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From clock to functional pacemaker

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Abstract

In mammals, the central pacemaker that coordinates 24-hr rhythms is located in the suprachiasmatic nucleus (SCN). Individual neurons of the SCN have a molecular basis for rhythm generation and hence, they function as cell autonomous oscillators. Communication and synchronization among these neurons are crucial for obtaining a coherent rhythm at the population level, that can serve as a pace making signal for brain and body. Hence, the ability of single SCN neurons to produce circadian rhythms is equally important as the ability of these neurons to synchronize one another, to obtain a bona fide pacemaker at the SCN tissue level. In this chapter we will discuss the mechanisms underlying synchronization, and plasticity herein, which allows adaptation to changes in day length. Furthermore, we will discuss deterioration in synchronization among SCN neurons in aging, and gain in synchronization by voluntary physical activity or exercise.

KEYWORDS

in vivo electrophysiology, networks, photoperiod, SCN, synchronization

1 | INTRODUCTION

The identification of the suprachiasmatic nucleus (SCN) as a discrete site comprising the master circadian pacemaker in the mammalian brain was a landmark discovery for contemporary circadian research in mammalian species (Moore & Eichler, 1972; Stephan & Zucker, 1972). In the SCN, circadian rhythmicity is generated at the single-cell level and isolated SCN neurons maintain their intrinsic rhythm (Welsh, Takahashi, & Kay, 2010). Generation of circadian rhythmicity is based on an intertwined negative feedback loop between "clock" genes and their protein products (Buhr & Takahashi, 2013; Hardin, Hall, & Rosbash, 1990; Schibler, 2007; Vosshall, Price, Sehgal, Saez, & Young, 1994; Zehring et al., 1984;). Thus, each SCN neuron functions as an autonomous single-cell oscillator, and the SCN network functions as a multi-oscillator system.

SCN cellular communication and synchronization are crucial in achieving robust rhythmicity at the SCN network level (Enoki, Ono, Kuroda, Honma, & Honma, 2017b; Enoki et al., 2017a; Herzog, Geusz, Khalsa, Straume, & Block, 1997; Herzog, Takahashi, & Block, 1998; Liu, Weaver, Strogatz, & Reppert, 1997; Mieda et al., 2015; Welsh, Logothetis, Meister, & Reppert, 1995). When multiple neurons are electrically active at about the same time of day (i.e., "synchronized"), the SCN's ensemble rhythm is strong; in contrast, if the neurons are not active at the same time (i.e., "desynchronized"), the

Abbreviations: E/I balance, excitatory/inhibitory balance; GABA, Gamma-Aminobutyric Acid; LD, light-dark; Opn4, melanopsin; pRGC, photosensitive retinal ganglion cell; SCN, suprachiasmatic nucleus; TTX, Tetrodotoxin; VIP, vasoactive intestinal peptide; VPAC2, Vasoactive intestinal peptide receptor 2.

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SCN's ensemble rhythm becomes weak or is lost, despite the presence of functional single cell oscillators. A weak ensemble rhythm exercises little control over bodily functions and results in poor rhythmicity at the organismal level. Thus, to obtain a recognizable rhythmic output, not only requires oscillatory behavior of individual neurons, but also sufficient synchronization amongst these neurons. Lack of either of these attributes leads to an absence of rhythmicity at the ensemble level of the SCN and to complete loss of pacemaker function (Figure 1).

In this chapter, we distinguish between "oscillator", "clock" and "pacemaker" (Meijer & Rietveld, 1989). We define an oscillator as a structure that is autonomously able to produce rhythms. Early evidence that the SCN functions as an oscillator come from in vitro studies, showing that in a continuous environment, the SCN neurons maintain to oscillate (Green & Gillette, 1982; Groos & Hendriks,1982), even when they were completely isolated (Welsh et al., 1995). A pacemaker is defined as a structure that has the ability to drive other structures rhythmic. Elegant proof for the pacemaker function of the SCN comes from transplantation studies, first developed in the Silver lab (Lehman et al., 1987), and later used to transplant SCN from animals with short or long freerunning rhythms to SCN ablated animals, resulting in full restoration of the transplanted properties (Ralph & Menaker, 1988). The term "clock" is loosely defined and often used. In fact, a clock is a chronometer, and is able to provide the actual time. In circadian research, this would be close to an entrained oscillator.

