The role of GABA in the regulation of the central circadian clock of the suprachiasmatic nucleus

Daisuke Ono[1], Ken-ichi Honma[2], Yuchio Yanagawa[3], Akihiro Yamanaka[1] and Sato Honma[2]

[1]Department of Neuroscience II, Research Institute of Environmental Medicine, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan
[2]Research and Education Center for Brain Science, Hokkaido University Graduate School of Medicine, Sapporo, 060-8638, Japan
[3]Department of Genetic and Behavioral Neuroscience, Gunma University Graduate School of Medicine, Maebashi, Gunma, Japan

*Corresponding author: Daisuke Ono (dai-ono@riem.nagoya-u.ac.jp)

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Highlights

- Almost all SCN neurons are GABAergic which show both excitatory and inhibitory functions
- GABA modulates cellular networks in the SCN depending on photoperiod
- GABA in the SCN might be important for circadian output rhythms

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 consist of thousands of individual neurons which show 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 GABAergic. Several studies have attempted to understand the roles of γ-amino butyric acid (GABA) in the SCN; however, precise mechanisms of GABA action in the SCN are still unclear. GABA exhibits excitability 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, results in the decoupling of circadian oscillators of the dorsal and ventral SCN. This is due to high intracellular chloride concentrations in the dorsal SCN. Since mice with functional GABA deficiency, such as vesicular GABA transporter (VGAT)-deficient and glutamate decarboxylase (GAD)-deficient mice are neonatal lethal, research has been limited to using a pharmacological approach. Furthermore, different recording methods have been used to understand the roles of GABA in the SCN. Excitability of GABA also changes during the postnatal period. Although there are still technical difficulties in understanding the functions of GABA in the SCN, technical developments may uncover new roles of GABA in circadian physiology and behavior.

Contents

1. 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, heat rate, breathing, and the sleep/wake cycle. Among them, circadian rhythms are defined as approximately 24 hour 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 (Moore and Eichler, 1972; Stephan and Zucker, 1972), and restored by implantation of the SCN (Lehman et al., 1987). Importantly, the restored circadian period was identical to that of the donor, rather than the host (Ralph et al., 1990; Sujino et al., 2003). 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 (Silver et al., 1996a).

The SCN contains approximately 20,000 neurons (Van den Pol, 1980) 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 (Herzog et al., 1998; Honma et al., 1998; Welsh et al., 1995). In cultured SCN slices, circadian rhythms synchronize with each other and express stable circadian rhythms (Herzog et al., 2004; Honma et al., 2004; Nakamura et al., 2002; Ono et al., 2013) (Figure 1). Cellular networks in the SCN are important for synchronization (Yamaguchi et al., 2003) and the stability of circadian oscillation in individual cells (Liu et al., 2007). Synchronized circadian rhythms within the SCN are responsible for coordinating

peripheral circadian oscillators (Yamazaki et al., 2000), and exhibiting of circadian behavior.

Circadian rhythms of individual SCN cells are generated by transcription-translation feedback loops involving several clock genes and protein products (Reppert and Weaver, 2002). 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*) (Bunger et al., 2000; Gekakis et al., 1998; Kume et al., 1999). The protein products of *Per* and *Cry* then suppress transactivation by the BMAL1/CLOCK heterodimer (Sato et al., 2006; Shearman et al., 2000). This clock machinery is widely observed on the single cell level.

2. Anatomical properties of the SCN

The SCN is divided into dorsal (shell) and ventral (core) subdivisions. Retinal projection is observed in the ventral SCN (Bryant et al., 2000; Tanaka et al., 1997). The SCN contains several neurotransmitters located within specific regions (Figure 2). This general organization has been studied in hamsters, mice, rats, and humans (Abrahamson and Moore, 2001; Card and Moore, 1984; Mai et al., 1991; Moore et al., 2002). Arginine vasopressin (AVP) neurons are located in the SCN shell. Conversely the SCN core expresses several neuropeptides. The most abundant cells in the SCN core are vasoactive intestinal peptides (VIP) neurons. Other cell types, such as gastrin releasing peptide (GRP) and calbindin (CalB) expressing cells, are also observed in the SCN core (Abrahamson and Moore, 2001; Silver et al., 1996b).

