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MINI-REVIEW

Role of GABA in the regulation of the central circadian clock of the suprachiasmatic nucleus

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Abstract
In mammals, circadian rhythms, such as sleep/wake cycles, are regulated by the central circadian clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN consists of thousands of individual neurons, which exhibit circadian rhythms. They synchronize with each other and produce robust and stable oscillations. Although several neurotransmitters are expressed in the SCN, almost all SCN neurons are γ-amino butyric acid (GABA)-ergic. Several studies have attempted to understand the roles of GABA in the SCN; however, precise mechanisms of the action of GABA in the SCN are still unclear. GABA exhibits excitatory and/or inhibitory characteristics depending on the circadian phase or region in the SCN. It can both synchronize and destabilize cellular circadian rhythms in individual SCN cells. Differing environmental light conditions, such as a long photoperiod, result in the decoupling of circadian oscillators of the dorsal and ventral SCN. This is due to high intracellular chloride concentrations in the dorsal SCN. Because mice with functional GABA deficiency, such as vesicular GABA transporter- and glutamate decarboxylase-deficient mice, are neonatal lethal, research has been limited to pharmacological approaches. Furthermore, different recording methods have been used to understand the roles of GABA in the SCN. The excitability of GABAergic neurons also changes during the postnatal period. Although there are technical difficulties in understanding the functions of GABA in the SCN, technical developments may help uncover new roles of GABA in circadian physiology and behavior.

Keywords Circadian rhythm · Suprachiasmatic nucleus · Clock gene · Cellular networks · GABA · Photoperiod

The central circadian clock: the suprachiasmatic nucleus (SCN)

Several physiological functions in our body exhibit oscillations on various time scales, including electrical activity in the brain, heart rate, breathing, and the sleep/wake cycle. Among them, circadian rhythms are defined as approximately 24-h oscillations in physiology and behavior. In mammals, the suprachiasmatic nucleus (SCN) of the hypothalamus is known as the central circadian pacemaker. Circadian behavioral rhythms were abolished by SCN lesions [71, 105], and restored by implantation of the SCN [56]. Importantly, the restored circadian period was identical to that of the donor, rather than the host [91, 107]. In addition, implantation of the SCN contained in a semipermeable polymeric capsule also restored circadian behavioral rhythms in SCN-lesioned hamsters, indicating that diffusible signals from the SCN control circadian behavior [102].

The SCN contains approximately 20,000 neurons [113] that have heterogeneous circadian properties. In dispersed SCN cell culture, individual SCN neurons exhibit autonomous oscillations. However, the circadian period, phase, and amplitude, differ from cell to cell [34, 37, 120]. In cultured SCN slices, circadian rhythms of individual cells synchronize with each other and they express stable circadian oscillations [33, 36, 79, 84] (Fig. 1). Cellular networks in the SCN are important for synchronization [121] and the stability of circadian oscillation in individual cells [59].

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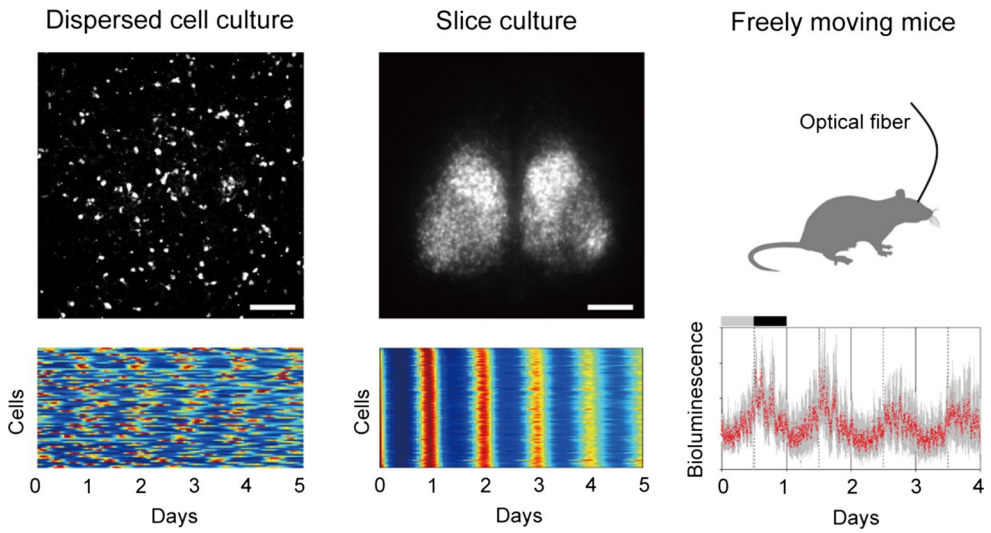