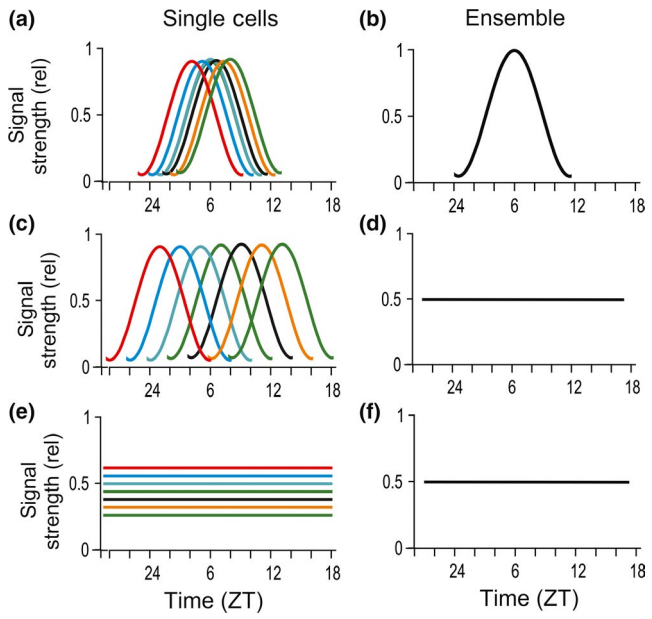

FIGURE 1 Based on molecular feedback loops, individual cells are autonomous oscillators. The cellular oscillations are a prerequisite to obtain rhythmicity at the ensemble level (b), as is the synchrony among the cellular oscillations (a). An absence in either of them (c, e) will result in lack of rhythmicity at the tissue level (d, f)

2 | NEURONAL SYNCHRONIZATION: MEASURING DAYLENGTH AND COPING WITH AGING

The SCN neuronal network gains important additional attributes through the plasticity within the network, such as precision (Herzog, Aton, Numano, Sakaki, & Tei, 2004; Shirakawa, Honma, Katsuno, Oguchi, & Honma, 2000), robustness (vanderLeest, Rohling, Michel, & Meijer, 2009) and also the ability to code for daylength (VanderLeest et al., 2007). Electrophysiological studies have shown that a clustering of the phases of single cell oscillations (synchronization) leads to a narrow peak of the rhythm of the whole SCN network, representing short days, while dispersal of activity patterns of SCN neurons (desynchronization) leads to a broader ensemble peak, representing long days (Coomans, Ramkisoensing, & Meijer, 2015; Ramkisoensing & Meijer, 2015). The electrical activity patterns of single SCN neurons are less affected by photoperiod (VanderLeest et al., 2007) and the single-cell waveform changes reported are not sufficient to explain adaptation of the ensemble output to day-length (Brown & Piggins, 2009). Similarly studies of clock gene expression show a change in the distribution of peak expression in response to different photoperiods (Buijink et al., 2016; Naito, Watanabe, Tei, Yoshimura, & Ebihara, 2008), but also reveal spatial differences between dorsal and ventral (Evans, Leise, Castanon-Cervantes, & Davidson, 2013), and between caudal and rostral SCN (Hazlerigg, Ebling, & Johnston, 2005; Inagaki, Honma, Ono, Tanahashi, & Honma, 2007). Thus, while the rhythm generating properties arise in individual cells, seasonal encoding depends on the integrity and plasticity of the SCN network. The phase distribution among SCN neurons governs the seasonal dependent changes in the duration of activity and rest (Houben, Deboer, van Oosterhout, & Meijer, 2009; VanderLeest et al., 2007). Of note, seasonal changes in physiology such as gonadal function and prolactin surge, require modulation of pineal melatonin and hypothalamic estrogen receptor stimulation respectively, the latter by direct output of the SCN (Coomans et al., 2015).

In nocturnal rodents, multiple signaling processes (e.g., VIP, GABA, gap junctions) affect the synchronization of SCN neurons. GABA is expressed in almost all SCN neurons, but its effect on the network is still under debate (Ono, Honma, Yanagawa, Yamanaka, & Honma, 2018) and has been found to be synchronizing (Liu & Reppert, 2000) as well as destabilizing (Freeman, Krock, Aton, Thaben, & Herzog, 2013) the neuronal network of the SCN. However, adaptation of the SCN rhythm to a new phase of an abruptly shifted light regime requires GABAergic communication between the dorsal (shell) and ventral (core) part of the SCN (Albus, Vansteensel, Michel, Block, & Meijer, 2005). Interestingly,

this coupling of shell and core SCN involves both inhibitory plus excitatory action of GABA, the latter being strongest during the night (Albus et al., 2005; Choi et al., 2008). The ability to act both inhibitory and excitatory as the predominant neurotransmitter in the SCN suggests that GABA contributes to the excitatory to inhibitory (E/I) balance of the SCN network. The E/I balance in neuronal networks is a fundamental feature that determines many network properties including synchrony. The photoperiod-induced state of synchrony in the SCN is indeed correlated by a change in E/I balance (Farajnia, van Westering, Meijer, & Michel, 2014b), but the causal link needs to be confirmed. The daily variations in E/I balance are not restricted to SCN and are also reported in humans (Chellappa et al., 2016).

