The circadian clock: A tale of genetic-electrical interplay and synaptic integration

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Abstract

Pioneering work in *Drosophila* uncovered the building blocks of the molecular clock, consisting of transcription-translation feedback loops (TTFLs). Subsequent experimental work demonstrated that the mammalian TTFL is localized in cells and tissues throughout the brain and body. Further research established that neuronal activity forms an essential aspect of clock function. However, how the membrane electrical activity of clock neurons of the suprachiasmatic nucleus collaborate with the TTFL to drive circadian behaviors remains mostly unknown. Intercellular communication synchronizes the individual circadian oscillators to produce a precise and coherent circadian output. Here, we briefly review significant research that is increasing our understanding of the critical interactions between the TTFL and neuronal and glial activity in the generation of circadian timing signals.

TTFL and membrane signaling

Individual neurons in the suprachiasmatic nucleus (SCN) contain molecular circadian clocks, consisting of the *Period1/2* (*Per1/2*), *Cryptochrome1/2* (*Cry1/2*), *Clock* and *Bmal1* genes. The molecular circadian signal is translated into a circadian pattern of action potential firing **and, in some SCN neurons, electrical silencing by hyper- and hypo-excitation [1]**, which is required for the generation of circadian behaviors. The interaction between SCN neuron electrical activity and the molecular clock, however, is more **complicated** than just a driven rhythm.

Inhibiting action potential firing in the SCN with tetrodotoxin (TTX) abolishes behavioral circadian rhythmicity but does not affect the timing of the circadian clock [2]. Therefore, SCN action potential firing is an output of the circadian clock required to drive circadian behaviors, but pacemaker timing persists in the absence of action potential activity at the single cell level. However, TTX application to SCN brain slices for an extended period of time produces a rundown of the molecular clock amplitude [3]. This suggests that for proper clock functioning the membrane electrical excitability feeds back onto the molecular clock machinery to support and maintain circadian oscillations [3]. Two **essential** and unresolved experimental questions in circadian biology are how the molecular clock communicates with the membrane ion channels that regulate membrane excitability, and how the membrane electrical activity signals to the molecular circadian clock. Knowledge of these mechanisms will provide insights into

how the molecular clock appropriately synchronizes its activity across the SCN network and outputs its collective phase to downstream targets. In addition, this information will provide a mechanistic understanding of how environmental and internal physiological signals influence the timing precision in SCN clock cells (Figure 1). Indeed, an ingenious study in flies has unequivocally shown that the membrane activity feeds back to impose time-of-day stamps onto the molecular clock programs [4]. **In mammals, the *Fbxl3*^{Afh} mutation delays the degradation of CRY1/2 and increases the circadian period [5]. The disrupted molecular clock alters the membrane excitability and GABA neurotransmission of the SCN neurons.**

Recent work has provided new insights into possible signaling pathways regulating **the** circadian rhythm in SCN neuronal activity. It had been postulated that the **excitable states** represent a balance between the depolarizing activity of voltage-gated sodium channels and hyperpolarizing potassium channels [6-8]. Recently, a voltage-independent sodium conductance, mediated by the NA/NALCN ion channel, has been shown to depolarize SCN neurons [9]. This current is driven by the rhythmic expression of NCA **Localization Factor**-1, providing an example of signaling pathway linking the molecular clock to ion channel function [9]. This channel pathway is a new addition to the families of cation channels that provide depolarizing forces to SCN neurons during the day, elevating their resting membrane potential and increasing firing rate [10-12]. The L-type calcium channel is another key cation channel involved in sustaining excitation in SCN neurons **[1,8].** Recently, L-type calcium channel activity has also been shown to be under the direct control of the TTFL component REV-ERBα [13], a negative feedback loop in the molecular circadian clock. This provides yet another example of a pathway where **the** TTFL can influence ion channel activity. Some potassium channels may reduce their conductivity to support such depolarization during the day [10-12]. For example, a reduction in the activity of the small-conductance calcium-activated potassium channels transits a proportion of daytime SCN neurons into hyperexcitation and depolarization blockade states, where they ceased firing [1,14].

In the evening, the activity of a number of potassium currents peaks to hyperpolarize and silence clock neurons [10-12]. Reduction of *Per1* activity by antisense oligonucleotides suppressed firing of SCN neurons by reducing intracellular calcium

levels and the conductance of the large calcium-activated potassium currents (BK; [15]). Indeed, the pharmacological blockade of BK channels mimicked the effects of the antisense on SCN firing rate [15]. Circadian clock regulation of BK channels and their biophysical properties, particularly the rate of inactivation, alters the subthreshold membrane properties contributing to the day-night firing rhythm [16,17]. This permits SCN neurons to readily fire action potentials during the day and make them less likely to spike at night. Additional evidence for this inactivation has also been provided for the fast-delayed rectifier potassium channels [18].