Fig. 1 PER2::LUC rhythms in dispersed SCN cells, SCN slices, and the SCN of freely moving mice. *Per2* protein fusion luciferase activity was measured using bioluminescence imaging from dispersed (left) and slice (middle) SCN culture using an EM-CCD camera. Circadian rhythms of individual SCN cells in dispersed (lower left) and slice (lower middle) cultures are expressed as pseudo colors, in which red and blue indicate peak and trough circadian rhythm phases. PER2::LUC rhythms in the SCN in freely moving mice were measured using an optical fiber under constant darkness for 4 days. Neuronal networks are important for SCN cellular circadian rhythm synchronization. Scale bars represent 200 μm

Synchronized circadian rhythms within the SCN are responsible for coordinating peripheral circadian oscillators [123], and exhibiting circadian behavior. In the SCN, there are multiple oscillators. Environmental light/dark conditions change the coupling between these regional oscillators in the SCN, which is critical for the output for the circadian behavior and peripheral clock [35, 41, 78, 82, 114].

Circadian rhythms of individual SCN cells are generated by transcription-translation feedback loops involving several clock genes and protein products [93]. In this feedback loop, the positive elements, BMAL1 and CLOCK form a heterodimer that initiates the transcription of genes that contain E-box enhancer sequences, including *Period* (*Per*) and *Cryptochrome* (*Cry*) [12, 23, 53]. The protein products of *Per* and *Cry* then suppress transactivation by the BMAL1/CLOCK heterodimer [96, 100]. This clock machinery is widely observed on the single cell level.

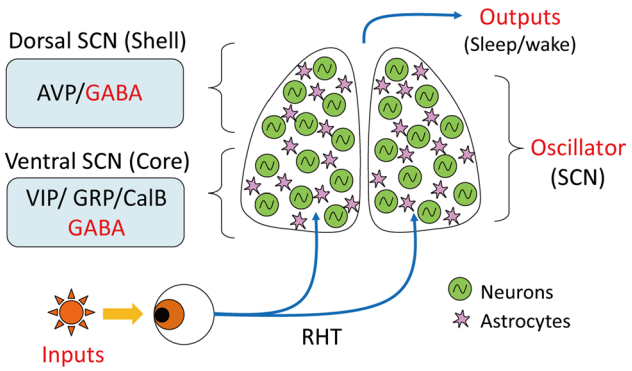

Fig. 2 Schematic diagram of circadian organization in the SCN. Light input is transmitted to the ventral SCN through the retinohypothalamic tract (RHT). Several neuropeptides are expressed in the SCN and almost all SCN cells are GABAergic neurons. Circadian rhythms in individual SCN cells synchronize via neurotransmitters, and synchronized circadian rhythms in the SCN regulate behaviors such as sleep and wake cycles

Anatomical properties of the SCN

The SCN is divided into dorsal (shell) and ventral (core) subdivisions. Retinal projections to the ventral SCN [11, 108] and dorsal SCN [29] are observed. The SCN contains several neurotransmitters located within specific regions (Fig. 2). This general organization has been studied in hamsters, mice, rats, and humans [1, 14, 62, 74]. Arginine vasopressin (AVP) neurons are located in the SCN shell. Conversely, the SCN core expresses several neuropeptides, such as vasoactive intestinal peptides (VIP), gastrin-releasing peptide (GRP), and calbindin [1, 103].

Remarkably, almost all SCN neurons express γ-amino butyric acid (GABA) [1, 73]. GABAergic neurons in the SCN contain GABA vesicular transporters (VGAT), [8] and GABA synthesizing enzymes, such as glutamate decarboxylase (GAD) [73, 83]. Two distinct isoforms of GAD (GAD65 and GAD67) are found within the SCN, and they have different molecular weights and subcellular distributions. A

study using immunohistochemistry determined that GAD65 and GAD67 are highly expressed in the ventral SCN of rats, similar to the distribution of VGAT [8]. GABA receptors, $GABA_A$ and $GABA_B$, are also found in the SCN [8, 21, 106]. $GABA_B$ receptors are mainly expressed in the dorsal area in the SCN, which generally corresponds to the region of AVP positive neurons [8].