VIP promotes phase synchronization by acting through the VPAC2 receptor (Aton, Colwell, Harmar, Waschek, & Herzog, 2005; Colwell et al., 2003; Harmar et al., 2002; Hastings, Brancaccio, & Maywood, 2014). The strong indications for VIP as a synchronizer of SCN neurons raises the question of whether VIP is involved in photoperiodic encoding by the SCN. The SCN activity of VIP-deficient animals measured in light-dark regimes of 12:12 (equinox), 16:8 (long day) or 8:16 (short day) is similar to WT littermates (Lucassen et al., 2012). However, upon release into constant darkness, the rhythm of the VIP-deficient SCN deteriorates almost immediately and is of poor quality, consistent with recordings in SCN slices (where photic information is also absent). The total loss of photoperiodic memory in constant darkness shows that VIP is indispensable for encoding day length information. Interestingly, a recent report shows that the ability of VIP to synchronize the SCN network depends on mTOR signaling (Liu et al., 2018) and the latter is also implied as a key complex involved in the effect of aging on clock function (Orozco-Solis & Sassone-Corsi, 2014).

2.1 | Desynchrony in aging: Pathology or adaptation?

Age related changes in the circadian system often lead to disruptions of the sleep-wake pattern (Hofman & Swaab, 2006), difficulties synchronizing the endogenous clock to a change in the light-dark cycle (Biello, 2009; Froy, 2011; Turek, Penev, Zhang, van Reeth, & Zee, 1995; Valentinuzzi, Scarbrough, Takahashi, & Turek, 1997) or adjusting to shift-work schedules (Härmä, Waterhouse, Minors, & Knauth, 1994). These debilitating conditions can often cause personal and social distress. In the US, 40%–70% of the elderly population suffers from chronic sleep disturbances with 1.5 times higher prevalence when living in long-term care facilities and occurs 2.5 times more often in women (Byles, Mishra, & Harris, 2005; Kamel & Gammack, 2006) for review (Van Someren, 2000). Sleep disturbances in the elderly include long latencies falling asleep, difficulties in maintaining sleep, and early waking times. In addition studies suggest that dysfunctional circadian clocks promote age-related decline in brain function as well as neurodegenerative diseases (Hood & Amir, 2017). One hallmark of an old clock is a weakening of the central timing signal generated by the SCN (Farajnia, Deboer, Rohling, Meijer, & Michel, 2014a; Oster, Baeriswyl, Van Der Horst, & Albrecht, 2003; Satinoff et al., 1993). Identification of the parts of the biological clockwork affected by aging is therefore prerequisite for development of intervention strategies.

The aged SCN shows attenuated amplitude of circadian oscillations in electrical activity (Nakamura et al., 2011) and neurotransmitters, e.g., vasoactive intestinal peptide (VIP), GABA and vasopressin (Cayetanot, Bentivoglio, & Aujard, 2005; Hofman & Swaab, 2006; Nygard, Hill, Wikstrom, & Kristensson, 2005; Palomba et al., 2008; Roozendaal, van Gool, Swaab, Hoogendijk, & Mirmiran, 1987). This attenuated signal may not be sufficient to control the phase of circadian rhythms in other brain areas and in organs like liver, kidney and intestine. While it is possible that this reduction in output signal of the SCN is caused by a lack of synchronization of pacemaker neurons within the SCN (Farajnia et al., 2012; Watanabe, Shibata, & Watanabe, 1995), there is also evidence that the physiology of the SCN neuron itself is impacted (Aujard, Herzog, & Block, 2001; Farajnia et al., 2012).

Ex vivo recordings of population activity and also of subpopulation and single cell activity in SCN of old mice revealed two clusters of neurons, one active during the day and another one in antiphase during the night (Farajnia et al., 2012). SCN activity in young control SCN only clustered around the middle of the day. It is noteworthy that aging does not lead to random temporal distribution of neuronal activity in the SCN, but seems to resemble some re-organization which still generates a rhythmic pattern of neuronal activity albeit with reduced amplitude. The idea that aging does not lead to a total loss of function and temporal chaos, but to re-organization of gene expression patterns and rhythms in cell metabolism is gaining support from recent studies (Chen et al., 2016; Sato et al., 2017).

Aging is clearly associated with alterations at the network level, in the sense that the antiphase oscillations diminish SCN amplitude. However, the very presence of a residual, but sufficiently synchronized, network can partly rescue age-related deterioration occurring at the single cell level (Farajnia et al., 2014a). Among these cellular deficits are changes in the circadian regulation of ion channel function and of Ca^{2+} homeostasis (Farajnia, Meijer, & Michel, 2015) will not only affect neuronal communication, but also the basic molecular mechanisms of rhythm generation (Lundkvist, Kwak, Davis, Tei, & Block, 2005), potentially leading to a downward spiral in function. This is in line with studies on aging neurons in other brain areas often showing not only reduced synaptic

density and efficiency (Burke & Barnes, 2010), but also exhibiting changes in Ca^{2+} homeostasis and Ca^{2+} signaling (Toescu, Verkhratsky, & Landfield, 2004). The latter seem to form a common link in cellular dysfunctions underlying several neurological diseases like bipolar disorders, schizophrenia, and Alzheimer's disease (Berridge, 2012). Therefore, understanding the cellular mechanisms of aging-associated clock disorders will allow targeting and restoration of circadian dysfunction and at least alleviate the symptoms of neurodegenerative diseases (Musiek, Xiong, & Holtzman, 2015). In addition, it may also offer mechanistic insights related to other neurological diseases with similar compromised Ca^{2+} homeostasis.