One of the **significant** challenges facing circadian biologists is that SCN neurons are exceedingly heterogeneous. Indeed, with this diversity comes complexity and the daunting task of categorizing and cataloguing neurons based on their intrinsic excitability [19]. That is, grouping neurons that show similar firing patterns when manually presented with depolarizing stimuli in the absence of synaptic communication. **This measure will naturally be aided by targeted recordings in identified SCN neuronal populations, using animals expressing appropriate genetic fluorescence reporters. For example, previous work in the SCN indicated that when presented with a depolarizing pulse, *Per1*-EGFP positive neurons (presumed clock neurons) show firing characteristics that are broadly different from neurons in which EGFP could not be detected (presumed non-clock cells) [1]. This form of neuronal targeting can be used in new models, such as the *Per1*-Venus mouse [20], and animals in which the SCN's peptidergic populations can also be labelled. It is noteworthy, however, that recent work suggests that *Per1*- and *Per2*-containing neurons in the SCN form different but overlapping neuronal population, adding complexity to analysis [21]. Nevertheless**, we now **also** understand that similar classes of neurons can generate comparable action potential firing patterns using different complements of ion channels [22]. **Ion channel expression is regulated by homeostatic mechanisms that couple channel expression to specific neuronal activity patterns [22].** Therefore, even similar types of SCN neurons may recruit subtly different ionic mechanisms in order to regulate the circadian activity**, and these ion channel activity patterns may show day-night expression patterns.**

Cell to cell signaling

Indeed, the governing principle in SCN circadian rhythm generation is the maintenance of synchrony and appropriate phase among its neurons. GABAergic neurotransmission is a fundamental component of the SCN neural network, and virtually all SCN neurons communicate using GABA [23,24]. GABA regulates many functions in the SCN, including light-induced phase shifts, synchronization of the dorsal and ventral SCN, and the sensitivity of the circadian clock to light-entraining signals. The specific role of GABA neurotransmission in maintaining cellular synchrony remains controversial (see recent reviews by [23-25]). GABA acts on synaptic $GABA_A$ receptors to mediate fast "phasic" signaling between SCN neurons, and extrasynaptic $GABA_A$ receptor activation provides SCN cells with a "tonic" $GABA_A$ current [26]. As a network, activity signaling by the tonic GABA-$GABA_A$ receptor current is a strong candidate to regulate the coupling strength between individual SCN neuronal oscillators [27,28]. Modulation of the intracellular chloride concentration ($[Cl^-]_i$) regulates the strength of the GABA currents by altering the magnitude of the current flowing through open GABA-activated channels at a given membrane potential [29]. In the adult SCN, GABA acts both as an inhibitory or excitatory neurotransmitter depending on the postsynaptic $[Cl^-]_i$ concentration and the time-of-day [29-33]. Excitatory GABA neurotransmission is more prevalent in the dorsal SCN compared to the ventral SCN, and the overall consensus is that there is more excitatory GABA transmission during the night than during the day [29-33]. The regional differences in GABA activity may reflect different intracellular Cl^- regulation in arginine vasopressin (AVP)- and vasoactive intestinal polypeptide (VIP)-expressing SCN neurons [34]. Computer simulations and physiological recordings suggest that this inhibitory-excitatory switch in GABA action is an essential component of the SCN network activity, regulating period length and photoperiod encoding [27,28,35].

Glia

Astrocytes in the SCN also express a molecular TTFL circadian clock and play an essential role in regulating activity and entrainment of the SCN clock [36-38]. There is one astrocyte for every three SCN neurons, and these glial cells have a soma and a large number of fine processes that encase **the** neurons and synapses [39,40]. These fine processes allow a single astrocyte to influence the activity of a significant number of synapses (Figure 1). Modulation of astrocyte activity can alter the timing of the

circadian clock. In *Drosophila,* inhibition of intracellular calcium signaling in astrocytes disrupts functional circadian rhythms [41,42]. Genetic deletion of BMAL1 in astrocytes lengthens the circadian period and represses the rhythmic expression of VIP and several clock genes [37,38,43].

Astrocytes modify neuronal activity and neurotransmission through a number of mechanisms including the release of transmitter substances (gliotransmitters), such as adenosine triphosphate (ATP) and glutamate [44]. ATP is released by astrocytes in a circadian pattern with the maximum concentration observed in the middle of the night [45-47]. ATP can activate ionotropic P2X receptors to potentiate GABA release from SCN synapses and metabotropic P2Y receptors to inhibit GABA release [48,49]. Enzymatic conversion of ATP to adenosine leads to activation of presynaptic adenosine A1 receptors located on terminals of the retinohypothalamic tract (RHT). This reduces light-induced phase changes by decreasing glutamate release by the RHT [50,51]. These data indicate that the role of glial-released ATP depends on the location and timing of its release. SCN astrocytes also release glutamate in a circadian manner, with higher concentrations reported during the subjective night [37]. The glutamate may act on presynaptic NMDA receptors containing NR2C subunits to facilitate GABA release [37].