The timing of GABA synthesis, trafficking, and release are important for the modulation of circadian rhythms in the SCN. It was reported that mRNA levels of the GABA synthesizing enzyme GAD65 were higher in the light than in the dark period, but mRNA levels of GAD67 were not rhythmic in the SCN [38]. Another group also reported that both GAD65 and GAD67 mRNA showed circadian oscillations under constant darkness [13]. VGAT contents in the SCN did not exhibit circadian rhythms, but expression levels were attenuated under constant darkness compared with light dark conditions [18]. Furthermore, intercellular signals, such as VIP, could modulate GABA release in the SCN [24, 44].

Excitatory and/or inhibitory effects of GABA in the SCN

Although the effects of GABA on cellular activity in the SCN have been studied for more than 30 years, results remain controversial. For example, one study demonstrated that GABA had inhibitory effects on almost all SCN neurons [65], while several studies have reported that GABA has both excitatory and inhibitory effects in the SCN [15, 22, 57, 101, 116]. Wanger et al. reported that in SCN tissue slices, the application of GABA decreased firing frequency at night, and increased firing frequency during the day. This result indicates that the effects of GABA in SCN neurons are dependent on the circadian phase [116]. However, some investigators have reported no difference in the excitatory and inhibitory effects of GABA during the subjective day compared to night [15, 26, 57, 58, 65]. Similar to the application of GABA, GABA agonists, such as muscimol and baclofen, induce excitatory or inhibitory effects in the SCN [26, 39, 70], while GABA has also been shown to produce either excitatory or inhibitory effects on firing across the circadian day [22]. These effects were inhibited by the $GABA_A$ receptor antagonist bicuculline [2, 15, 22, 26, 65]. Regional differences in the effects of GABA in the SCN have also been reported. Short-term application of bicuculline increased neuronal activity in the ventral SCN, but decreased activity in dorsal SCN slices [2]. These pharmacological approaches have shown inconsistent observations regarding the acute effects of GABA in the SCN. Furthermore, these agents may represent non-specific pharmacological actions. For example, the $GABA_A$ receptor antagonist, bicuculline is generally known to block small-conductance calcium-activated potassium channels [45, 51] and does so in SCN neurons [110]. It is necessary to consider this non-specific effect of these drugs.

Coupling circadian oscillators in the SCN

Individual SCN neurons exhibit autonomous circadian rhythms even when isolated in culture [119, 120]. The circadian period, phase, and amplitude differ from each other in dispersed cell culture, although their rhythms are synchronized in SCN slices [33, 36, 79, 84] and in vivo [42, 122]. Due to the involvement of heterogeneous oscillators in the SCN, individual SCN cells must couple to each other. Synchronized circadian rhythms in the SCN entrain light–dark cycles to adapt to environmental light–dark conditions. It is thought that GABA may be involved in mediating circadian rhythm coupling in individual SCN neurons.

Synchronizer or destabilizer of cellular circadian rhythms

Liu and Reppert demonstrated that the application of GABA on dispersed SCN cultured cells completely inhibited spontaneous firing at all circadian phases. The application of GABA after the peak phase of neuronal activity rhythms induced a large phase delay. The $GABA_A$ agonist muscimol induced phase shifts of neuronal activity rhythms in SCN neurons, whereas the application of the $GABA_B$ agonist bacrofen had no effect, suggesting that this phase-dependent shift is mediated by $GABA_A$ receptors. Additionally, GABA was applied daily to dispersed SCN cultured cells to assess the effects of circadian rhythms in individual SCN neurons. After administration of GABA for 3 h every 24 h for 5 days, circadian rhythms of spontaneous firings in individual SCN neurons synchronized. This result has been interpreted as evidence that the daily application of GABA synchronized circadian firing rhythms in the dispersed SCN cell culture [60] (Fig. 3a). However, these data do not exclude the effects of other transmitters, such as neuropeptides.