3 | PHOTIC ENTRAINMENT

Environmental light is the major synchronizing or entrainment signal for the mammalian SCN (Foster & Roenneberg, 2008). For stable entrainment, it is essential that the effects of light on the phase of the SCN rhythm are dependent on the timing of light exposure. Not only is the time-dependency of the effect of light a prerequisite in order for the SCN to entrain to the external LD cycle (Pittendrigh & Daan, 1976), but the phase dependency is also qualitatively similar among all living organisms: light induces phase delays in the early evening and phase advances at the end of the evening. In addition light exposure will also effect the speed the internal clock, leading for instance to increased period length of circadian rhythms in locomotor activity in nocturnal animals. This effect of light is also thought to contribute to synchronization of the clock to environmental light/dark cycles (Daan, 1977; Pittendrigh, 1981).

Incoming light is detected in the eye by three classes of retinal photoreceptors: photosensitive retinal ganglion cells (pRGCs), which utilize the photopigment melanopsin, and rods and cones in the outer retina (Guler et al., 2008). Until recently, it was believed that melanopsin was the most important photopigment for entraining the SCN to the external light-dark cycle. However, evidence from our laboratory and others suggests that melanopsin is not required for photic entrainment and classic photoreceptors also play a role in this process (Panda et al., 2003; Ruby et al., 2002; van Diepen, Ramkisoensing, Peirson, Foster, & Meijer, 2013; van Oosterhout et al., 2012a). Supporting findings are: (a) melanopsin deficient animals entrain to light-dark cycles (Panda et al., 2003), (b) the SCN of melanopsin deficient mice ($Opn4^{-/-}$) shows sustained light responses (van Diepen et al., 2013; van Oosterhout et al., 2012a), (c) melanopsin deficient mice show phase shifting responses that are about 50% of those in WT mice (Ruby et al., 2002), and iv) "rod-only" mice show changes in period when brought under varying low illumination levels (Altimus et al., 2010). The classical photoreceptors project to pRGCs and thus require the presence of these cells, but not of melanopsin. The implication of this finding is that in addition to stimulation of melanopsin by blue light (480 nm wavelength light), other colors can also signal to the SCN, through the activation of rods and cones, that in turn project to pRGCs. This is of importance for nocturnal animals that are increasingly threatened by environmental light pollution and are affected by light at night (Coomans et al., 2013; Lucassen et al., 2016; Ohta, Mitchell, & McMahon, 2006; Stenvers et al., 2016). Additionally this is important for stimulating human clock function by light during the day, and by diminishing light stimulation at night. For instance, it is not sufficient to abandon blue light exposure during the night in order to mimic darkness, while vice versa, all wavelengths of light will help to stimulate our clocks during the day.

Light information, once processed by retinal ganglion cells is transmitted to the SCN primarily via the retinohypothalamic tract, which releases glutamate and PACAP at its terminals (Antle, Smith, Sterniczuk, Yamakawa, & Rakai, 2009; Meijer, Albus, Weidema, & Ravesloot, 1993; Michel, Itri, Han, Gniotczynski, & Colwell, 2006; Morin & Allen, 2006). This input directly affects SCN electrical discharge (Brown, Wynne, Piggins, & Lucas, 2011; Groos & Mason, 1978; Meijer, Watanabe, Schaap, Albus, & Detari, 1998). The majority of the light responsive SCN neurons respond in a sustained manner to light; that is, their discharge is altered for the full duration of the light stimulus. This response type is more typical for areas involved in non-visual light processing, than for areas involved in pattern recognition (vision). The sustained response is of functional importance as it allows the SCN to "see" the length of the day or the duration of light exposure. It also explains that light stimuli of longer duration lead to larger magnitude phase shifts, as compared to short duration light pulses (Meijer, Rusak, & Ganshirt, 1992; Nelson & Takahashi, 1991). Because the sustained discharge level is dependent on the light intensity, the SCN neurons function as luminance detectors around dawn and dusk (their working range), and work to track environmental light intensities. Aside from detecting luminance, some SCN neurons also respond to colors (Walmsley et al., 2015).

In the rat, hamster, and mouse about 25% of the SCN cells are responsive to retinal illumination (Groos & Mason, 1980; Meijer, Groos, & Rusak, 1986), and the majority of these responsive cells are activated by light. This is consistent with the excitatory action of glutamate, which mediates these responses.