Remarkably, almost all SCN cells are γ-amino butyric acid (GABA)-positive neurons (Abrahamson and Moore, 2001; Moore and Speh, 1993). SCN GABAergic neurons contain GABA vesicular transporters (VGAT), (Belenky et al., 2008), and GABA synthesizing enzymes, glutamate decarboxylase (GAD) (Moore and Speh, 1993; Okamura et al., 1989). Two distinct isoforms of GAD (GAD65 and GAD67) are found within the SCN. They are encoded by two different genes, and have different molecular weights, and subcellular distributions. A study that used immunohistochemistry determined that GAD65 and GAD67 are localized in the SCN of rats (Belenky et al., 2008). GABA receptors, $GABA_A$ and $GABA_B$, are also found in the SCN (Belenky et al., 2008; Francois-Bellan et al., 1989; Strecker et al., 1997).

3. 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 in the almost all SCN neurons (Mason et al., 1991). Conversely, several studies have reported that GABA has both excitatory and inhibitory effects in the SCN (Choi et al., 2008; Freeman et al., 2013; Liou and Albers, 1990; Shirakawa et al., 2000; Wagner et al., 1997). Wanger et al. reported that in SCN slice tissue, GABA application decreased firing frequency during night, and increased firing frequency during the day. This result indicates that the effects of GABA in SCN neurons are dependent on circadian phase (Wagner et al., 1997). However, some investigators have reported that there is no difference in the excitatory and

inhibitory effects of GABA during the subjective day compared to night (Choi et al., 2008; Gribkoff et al., 1999; Liou and Albers, 1990; Liou et al., 1990; Mason et al., 1991). Similar to GABA application, GABA agonists such as musimol and baclofen, induce excitatory or inhibitory effects in the SCN (Gribkoff et al., 1999; Ikeda et al., 2003a; Moldavan et al., 2006), and the $GABA_A$ antagonist bicuculline has also been shown to produce both excitatory and inhibitory effects (Albus et al., 2005; Choi et al., 2008; Freeman et al., 2013; Gribkoff et al., 1999; Mason et al., 1991). Regional differences in GABA effects in the SCN have also been reported. Short term of application of bicuculline increased neuronal activity in the ventral SCN, but decreased activity in dorsal SCN slices (Albus et al., 2005). These pharmacological approaches have not shown consistent observations of the effects of GABA in the SCN.

4. Coupling circadian oscillators in the SCN

Individual SCN neurons exhibit autonomous circadian rhythms even when isolated in culture (Webb et al., 2009; Welsh et al., 1995). Circadian period, phase and amplitude differ from each other in dispersed cell culture, although their rhythms are synchronized in SCN slices (Herzog et al., 2004; Honma et al., 2004; Nakamura et al., 2002; Ono et al., 2013) and *in vivo* (Inouye and Kawamura, 1979; Yamazaki et al., 1998). 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 (LD) cycles to adapt environmental light-dark condition. It is thought that GABA may be involved in mediating circadian rhythm coupling in individual SCN neurons.

4-1) Synchronizer or destabilizer of cellular circadian rhythms

Liu and Reppert demonstrated that GABA application to dispersed SCN cultured cells completely inhibited spontaneous firing at all circadian phases. Application of GABA after the peak phase of neuronal activity rhythms induced a large phase delay. Application of the $GABA_A$ agonist, muscimol, induced phase shifts of neuronal activity rhythms in SCN neurons, whereas 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 hours, every 24 hours for 5 days, circadian rhythms of spontaneous firings in individual SCN neurons synchronized each other. This result indicates that daily application of GABA synchronized circadian firing rhythms in the dispersed SCN cell culture (Liu and Reppert, 2000) (Figure 3A).

