Ivanov, Buijs, & Shea, 2007).

4.1. Example 1: *In vivo* electrophysiology studies of photic entrainment

Studying the effect of light on the SCN requires *in vivo* recordings in which the circuitry between the retina and SCN is preserved. The retina projects both directly to the SCN via fibers that contain glutamate and PACAP, and indirectly via the intergeniculate leaflet (for review, see Morin & Allen, 2006). To perform *in vivo* recordings in anesthetized animals, the anesthetic of choice is usually urethane, as it has minimal effects on the hypothalamus. The advantage of recording from anesthetized animals is that it enables the researcher to record from individual neurons. Single-unit recordings in anesthetized hamsters, rats, and mice revealed that approximately 25% of SCN neurons respond acutely to retinal stimulation (Brown, Wynne, Piggins, & Lucas, 2011; Cui & Dyball, 1996; Groos & Mason, 1982; Meijer, Groos, & Rusak, 1986; Nakamura, Fujimura, Ebihara, & Shinohara, 2004). The majority of light-responsive SCN neurons are activated tonically by light, whereas a minority of cells is light suppressed. At the end of the experiment, small microelectrolytic lesions can be made by passing weak electrical current through the electrode. Light-responsive neurons appear to be located in the retino-receptive area of the SCN, suggesting that these neurons are driven directly by retinal ganglion cells.

Light responses have also been measured in freely moving animals (Meijer, Watanabe, Schaap, Albus, & Detari, 1998; van Diepen, Ramkisoensing, Peirson, Foster, & Meijer, 2013; van Oosterhout, Fisher, et al., 2012). Such recordings are usually performed using stationary electrodes that typically record from a population of neurons. Results obtained from recording freely moving animals are consistent with results obtained using anesthetized animals; specifically, the SCN has the remarkable ability to respond tonically (without adaptation) to light (Meijer et al., 1998). In addition, *in vivo* recordings have enabled researchers to measure light

responses over several circadian cycles; such recordings revealed that light sensitivity is greater during the night than during the day. However, a light response can be observed during the day if sufficient light intensity is used; but despite this, these responses do not induce a phase shift. Thus, the acute light response in the SCN is clearly necessary—but not sufficient— to induce a phase shift.

The use of retina-specific transgenic animals facilitated the identification of the retinal photopigments that signal to the SCN. For example, melanopsin is considered to play a major role in detecting irradiance. Melanopsin-deficient ($Opn4^{-/-}$) animals have a decreased phase shift in response to light (Panda et al., 2003; Ruby et al., 2002). Recordings from the SCN of $Opn4^{-/-}$ animals revealed a surprisingly large response to ultraviolet, blue, and green wavelengths. Whether these effects are mediated by rods or cones is not clear; however, some evidence suggests that both rods and cones can transmit light information to the SCN (van Diepen et al., 2013; van Oosterhout, Lucassen, et al., 2012), while other studies indicate that rods are most important for entrainment (Altimus et al., 2010).

4.2. Example 2: *In vivo* studies indicate influence of behavioral activity and sleep stage on the SCN

Simultaneous recordings of SCN electrical activity and behavioral activity of rats, hamsters, and mice: such recordings revealed that behavioral activity and arousal state have a direct impact on the level of electrical activity in the SCN (Deboer, Vansteensel, Detari, & Meijer, 2003; Meijer, Schaap, Watanabe, & Albus, 1997; Schaap & Meijer, 2001; van Oosterhout, Lucassen, et al., 2012; Yamazaki, Kerbeshian, Hocker, Block, & Menaker, 1998). Episodes of increased behavioral activity—assessed using passive infrared recording, measuring wheel-running activity, or analyzing video recordings of the animals—are correlated with an acute suppression of electrical activity within the SCN (Fig. 3). The suppressed activity lasts for the entire duration of the behavioral activity, and after behavioral activity returns to baseline, the electrical activity gradually returns to baseline levels, albeit with a surprisingly long time course (with a time constant on the order of 600 s). This suppressed electrical activity in the SCN can occur both during the night and during the day. Video recordings revealed that types of activity that involve high-intensity levels of behavior (e.g., walking, digging, running, etc.) cause a larger suppression than low-intensity behaviors such as eating and drinking (van Oosterhout, Lucassen, et al., 2012). Even extremely brief periods of behavioral activity can suppress electrical activity in the SCN.