Monitoring gene expression or protein products of clock genes in the SCN using bioluminescence reporters has provided insights into the understanding of circadian rhythms [121, 123, 125]. Based on the evidence for the role of GABA in the synchronization of circadian rhythms in the SCN [60], antagonizing GABA receptors may induce desynchronization of cellular circadian rhythms. However, long-term application of $GABA_A$ (bicuculline) and $GABA_B$ (saclofen) receptor antagonists into the *Per1* promoter-driven luciferase reporter (*Per1-luc)* SCN slices gradually increased the amplitude of circadian rhythms compared with controls at the tissue level [4]. This increased amplitude of circadian rhythms was due to increased amplitude

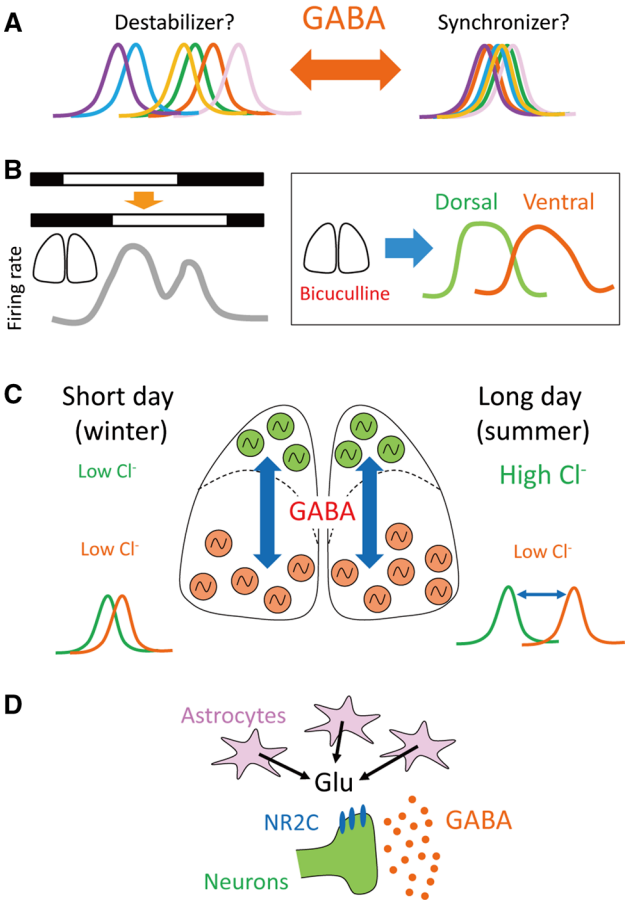

Fig. 3 Possible roles of GABA in SCN cellular coupling. **a** GABA acts as both a synchronizer and destabilizer. **b** A phase delay shift results in bimodal neuronal activity in the intact SCN slice; however, after the application of $GABA_A$ receptor antagonists, circadian rhythms in the dorsal and ventral SCN are dissociated. **c** Long-day photoperiods change the intracellular chloride concentration in the dorsal SCN and decoupled circadian rhythms between the dorsal and ventral SCN. **d** Hypothetical model of the astrocytic-neuronal intercellular axis in the SCN

of cellular circadian rhythms and decreased cycle-to-cycle period variation (increased precision of cellular rhythms). GABA receptor antagonists (bicuculline and saclofen) also increased the amplitude of circadian firing rhythms in dispersed cultured cells [4]. Similarly, blocking $GABA_A$ receptor signaling with the application of gabazine onto the *Per2* protein fusion luciferase reporter (PER2::LUC) SCN slices decreased circadian period variability in individual cells compared to vehicle-treated controls [22]. These results suggest that GABA destabilizes circadian oscillations in the SCN (Fig. 3a).

Coupling of dorsal and ventral circadian oscillators

Constant light causes splitting of circadian behavioral rhythms [89]. The circadian rhythms of the shell and core, or left and right SCN, exhibited a 180° antiphase during splitting under constant light conditions [82, 124]. Another study showed that an abrupt change in the light–dark cycle disrupted synchronous oscillation of circadian components in the rat SCN [76]. After a phase delay shift of light–dark cycles, clock gene expression rhythms shifted rapidly in the ventrolateral SCN, whereas this shift occurred more slowly in the dorsomedial SCN [76]. Several researchers have demonstrated a role of GABA in the re-synchronization of the dorsal and ventral regions of the SCN after manipulation of environmental light–dark cycles.