Monitoring gene expression or protein products of clock genes in the SCN using bioluminescence reporters has provided several insights to the understanding of circadian rhythms (Yamaguchi et al., 2003; Yamazaki et al., 2000; Yoo et al., 2004). Taking advantage of these bioluminescence tools, several characteristics of circadian rhythms have been discovered. Based on the evidence for the role of GABA in the synchronization of circadian rhythms in the SCN (Liu and Reppert, 2000), antagonizing GABA receptors may induce desynchronization of cellular circadian rhythms. However, long-term application of $GABA_A$ and $GABA_B$ antagonists into *Per1-luc* (*Per1* promotor driven

luciferase reporter) SCN slices, gradually increased the amplitude of circadian rhythms compared to controls at the tissue level (Aton et al., 2006). This increased amplitude of circadian rhythms was due to increased amplitude of cellular circadian rhythms and decreased cycle-to-cycle period variation (increased precision of cellular rhythms). GABA receptor antagonists also increased the amplitude of circadian firing rhythms in dispersed cultured cells (Aton et al., 2006). Similarly, blocking $GABA_A$ receptor signaling with application of gabazine onto PER2::LUC (*Per2* protein fusion luciferase reporter) SCN slices decreased circadian period variability in individual cells as compared to vehicle-treated controls (Freeman et al., 2013). These results suggest that GABA destabilizes circadian oscillations in the SCN (Figure 3A).

4-2) Coupling of dorsal and ventral circadian oscillators

Constant light causes splitting of circadian behavioral rhythms (Pittendrigh and Daan, 1976). The circadian rhythms of the shell and core, or left and right SCN exhibited a 180-degree antiphase during splitting under constant light condition (Ohta et al., 2005; Yan et al., 2005). Another study showed that an abrupt change in the light-dark cycle, disrupted synchronous oscillation of circadian components in the rat SCN (Nagano et al., 2003). 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 (Nagano et al., 2003). Several researchers have demonstrated a role for 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 a GABA's role in coupling dorsal and ventral SCN circadian rhythms in acute SCN slices. Measurements of neuronal activity in the SCN following a 6-hour 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 significantly advanced compares with that of the dorsal SCN. Continuous application of a $GABA_A$ receptor antagonist produced similar results (Figure 3B). These results indicate that GABA is important for coupling circadian rhythms in the dorsal and ventral SCN (Albus et al., 2005).

4-3) Photoperiodic changes in SCN cellular networks

Environmental light-dark conditions are changed depending on season. This photoperiodic change is important for seasonal reproduction in some animals (Nakao et al., 2008; Yoshimura et al., 2003). The long day photoperiod also changes cellular networks in the SCN, and decouples circadian oscillatory cell groups (Hazlerigg et al., 2005; Inagaki et al., 2007; Naito et al., 2008). Recently the role of $GABA_A$ receptors in coupling SCN dorsal and ventral circadian oscillators was reported to occur during the long day photoperiod (Evans et al., 2013) (Figure 3C). Using PER2::LUC reporter mice, they exposed mice to 20 hours of light and 4 hours of dark (LD20:4), and measured PER2::LUC bioluminescence from the SCN slice. In these conditions (LD20:4), the circadian phase of the SCN core was advanced compared to that of the shell. This phase difference between dorsal and ventral region in the SCN gradually

retuned 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, bicuculine, was applied SCN cells in slice. Bicuculline application attenuated re-synchronization when the circadian phase between dorsal and ventral regions was out of phase, but not in-phase. These results suggest that $GABA_A$ signaling contributes to the synchronization of circadian rhythms between the dorsal and ventral SCN in a state-dependent manner. These results also suggest that GABA is sufficient for the synchronization of dispersed SCN cells (Liu and Reppert, 2000), and that absence of GABA does not desynchronize cellular rhythms under steady-state networks in the SCN (Aton et al., 2006).