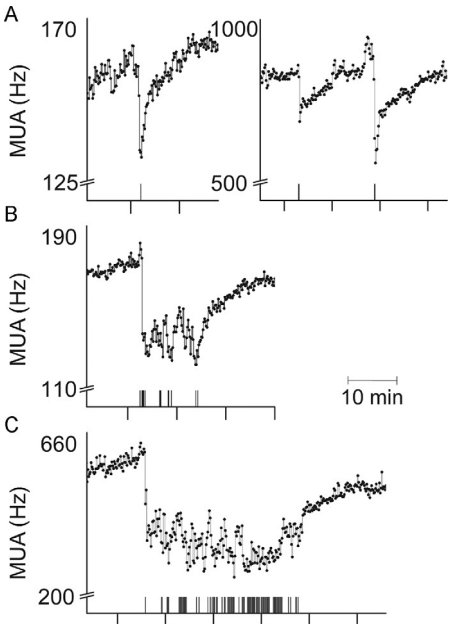

Figure 3 Three examples showing suppression of SCN multiunit activity (MUA) in response to increased behavioral activity. MUA is plotted as a function of time (time bar indicated in the figure). Behavioral activity is plotted as bars on top of the *x*-axis. The simultaneous recording of MUA and behavioral activity allowed detection of the response of the SCN to behavioral activity. (A) Even a brief bout of behavioral activity can cause a significant suppression of electrical activity. (B) Longer periods of behavioral activity prolong suppression of SCN activity. (C) Suppression is sustained also for longer periods of activity. *Reproduced from van Oosterhout, Lucassen, et al. (2012) in accordance with the Creative Commons Attribution License.*

Interestingly, increased behavioral activity does not suppress all neurons in the SCN. In hamsters, many neurons were suppressed (Yamazaki et al., 1998), whereas only 30% and 20% of neurons were suppressed in mice (van Oosterhout, Lucassen, et al., 2012) and rats (Schaap & Meijer, 2001), respectively.

This suppression of SCN activity by behavioral activity has consequences on the function of the SCN. If an animal is active during the active phase, suppression will cause a further decrease in electrical activity in the animal's SCN. Hence, any behavioral activity that is controlled by the SCN will in turn affect the SCN, thereby increasing its amplitude. The SCN is therefore subject to input from other networks and should be regarded as a team player within a larger network (van Oosterhout, Lucassen, et al., 2012).

Simultaneous recordings of EEG and SCN electrical activity revealed that an animal's arousal state can also affect SCN activity. During non-REM sleep, the SCN's electrical activity is reduced; in contrast, during REM sleep, the SCN's electrical activity increases (Deboer et al., 2003). Sleep deprivation can have lasting effects on the SCN, decreasing the amplitude of electrical activity (Deboer, Detari, & Meijer, 2007). The feedback mechanisms by which sleep affects the SCN are not entirely understood. An interesting consequence of this feedback to the SCN is that variability in the electrical activity rhythm is strongly enhanced *in vivo* (Fig. 4A). Thus, although the *in vitro* recordings revealed a clear circadian pattern with extremely low variability, the *in vivo* recordings revealed more complexity and are characterized by high frequency fluctuations that are superimposed on the circadian waveform (Meijer et al., 1997).