Conclusion and perspectives

Emerging work in the circadian field is revealing a collaborative, but intricate and complex relationship between the molecular clockwork and the electrical activity in SCN neurons. Progress in understanding the mechanistic nature of this relationship is slow because despite our vast understanding of the cell-autonomous processes causing daily oscillations in clock gene expression, our knowledge of how the molecular clockwork interacts with the membrane to regulate **the** excitability of SCN neurons is severely lacking. Feedback cues from the environment and internal physiology signal to SCN neurons**. However,** here too, the mechanisms involved in this electrical-genetic interaction remain elusive. Despite these knowledge gaps, **the** intracellular calcium level and the activity of its associated signaling pathways are known to play important roles. Here, a **critical** observation is that both the intracellular and extracellular sources of calcium are important for circadian rhythm generation and communication in the SCN [52-55].

Indeed, evidence in mammals and *Drosophila* supports the concept that the plasma membrane is not merely the proximal target of the molecular clock, but its excitability is integral to clock function. Across this partnership also lies the glial circadian clock. As in neurons, the relationship between the molecular clock and glio-physiology remains poorly understood. Uncovering the signaling pathways and mechanisms involved are daunting challenges, but a necessary task if we are to understand how circadian rhythms are generated and communicated in the SCN and across the brain.

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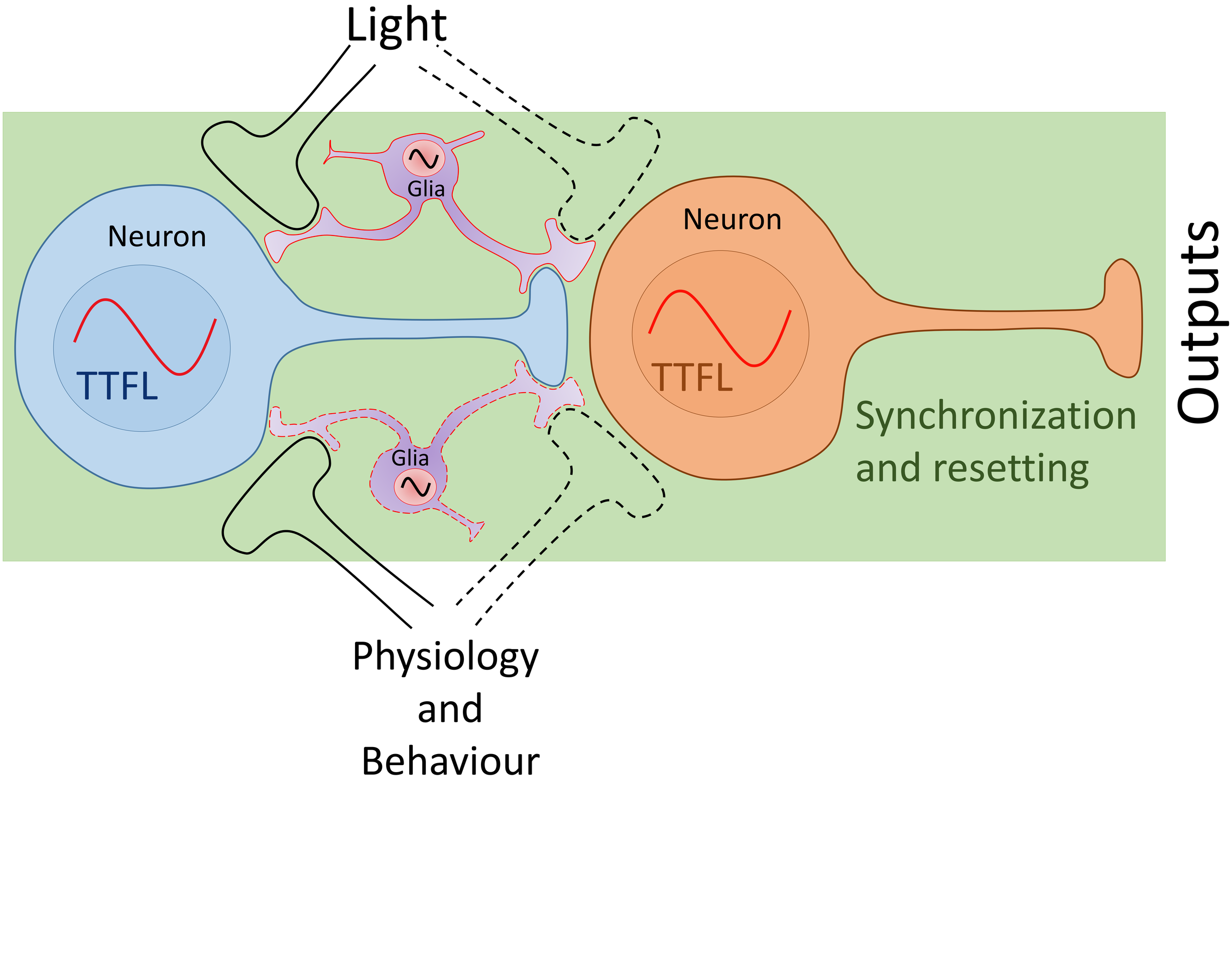
**This study uses ex vivo methods to simultaneously monitor intracellular calcium levels, clock gene activity and spontaneous spiking in SCN slices. The overall conclusions support previous work, demonstrating that in SCN neurons, intracellular calcium rhythms are dependent on both intracellular release and extracellular entry of calcium.


Light
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