4-4) Intracellular chloride concentrations and cellular coupling

Long day photoperiods also change the excitability (Farajnia et al., 2014), and levels of chloride transporter expression in SCN neurons. This determines the difference in circadian phase and period between the dorsal and ventral SCN (Myung et al., 2015) (Figure 3C). It was observed that under long day conditions, the phase difference in circadian *Bmal1-Eluc* (*Bmal1* promotor driven luciferase reporter) rhythms between the dorsal and ventral SCN were increased, and the circadian period of the dorsal SCN was decreased compared to that of the ventral SCN. Myoung et al also measured intracellular chloride concentrations in the SCN, using N-(ethoxycarbonylmethyl)-6-methoxyquinoliniumbromide (MQAE) 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 gastrin-releasing peptide (GRP) neurons, whereas NKCC1 is expressed in VIP, GRP, and VP neurons within the SCN (Belenky et al., 2010). Recently, Klett and Allen reported that intracellular chloride concentrations were higher during the day than at night in both AVP and VIP positive neurons (Klett and Allen, 2017). 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.

4-5) 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 (Prolo et al., 2005). In addition, astrocytes co-cultured with adult SCN explants significantly sustained the rhythms of astrocytes, suggesting that diffusible factors from the SCN are sufficient to entrain circadian oscillations in astrocytes (Prolo et al., 2005). VIP expressed in SCN neurons entrains astrocyte circadian rhythms (Marpegan et al., 2009). It is known that astrocytes release ATP into the extracellular space. Cultured astrocytes display daily oscillations of extracellular ATP concentrations that are under circadian control (Marpegan et al., 2011). Astrocytes regulate neuronal networks through the

reuptake and release of various transmitters including glutamate (Halassa and Haydon, 2010; Perea et al., 2009). 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 (Barca-Mayo et al., 2017; Brancaccio et al., 2017; Tso et al., 2017). Astrocyte specific deletion of the *Bmal1* gene reduced expression of astrocytic GABA transporter (GAT)1 and GAT3, suggesting a potential impairment in the clearance of extracellular GABA released by neurons (Barca-Mayo et al., 2017). 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 activation of NMDA receptors (NR2C), which subsequently facilitates neuronal GABA release (Brancaccio et al., 2017) (Figure 3D). Since the peak phase of circadian calcium rhythms in astrocytes were observed during the night, GABA release from neurons via glutamate release from astrocytes would increase during the night. As aresuls, neuronal activity would decrease during the night.

5. Circadian outputs and GABA functions

Circadian rhythms in SCN neurons synchronize each other via several neurotransmitters, such as AVP, VIP, GRP (Aton et al., 2005; Maywood et al., 2011; Maywood et al., 2006). 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 (Schwartz et al., 1987). Optogenetic stimulation of the SCN *in vivo* modulated circadian behavioral rhythms (Jones et al., 2015). GABA may therefore regulate the transmission of circadian output from the SCN by changing neuronal excitability.

Identification of SCN circadian outputs is important for understanding the mechanisms underlying the regulation of behavioral rhythms. Previously, SCN efferent projections have been investigated (Kalsbeek et al., 2006; Leak and Moore, 2001; Stephan et al., 1981; Watts and Swanson, 1987; Watts et al., 1987). Neurons in the dorsal SCN project densely to the preoptic area (POA), paraventricular nucleus (PVN), dorsomedial hypothalamus (DMH), and the 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) (Leak and Moore, 2001). Interestingly, the circadian rhythms of neuronal activity outside the SCN *in vivo* were in antiphase compared to the SCN in nocturnal animals (Inouye and Kawamura, 1979; Nakamura et al., 2008). 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 to that of the SCN (Sato and Kawamura, 1984), suggesting that circadian information from the SCN is transferred to output brain areas, and that this mechanism is different in nocturnal and diurnal animals. However, the mechanisms of switching day-night information from the SCN have not been

identified.