Albus et al. revealed the role of GABA in coupling dorsal and ventral SCN circadian rhythms in acute SCN slices. Measurements of neuronal activity in the SCN following a 6-h phase delay in the light/dark schedule revealed bimodal patterns of activity rhythms. Furthermore, when the SCN was cut horizontally, separating the slice into dorsal and ventral areas, the peak phase of neuronal activity rhythms in the ventral SCN was significantly advanced compared with that in the dorsal SCN. Continuous application of a $GABA_A$ receptor antagonist (bicuculline) yielded similar results [2] (Fig. 3b). These results indicate that GABA is important for coupling circadian rhythms in the dorsal and ventral SCN.

Photoperiodic changes in SCN cellular networks

Environmental light–dark conditions change depending on the season. This photoperiodic change is important for seasonal reproduction in some animals [81, 126]. The long-day photoperiod also changes cellular networks in the SCN and decouples circadian oscillatory cell groups [30, 41, 77]. Recently, the role of $GABA_A$ receptors in coupling SCN dorsal and ventral circadian oscillators was reported to occur during the long-day photoperiod [19] (Fig. 3c). PER2::LUC reporter mice were exposed to 20 h of light and 4 h of dark (LD20:4), and the PER2::LUC bioluminescence was measured from the SCN slice. In these conditions, the circadian phase of the SCN core was advanced compared with that of the shell. This phase difference between the dorsal and ventral region in the SCN gradually returned to an organizational state similar to that observed under LD12:12 conditions. To investigate the role of $GABA_A$ signaling in the re-synchronization of dorsal and ventral oscillators after LD20:4 conditions, the $GABA_A$ receptor antagonist, bicuculline, was applied to the SCN slice. Bicuculline attenuated the re-synchronization when the circadian phase between dorsal and ventral regions was out of phase, but not when it was in-phase. These results were interpreted as evidence that $GABA_A$ signaling contributes to the synchronization of circadian rhythms between the dorsal and ventral SCN in a state-dependent manner. An alternative interpretation would suggest that GABA may acutely modulate firing without having much, if any, effect on circadian phase. These results

also suggest that GABA is sufficient for the synchronization of dispersed SCN cells [60] and that the absence of GABA does not desynchronize cellular rhythms under steady-state networks in the SCN [4].

Intracellular chloride concentrations and cellular coupling

Long-day photoperiods also change the excitability [20], and levels of chloride transporter expression in SCN neurons. This determines the difference in circadian phase and period between the dorsal and ventral SCN [75] (Fig. 3c). It was observed that under long-day conditions, the phase difference in *Bmal1* promoter-driven luciferase reporter *(Bmal1-Eluc)* circadian rhythms between the dorsal and ventral SCN were increased, and the circadian period of the dorsal SCN was decreased compared with the ventral SCN. Myung et al. also measured intracellular chloride concentrations in the SCN, using N-(ethoxycarbonylmethyl)-6-methoxyquinoliniumbromide fluorescence, and found that intracellular chloride concentrations were increased under long-day photoperiods. These results are due to a higher expression ratio of sodium/potassium/chloride cotransporter (NKCC1)/potassium/chloride cotransporters (KCC2), in the dorsal than the ventral SCN. Because NKCC1 is a chloride importer, a high ratio of NKCC1/KCC2 results in more GABA-induced excitation. KCC2 is expressed exclusively in VIP and GRP neurons, whereas NKCC1 is expressed in VIP, GRP, and AVP neurons within the SCN [7]. Recently, Klett and Allen reported that intracellular chloride concentrations were higher during the day than at night in both AVP- and VIP-positive neurons [52]. The prevalence of GABA excitation and inhibition is dependent on the level of chloride transporter expression and may affect the coupling of dorsal and ventral SCN circadian oscillations.