Recordings, carried out in the Rusak laboratory, in the day-active 13-lined ground squirrel, *Eutamias sibericus* (Meijer, Rusak, & Harrington, 1989), revealed that light-suppressed responses are relatively more prevalent than light-activated responses. These results were later confirmed in the

diurnal degus (Jiao, Lee, & Rusak, 1999) (Figure 2). It is not yet known how these inhibitory responses arise in the SCN of diurnal species, as glutamate, in physiological concentrations, is exclusively an excitatory transmitter. It seems likely, therefore, that the wiring of light recipient neurons to secondary neurons is different in the SCN of diurnals.

The differences in light response between nocturnal and diurnal animals may account for differences observed when diurnal and nocturnal animals are brought under conditions of continuous darkness or continuous light. In continuous light, nocturnal animals respond generally with a lengthening in their period, a decrease in rhythmicity and in some cases even a-rhythmicity. In contrast, several diurnal animals, such as Tupaia belangeri and Eutamias sibericus, show intact rhythms in constant light but a deterioration in rhythmicity when they are kept in constant dim light or darkness. These differences have received relatively little attention in the circadian field.

4 | FROM CLOCK TO PACEMAKER

Electrical activity is determined initially by the membrane potential of the cells. On average, the resting potential of SCN neurons fluctuates during the 24-hr period, ranging from −47 mV during the night to −36 mV during the day (Kuhlman & McMahon, 2004), contributing to the higher firing rate during the day. Electrical activity triggers the release of neurotransmitters and humoral factors, both within the SCN and at the nerve terminals that project to downstream brain areas. We investigated the extent to which the pattern in electrical activity of the SCN determines behavioral activity levels by simultaneous recordings of SCN electrical activity and behavior. We observed that the onset and offset of behavioral activity occur around half-maximum SCN electrical activity (Houben et al., 2009). Thus, the SCN appears to function as a gate, allowing behavioral activity when electrical activity is below the 50% level and suppressing it when SCN activity rises above the 50% activity level. The relation between SCN electrical activity and behavioral activity can be described with a probability function that is steep at the 50% electrical activity level, indicating that most transitions between rest and activity occur close to this point (Houben et al., 2009).

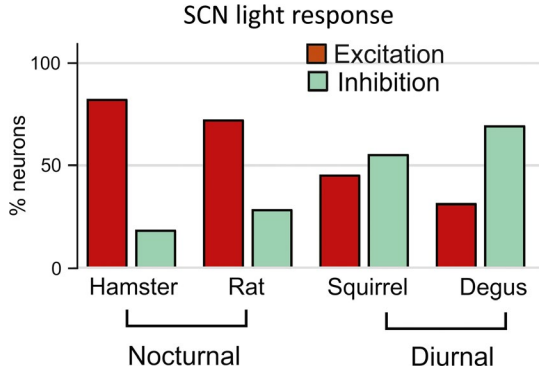

FIGURE 2 In nocturnal animals, such as the hamster and rat, the predominant light response is excitation of SCN neuronal activity, whereas in the 13-lined ground squirrel and degus, the predominant response is inhibition. Note that the proportion on the *y*-axis refers to the proportion of the total percentage of light responsive SCN cells in these animals (Based on; Meijer et al., 1986, 1989; Jiao et al., 1999)

Whether the temporal pattern of behavioral activity within the activity and resting period, (i.e., at a smaller time scale of min – hours) is also observed at the level of the SCN was investigated with microelectrode recordings of SCN neurons in freely moving mice. This was combined with exposure of the animals to short and long "T-cycles" (i.e., light/dark regimes with periods deviating from 24 hr). The use of T-cycles induced asymmetrical waveforms in SCN electrical activity, which was completely paralleled by an asymmetrical distribution of behavioral activity (Figure 3). Thus, the highest density in behavioral activity occurred during the lowest levels in electrical activity (Houben, Coomans, & Meijer, 2014).

Moreover, we observed that the waveform of the SCN rhythm depends on the ratio between the endogenous period (tau), and the period of the external cycle (T). When tau > T, such as in short external cycles of for instance 22 hr, the trough of the rhythm is skewed to the left and occurs shortly after the onset of darkness. When tau < T, the waveform is skewed to the right and the trough is about 6 hr later. When we gradually decrease the T cycle length from 26 to 24 hr, we observed almost identical trough times in the SCN rhythms at the end of the night. A sudden transition occurs when T is lowered further, from 24 to 22 hr, and the trough time flips over to the beginning of the night. And thus, it appears that a small change in tau, around $T = 24$ hr, results in major shifts in the trough of the SCN rhythm. This phenomenon can potentially explain the difference in preferred activity times between early and late chronotypes, or between adolescents and aged individuals. While the difference in their cycle length is only minor, the slight shift from tau > T (adolescents, late chronotypes) to tau < T (aged individuals, early chronotypes) impacts the SCN rhythm waveform, and hence the preferred time of being active in the evening or in the morning (Houben et al., 2014).