The PVN receives an efferent projection from the SCN. The PVN contains several neuropeptides such as corticotrophin releasing factor (CRF), oxytocin, and AVP, and regulates endocrine and autonomic functions. Electrical stimulation of the SCN evoked monosynaptic inhibitory postsynaptic potentials (IPSPs) as well as excitatory postsynaptic potentials (EPSPs) in the PVN, indicating that GABA and glutamate are important mediators of fast monosynaptic transmission from the SCN to PVN (Hermes et al., 1996a; Hermes et al., 1996b). Tousson and Meissl used a multi-electrode array dish to demonstrate that humoral factors are responsible for PVN (Tousson and Meissl, 2004). They observed circadian rhythms in PVN neuronal activity measured in brain slices containing both the PVN and SCN. When the SCN was removed from the brain slice, PVN circadian rhythms disappeared and 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 (Moore and Klein, 1974). Melatonin concentration is increased during the night, and decreased during the day. Blocking GABAergic transmission to the PVN results in inhibition of melatonin synthesis in the pineal gland (Kalsbeek et al., 1996; Kalsbeek et al., 2000), 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 (Liu et al., 1997; Scott et al., 2010).

Neuronal and humoral regulation of PVN neuronal activity by the SCN is important for temporal integration of physiological events.

6. 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 (Figure 4). Primary culture techniques (dispersed and slice) are useful for measuring SCN circadian rhythms. Usually neonatal SCN is used for this experiment (Herzog et al., 1998; Liu and Reppert, 2000; Nakamura et al., 2002; Welsh et al., 1995). Acute SCN slices from adult animals have also been used for recording circadian rhythms in the SCN (Albus et al., 2005; Green and Gillette, 1982; Wagner et al., 1997). Using neonatal SCN tissue allows us to measure circadian rhythms over long periods; however, the cellular properties and neuronal networks in the SCN may not be the same as the adult SCN. 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 developmental changes of chloride transporters modulate excitability to GABA depending on age (Ben-Ari, 2002). 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. Ono et al. demonstrated that *Cryptochrome* (*Cry*), a clock gene, is involved in cellular coupling in the adult SCN (Ono et al., 2013, 2016). GABA may have an important role in cellular coupling and circadian outputs in the SCN depending on developmental stage.

GABA functions have been studied using various recording methods, such as neuronal activity recording, calcium imaging, and bioluminescence imaging (Albus et al., 2005; Aton et al., 2006; Ikeda et al., 2003b; Irwin and Allen, 2009; Liu and Reppert, 2000; Shirakawa et al., 2000; Wagner et al., 1997). These recording methods are useful to measure circadian oscillations in the SCN, although circadian oscillation such as gene expression, intracellular calcium levels, and neuronal activity do not show 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-hour light/dark phase advance schedule. *Per1* and neuronal activity rhythms in SCN slices phase shifted immediately after the 6-hour phase advance, whereas *in vivo* neuronal activity did not immediately shift, indicating that neuronal activity was dissociated between brain slice and *in vivo* (Vansteensel et al., 2003). Ono et al., also demonstrated that circadian rhythms of the clock genes, *Per1* and *Bmal1,* were dissociated in cultured SCN slices (Ono et al., 2017). 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 recordings of circadian oscillation is a useful tool to understand the hierarchal structure of SCN networks, in addition to the roles of GABA within these networks.