These higher frequency fluctuations can be interpreted as noise (i.e., decreasing the reliability of the central pacemaker) or they can be interpreted as a functional modulation of the system. When the electrical activity data are analyzed as a time series, evidence emerges to support the second interpretation. Healthy systems show a proportional increase in variability (scale-free behavior) when analyzed over different time domains, as revealed by detrended fluctuation analysis. If one performs such analysis on the discharge patterns that were obtained *in vitro*, it becomes apparent that *in vitro* data lack scale-free behavior and that the circadian component in the signal is over-represented (Fig. 4B). In contrast, the temporal structure of the data obtained by *in vivo* recordings is scale free (Hu et al., 2012). These data support the notion that the seemingly noisy *in vivo* data, in fact, reflect a healthy system with appropriate fluctuations in all time domains. Fluctuations in all time domains may help the animal adapt and respond to distortions that occur in various time domains. This result is consistent with the interpretation that the SCN controls behavioral activity on a circadian time scale as well as on an ultradian time scale (Houben et al., 2014; Hu et al., 2007).

5. CONCLUSIONS

In this chapter, we described several methodologies that are suitable for performing a neurophysiological analysis of the circadian system at multiple levels. It is important to take all levels into account as higher levels of organization contribute substantially to the basic properties of the SCN. While rudimentary oscillators are obtained at the single-cell level, robustness, precision, entrainment, and adaptations to the changing seasons are

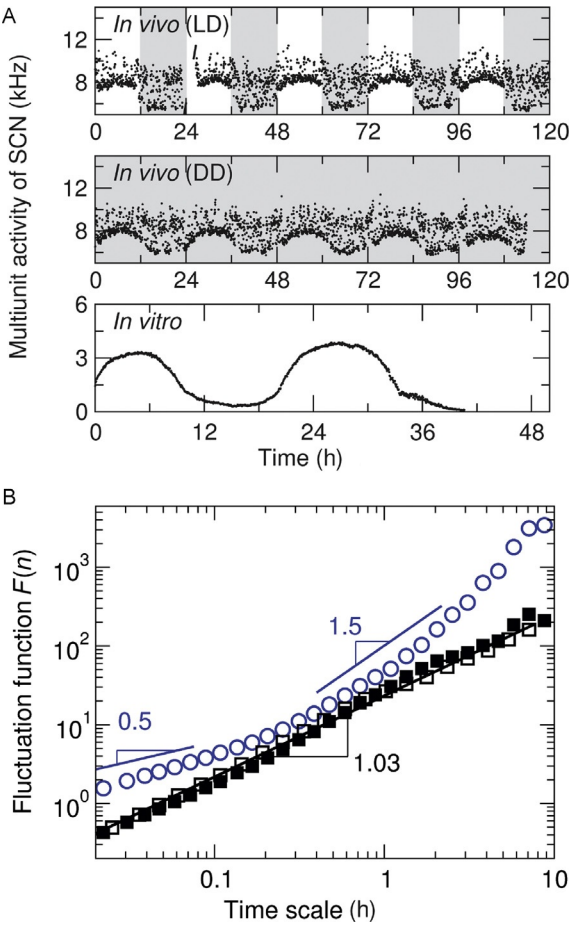

Figure 4 Variability and scale invariance in multiunit activity of SCN neurons. (A) Upper two panels: *In vivo* recordings of the rat SCN in a light–dark cycle (LD) and in continuous darkness (DD). Under both conditions, variability is superimposed on the circadian waveform. Lower panel: *in vitro*, when the SCN is isolated from other brain areas, the electrical activity rhythm lacks fluctuations in the shorter time domain, and only the circadian component remains. (B) SCN electrical activity has scale-invariant properties *in vivo* but not *in vitro*. Scale invariance is reflected by a slope of 1.0 on a log–log plot of the fluctuations as a function of time. Open circles reflect *in vitro* recordings ($N = 6$) and indicate complete loss of scale invariance. Open squares: *in vivo* recordings in LD ($N = 7$) and closed squares: *in vivo* recordings in DD ($N = 7$) both show a slope close to 1.0. *Reproduced from Hu et al. (2012) in accordance with the Creative Commons Attribution License.*

all examples of properties that arise at higher levels of organization. The continued development of methodologies in the fields of neuroscience and computational biology will help further reveal the basic principles underlying this remarkable timing system.

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