To further test the causality between electrical activity and activity levels, we injected TTX into the SCN in freely moving animals. TTX blocks sodium dependent action potentials, and this leads to a silencing of SCN electrical activity (Figure 4). Injections of TTX during the electrical active phase of the SCN (when nocturnal animals are resting) caused the immediate onset of behavioral activity. The suppressions in SCN electrical activity were confirmed by concurrent electrical activity recordings at the injection

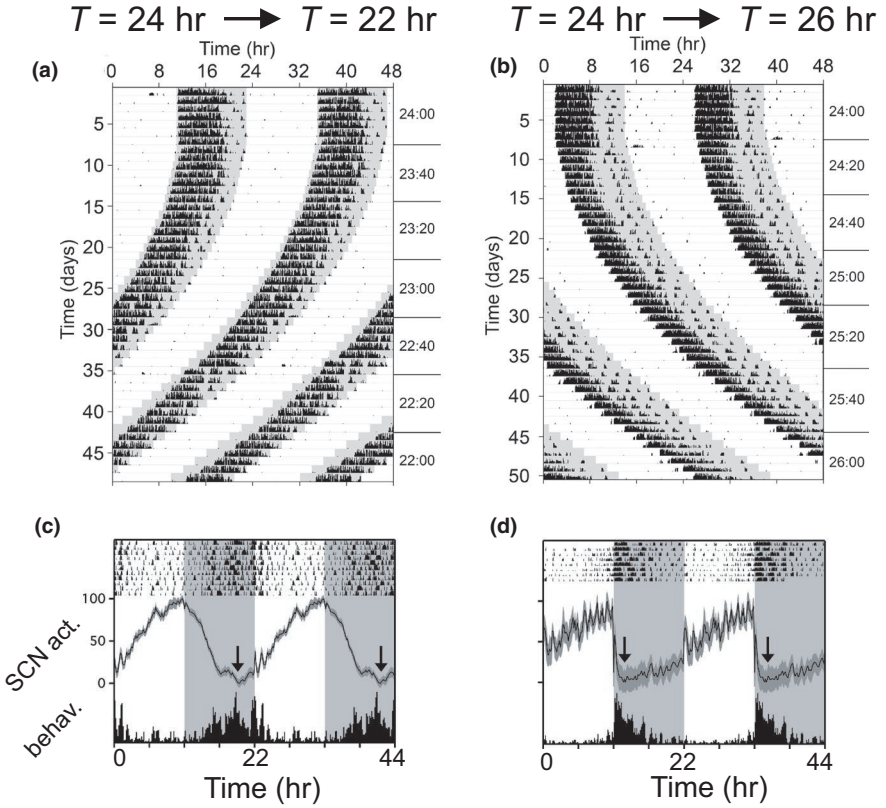

FIGURE 3 Behavioral activity recordings under gradually shortening T cycles (a, from 24 to 22 hr), or to gradually lengthening T cycles (b, from 24 to 26 hr). The behavioral activity pattern shows a shift from activity predominantly in the second half of the night when tau > T (a), and in the first half of the night when tau < T (b). The electrical activity waveform in the SCN is measured simultaneously with the behavioral activity. The trough in SCN electrical activity coincides with the peak in behavioral activity and is in the second part of the night (c), or in the first part of the night (d). The interpretation of these finding is that the level of electrical SCN activity is of importance for the behavioral activity pattern, and that low electrical activity levels trigger high behavioral activity levels, in nocturnal animals. Reproduced from (Houben et al., 2014) in accordance with the Creative Commons Attribution License

side, and were absent with control injections (Houben et al., 2014).

These experiments provide evidence that the SCN electrical activity has an acute impact on behavioral activity and also functions outside the circadian time domain to regulate temporal behavior. Thus, even at a small timescale (min – hours) the SCN electrical activity carries information that is significant for the behavioral activity structure. Of note, this does not mean that the temporal activity profile is solely determined by the SCN. The recordings are performed in conditions with minimal stress, with food availability ad libitum, etc. In real life, there are probably numerous factors that add to the profile. However, the identification of the relevance of electrical activity as a regulator for behavioral activity levels, was an unforeseen result, and is important for understanding the regulatory mechanisms underlying behavioral activity. The relevance of acute modulation of activity levels, such as they occur through "feedback" pathways to the SCN, will be discussed in the next section.

5 | FEEDBACK TO THE SCN BY INTERNAL AND BEHAVIORAL SIGNALS

The SCN clock is responsive to many stimuli, either arising from extra-SCN brain areas, from the animal's own behavioral activity and from the environment. While it was once deduced that a central clock, in order to function as a robust and precise pacemaker, should be responsive to few, if any, stimuli other than light, we now appreciate that the responsiveness to both internal and external cues in fact adds to the adaptive value of the circadian system.