Astrocytes and GABA

In addition to neurons, astrocytes in the SCN are involved in circadian rhythms. Individual astrocytes display circadian rhythms entrained to daily temperature cycles [90]. Moreover, astrocytes co-cultured with adult SCN explants sustained the rhythms of astrocytes, suggesting that diffusible factors from the SCN are sufficient to entrain circadian oscillations in astrocytes [90]. Furthermore, VIP expressed in SCN neurons entrains astrocyte circadian rhythms [63]. It is known that astrocytes release ATP into the extracellular space and it has been shown that cultured astrocytes display daily oscillations of extracellular ATP concentrations that are under circadian control [64]. Astrocytes also regulate neuronal networks through the reuptake and release of various transmitters including glutamate [27, 88]; however, the functional mechanisms of these transmitters in astrocytes have yet to be identified. Recently, three groups reported that astrocytes regulate SCN and behavioral circadian rhythms [5, 10, 112]. Astrocyte-specific deletion of the *Bmal1* gene led to reduced expression of astrocytic GABA transporter 1 and 3 (GAT1 and GAT3), suggesting a potential impairment in the clearance of extracellular GABA released by neurons [5]. Brancaccio et al. proposed a new model of circadian timekeeping in the SCN. They hypothesized that in the SCN, glutamate released from astrocytes maintains higher intracellular calcium levels, specifically in pre-synaptic terminals, through the activation of NMDA receptors (NR2C), which subsequently facilitates neuronal GABA release [10] (Fig. 3d). Because the peak phase of circadian calcium rhythms in astrocytes was observed at night, GABA release from neurons via glutamate release from astrocytes would increase at night, resulting in a decrease in neuronal activity at night.

Circadian outputs and GABA functions

Circadian rhythms in SCN neurons synchronize via several neurotransmitters, such as AVP, VIP, and GRP [3, 66, 67]. To regulate sleep/wake cycles, SCN circadian rhythms need to send outputs to peripheral circadian oscillators. Neuronal activity in the SCN is one of the most important input and/or output signals from molecular oscillations. Application of the sodium channel blocker, tetrodotoxin, into the SCN in vivo resulted in arrhythmicity in behavior without affecting the circadian oscillation [97]. In addition, optogenetic stimulation of the SCN in vivo modulated circadian behavioral rhythms [46]. GABA, therefore, may regulate the transmission of circadian output from the SCN by changing neuronal excitability. In contrast, it is also reported that GABA is involved in the synaptic plasticity changes in the retinohypothalamic tract-SCN synapses [69]. These results indicate that GABA modulates both input and output of circadian oscillation in the SCN.

Identification of SCN circadian outputs is important for understanding the mechanisms underlying the regulation of behavioral rhythms. Previously, SCN efferent projections have been investigated [50, 54, 104, 117, 118]. Neurons in the dorsal SCN project densely to the preoptic area, paraventricular nucleus (PVN), dorsomedial hypothalamus, and subparaventricular zone (SPVZ). In contrast, dense projections from the core are limited to the peri-suprachiasmatic area (PSCN), lateral SPVZ, and ventral tuberal area (VTU) [54]. Interestingly, the circadian rhythms of neuronal activity outside the SCN in vivo were in antiphase compared with the SCN in nocturnal animals [42, 80]. Importantly, in both diurnal and nocturnal animals, the peak phase of neuronal activity rhythms in the SCN is observed during the day. However, in diurnal animals, the peak phase of neuronal

activity outside of the SCN is in-phase compared with that of the SCN [95], suggesting that circadian information from the SCN is transferred to output brain areas, and that this mechanism is different in nocturnal and diurnal animals. The mechanisms for switching day-night information from the SCN, however, have not been identified.

The PVN receives an efferent projection from the SCN. The PVN contains several neuropeptides, such as corticotrophin-releasing factor, oxytocin, and AVP, and regulates endocrine and autonomic functions. Electrical stimulation of the SCN evoked monosynaptic inhibitory postsynaptic potentials, as well as excitatory postsynaptic potentials in the PVN, indicating that GABA and glutamate are important mediators of fast monosynaptic transmission from the SCN to the PVN [31, 32]. Tousson and Meissl used a multi-electrode array dish to demonstrate that humoral factors are responsible for circadian rhythms in the PVN [111]. They observed circadian rhythms in PVN neuronal activity measured in brain slices containing both PVN and SCN. When the SCN was removed from the brain slice, PVN circadian rhythms disappeared and were subsequently restored by co-cultured SCN grafts.