7. Perspectives

All studies that have addressed the function of GABA in the SCN have only used pharmacological approaches. Since mice with genetic GABA deficiency, such as VGAT deficient- and GAD65/67-deficient mice, can not survive after birth (Kakizaki et al., 2015; Saito et al., 2010), 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 in 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 (Bedont et al., 2014; Hatori et al., 2014; Lee et al., 2015; Mieda et al., 2015). Creating conditional KO mice is useful; however, there are currently no SCN-specific Cre driver mice. Although Neuromedin-S is specifically expressed in the SCN, not all SCN neurons expressed this neuropeptide (Lee et al., 2015). Recently a Crispr/Cas9 genome editing method has been developed, and is a powerful tool to manipulate genes of interest in various species (Cong et al., 2013). Adeno associated viral driven Crispr/Cas9 techniques may be applicable to the study of GABA function specifically in the SCN (Ran et al., 2015). 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,

glycoprotein-deleted (DG) rabies virus is a useful tool for studies of neural circuits (Osakada et al., 2011). In combination with Cre mice, we can identify direct input and output pathways of specific neurons (Schwarz et al., 2015). However, since neurons infected with rabies virus are killed by approximately 14 days after infection (Osakada et al., 2011), manipulation or recording of neuronal activity is limited to this short time window. However, recently self-inactivating rabies virus has been developed, which may allow us to further understand neuronal brain networks (Ciabatti et al., 2017). AAV-mediated retrograde tracing methods were also developed, which allow us to label, manipulate, and measure retrograde-labeled neurons (Tervo et al., 2016). 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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Figures

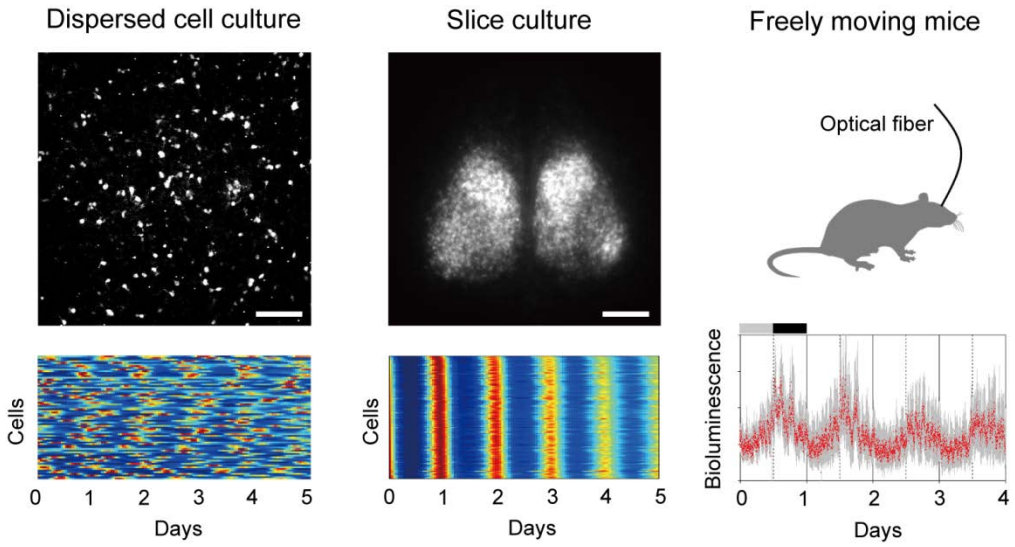

Figure 1: PER2::LUC rhythms in dispersed SCN cells, SCN slices, and the SCN of freely moving mice.

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) culture 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 four days. Neuronal networks are important for SCN cellular circadian rhythms synchronization. Scale bars are equal to 200 μm.