These responses are highly relevant and acutely modify the function of the SCN. As the several internal stimuli are themselves subject to circadian control by the SCN, their input to the SCN forms a positive feedback loop. The feedback to the SCN may not necessarily affect the molecular clock; rather, they may "simply" affect the level of the membrane potential. This is the reason their impact may have been

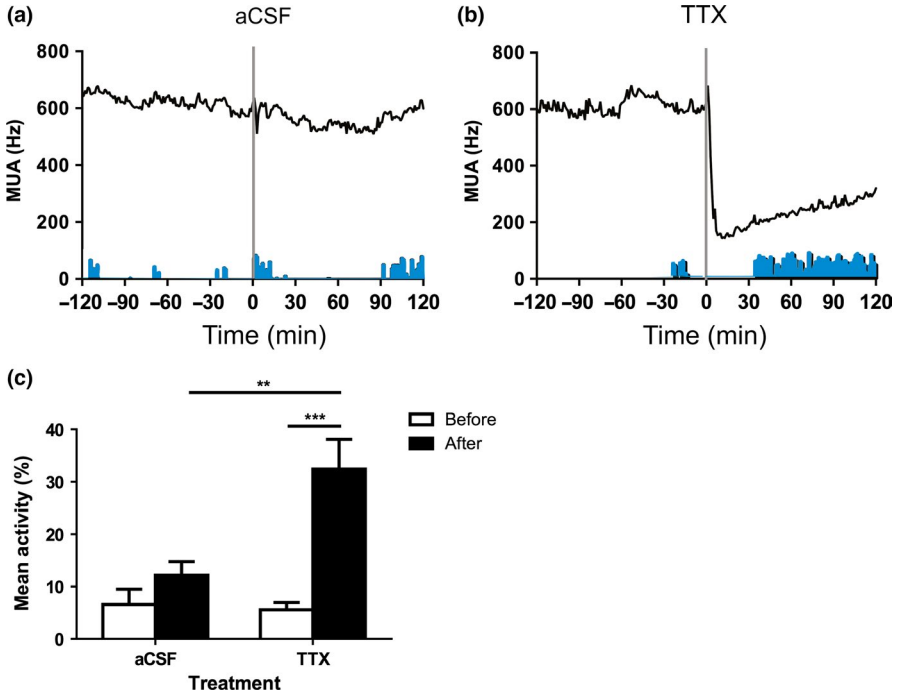

FIGURE 4 Suppressing SCN electrical activity stimulates behavioral activity. TTX was applied through a cannula aimed at the SCN, while simultaneously, the electrical activity was recorded in the SCN. TTX but not control injections resulted in a decrease in SCN electrical activity (a, b). The decrease in SCN electrical activity triggered behavioral activity, plotted in blue, below the electrical activity trace, that was significantly different from behavioral activity levels following control stimulation (c). Reproduced from (Houben et al., 2014) in accordance with the Creative Commons Attribution License

overlooked earlier, when the phase shifting effects of stimuli were the routine method of assessing their potential influence on the clock. The doctrine of making phase response curves has thereby hidden the possibility that stimuli have immediate effects on the SCN neurons, resulting in immediate changes in SCN electrical output, without shifting the phase of the molecular clock. For instance, the influence of sleep on SCN electrical activity has been elucidated with in vivo recordings of SCN electrical activity and simultaneous EEG recordings. The different sleep stages have an immediate effect on the SCN: REM sleep leads to an enhancement of SCN electrical activity, while NREM sleep leads to a reduction in SCN activity (Deboer, Vansteensel, Detari, & Meijer, 2003). Yet these normally occurring sleep stages have no phase shifting effects (Mistlberger et al., 2003).

Recently, interest has developed in the effect of exercise on the circadian system. Exercise strengthens the circadian system (Hughes & Piggins, 2012; Leise et al., 2013) and scheduled exercise can boost the circadian rhythm of VIP deficient mice (Schroeder et al., 2012). In rodents, running wheel activity feeds back to the central clock, leading to acute suppression in electrical activity (Schaap & Meijer, 2001; van Oosterhout et al., 2012b). Consequently, behavioral activity, which is itself under the direct control of the SCN, feeds back to the SCN, resulting in a positive feedback loop, that potentially enhances the rhythm amplitude of the SCN.