The SCN regulates circadian rhythms of plasma melatonin concentrations via a multi-synaptic pathway, including the PVN, sympathetic preganglionic neurons of the spinal cord, and noradrenergic sympathetic neurons of the superior cervical ganglion [72]. The melatonin concentration is increased at night and decreased during the day. Blocking GABAergic transmission to the PVN results in inhibition of melatonin synthesis in the pineal gland [48, 49], suggesting that GABAergic signals from the SCN have an inhibitory effect on melatonin synthesis during the day. Importantly, melatonin administration suppressed spontaneous firing in the SCN [61, 99]. Neuronal and humoral regulation of PVN neuronal activity by the SCN is important for temporal integration of physiological events.

Variety of circadian recording methods

Several studies have attempted to understand the function of GABA in the SCN, using both ex vivo and in vivo methods (Fig. 4). Primary culture techniques (dispersed and slice) are useful for measuring SCN circadian rhythms. Typically, the SCN from neonatal animals is used for this experiment [34, 60, 79, 120]; however, acute SCN slices from adult animals have also been used for recording circadian rhythms [2, 25, 116]. Neonatal SCN tissue allows researchers to measure circadian rhythms over long periods; however, the cellular properties and neuronal networks in the SCN may not be the same in the adult. In general, NKCC1 expression predominates in immature neurons, in which the intracellular concentration of chloride ions is relatively high, whereas KCC2 expression predominates in mature neurons. These

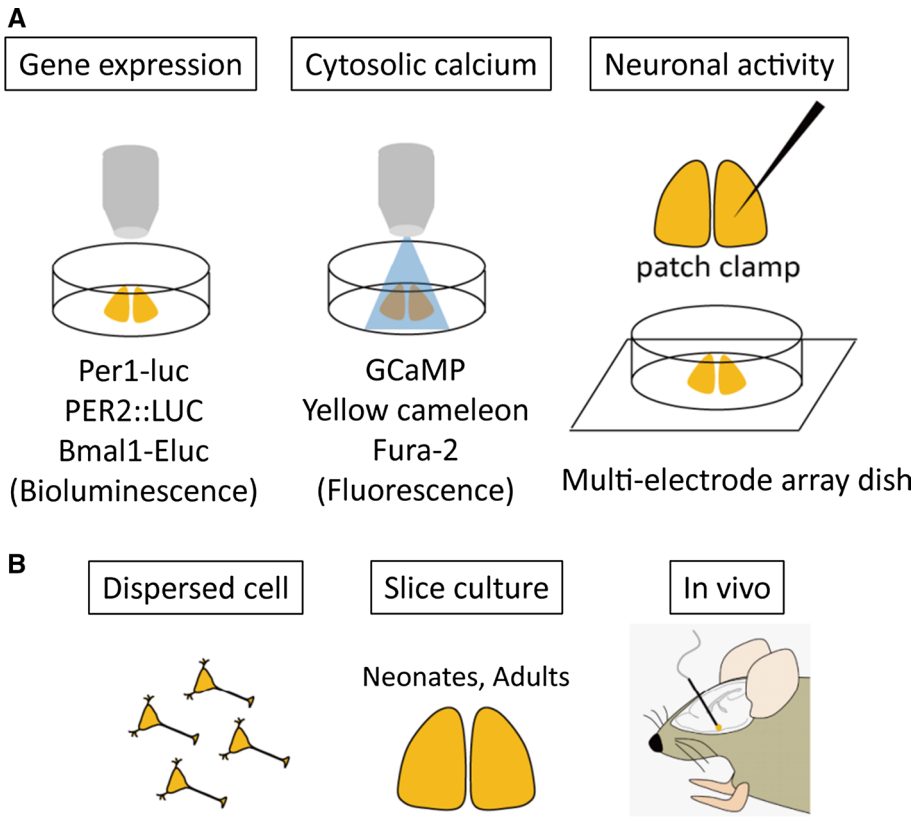

Fig. 4 Variety of circadian rhythm recording techniques. **a** Different parameters can be measured using different methods. Clock gene expression rhythms can be measured with bioluminescence reporters. Cytosolic calcium concentrations can be measured with fluorescent probes. Neuronal activity can be recorded using patch clamp or multi-electrode array dish techniques. **b** SCN cell preparations for recording circadian rhythms. Individual SCN properties may depend on the coupling strength and the age of mice

developmental changes of chloride transporters modulate excitability to GABA depending on age [9]. Developmental effects of GABA on SCN circadian oscillations have not been fully identified. Recently, however, we reported developmental changes of neuronal networks in the SCN, as Ono et al. demonstrated that *Cryptochrome* (*Cry*), a clock gene, is involved in cellular coupling in the adult SCN [84, 85]. GABA may have an important role in cellular coupling and circadian outputs in the SCN, depending on the developmental stage.