To investigate this, we provided running disks to animals, to allow for recording of electrical SCN activity, while simultaneously enabling the animals' display of disk running. We first recorded the mice without a disk, and then added disks to the cages for a week. We observed that the presence of a disk resulted in significantly higher levels of activity but also to more rest during the resting phase. Furthermore, we noted that running wheel behavior resulted in an increased rhythm amplitude of SCN electrical activity (Figure 5, van Diepen and Meijer, unpublished results). We conclude that the higher amplitudes are caused by activity induced suppressions during the trough of the electrical activity rhythm (when the animal is active) and a lack of behaviorally induced suppressions during the resting phase. Note that animals run in wheels voluntarily, even when wheels are placed outdoors and non-captive animals are recorded (Meijer & Robbers, 2014). In laboratory conditions, mice run about 6–8 km per day (Farajnia et al., 2012), which is similar to the distances mice run in the wild (Krebs, Kenney, & Singleton, 1995). Providing running wheels are likely essential in obtaining healthy animal models. Thus performing experiments while animals have unnaturally low levels of behavioral activity, will affect both the welfare of the animal as well as the validity of the animal model and should be avoided (Gu et al., 2015). It is important to distinguish between voluntary and involuntary activity. When activity levels are increased by

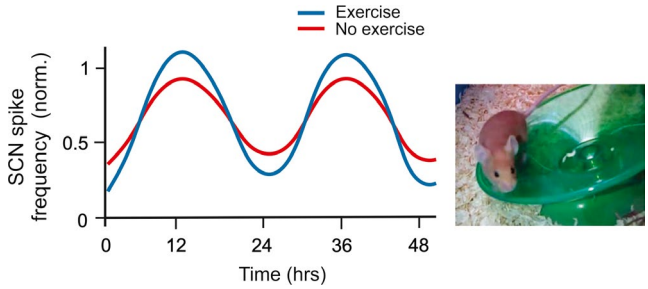

FIGURE 5 SCN recordings in the presence and absence of a running disk. The presence of a running disk resulted in enhanced amplitude of the SCN electrical activity rhythm (Van Diepen and Meijer, unpublished results)

the experimental paradigm, the electrical activity of the SCN increased instead of decreased. Moreover, voluntary activity results in phase shifts, while involuntary (imposed) activity does not (Webb, Antle, & Mistlberger, 2014). Apparently, involuntary behavior exploits neuronal pathways that are different from those that are activated by voluntary behavior.

Aged animals displayed a lower amplitude of the SCN electrical activity rhythm in vitro (Farajnia et al., 2012). The decrease in amplitude was attributable in the first place to a higher trough of the rhythm, explainable by a subpopulation of neurons that peaked in antiphase. It may not be surprising, therefore, that especially a lowering of the trough, induced by enhanced behavioral activity levels during the active phase (night), may boost the strength of the rhythm in aged mice. The challenge, then, is to create situation or condition in which voluntary activity will take place in the aged, rather than to impose an activity program on them.

6 | PERSPECTIVES

Circadian clocks in multicellular organisms have evolved over more than 500 million years in order to provide a temporal order to physiological functions and behavior. How robust should this timing system be, in order to have adaptive significance? Over the past decades, we have faced a "paradigm shift" with respect to our view on this question. While we initially reasoned and expected that the SCN clock should be largely unresponsive to cues other than light, in order to be a robust predictor of time, we have now learned that the SCN is responsive to many cues, both from the internal and external world.

In this review we have discussed the plastic organization of the SCN network, which serves to obtain a sufficiently high amplitude based on the activity of many single cell oscillators. Moreover, plasticity at the level of synchronization is a biological strategy to adapt to the length of the day, with narrow phase distributions in short days, and broad phase distributions in long days. The ensemble activity of the SCN is synchronized

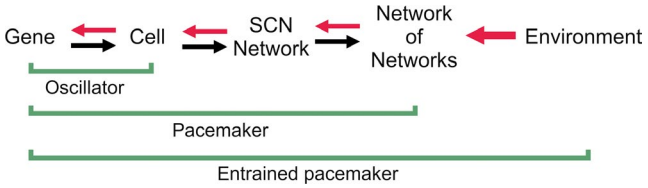

FIGURE 6 Bidirectional influences between the molecular machinery in oscillatory cells and integrative functions that evolve at the network levels, or even results from environmental input

to the environmental light dark cycle, and is a pacemaker signal for downstream areas, rendering rhythmicity in brain and body (Figure 6). Our increasing understanding of the SCN as a pacemaker will help the elderly, who suffer from lack of rhythmicity and sleep disorders. A lack of SCN rhythmicity can result from insufficient amplitude of single cell oscillations and/or from insufficient synchrony among them. Physical activity —during the normal activity phase of the animal— strengthens the function of the SCN, by acute suppression of electrical activity that is superimposed on the trough of the rhythm (leading to a deeper trough). Hence, the individual neurons of the SCN, that oscillate on the basis of a feedback loop between clock genes and protein products, are part of a larger networks with feedback pathways, not only within the SCN, but also involving other brain areas, physiology, and behavior. In healthy individuals, this is a positive feedback loop, in which properly timed behavioral activity and the SCN rhythm strengthen each other. In pathological conditions, a lack of physical activity, a sedentary life style, or shift work will lead to further decline in pacemaker activity. It is all in the loop.

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CONFLICT OF INTEREST

The author declares to have no conflict of interest.

AUTHOR CONTRIBUTIONS

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