GABA functions have been studied using various recording methods, such as neuronal activity recording, calcium imaging, and bioluminescence imaging [2, 4, 40, 43, 60, 101, 116]. These recording methods are useful for measuring circadian oscillations in the SCN. However, circadian oscillation in measurements such as gene expression, intracellular calcium levels, and neuronal activity do not show the same properties. For instance, Vansteensel et al. measured *Per1-luc* and neuronal activity rhythms in both acute SCN slices and in vivo, during a 6-h light/dark phase advance schedule. *Per1* and neuronal activity rhythms in SCN slices demonstrated a phase shift immediately after the 6-h phase advance, whereas in vivo neuronal activity did not immediately shift, indicating that neuronal activity was dissociated between brain slices and in vivo [115]. Ono et al. also demonstrated that circadian rhythms of the clock genes, *Per1* and *Bmal1,* were dissociated in cultured SCN slices [86]. We have successfully simultaneously measured *Per1*, *Bmal1*, cytosolic calcium ions, and neuronal activity in SCN slices and found that the circadian period of *Bmal1-ELuc* rhythms is shorter than that of *Per1-luc* rhythms. Furthermore, the circadian period of calcium and neuronal activity rhythms were intermediate between *Per1* and *Bmal1* rhythms. Simultaneous and multifunctional recording of circadian oscillation is a useful tool for understanding the hierarchal structure of SCN networks in addition to the roles of GABA within these networks.

Perspectives

All studies that have addressed the function of GABA in the SCN have only used pharmacological approaches. Because mice with genetic GABA deficiencies, such as VGAT- and GAD65/67-deficient mice, cannot survive after birth [47, 94], it is difficult to assess the roles of GABA in the SCN using a genetic approach. It is possible to measure SCN circadian rhythms obtained from embryos of VGAT- or GAD65/67-deficient mice. However, this slice culture approach is not enough to understand mechanisms of circadian rhythms as they relate to behavior. A conditional knockout method could potentially provide a solution to this problem. Recently, several Cre driver mice have been used to conditionally disrupt genes of interest in the SCN [6, 28, 55, 68]. Creating conditional KO mice is useful; however, there are currently no SCN-specific Cre driver mice. While Neuromedin-S is specifically expressed in the SCN, not all SCN neurons express this neuropeptide [55]. Recently, a Crispr/Cas9 genome editing method, a powerful tool for manipulating genes of interest in various species [17], was developed and has been used to manipulate signaling in specific cell types of the SCN [112]. Adeno-associated viral driven Crispr/Cas9 techniques may be applicable to the study of GABA function specifically in the SCN [92]. Since transfection and expression efficiency are not perfect, however, it is difficult to control viral diffusion outside of the SCN. Therefore, alternative methods are required for SCN-specific functional GABA deficiency.

To understand the roles of GABA in circadian physiology and behavior, it is important to identify neuronal networks and output pathways from the SCN. Several new methods have been developed to accomplish this. For example, the glycoprotein-deleted (DG) rabies virus is a useful tool for studies of neural circuits [87]. In combination with Cre mice, we can identify direct input and output pathways of specific neurons [98]. However, because neurons infected with the rabies virus are killed by approximately 14 days after infection [87], manipulations or recordings of neuronal activity are limited to this short time window. Recently, self-inactivating rabies virus has been developed, which may allow us to further understand neuronal brain networks [16]. AAV-mediated retrograde tracing methods have also been developed, which could allow us to label, manipulate, and measure retrograde-labeled neurons [109]. Identification of a local GABA circuit in the SCN and long-range brain networks may be important for the understanding of circadian physiology and behavior in the future.

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Compliance with ethical standards

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

Conflict of interest The authors declare no conflicts of interest.

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