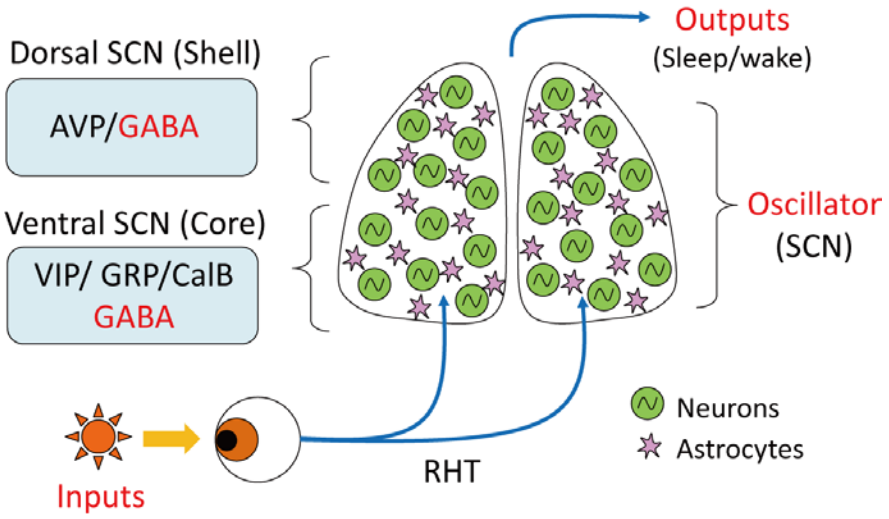

Figure 2: Schematic diagram of circadian organization in the SCN

Light input is transmitted to the ventral SCN through the retino-hypothalamic tract (RHT). Several neuropeptides are expressed in the SCN. 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.

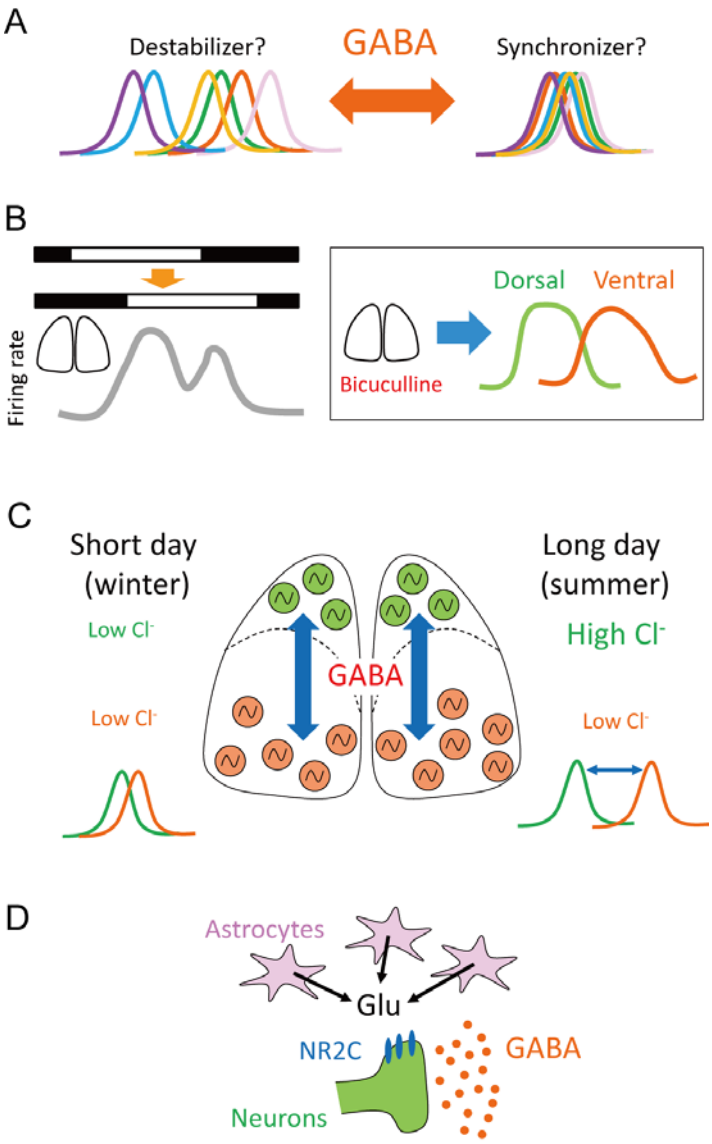

Figure 3: Possible roles of GABA in SCN cellular coupling

(A) GABA acts as both a synchronizer and destabilizer (B) Phase delay shift result in bimodal neuronal activity in the intact SCN slice; however, after 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.

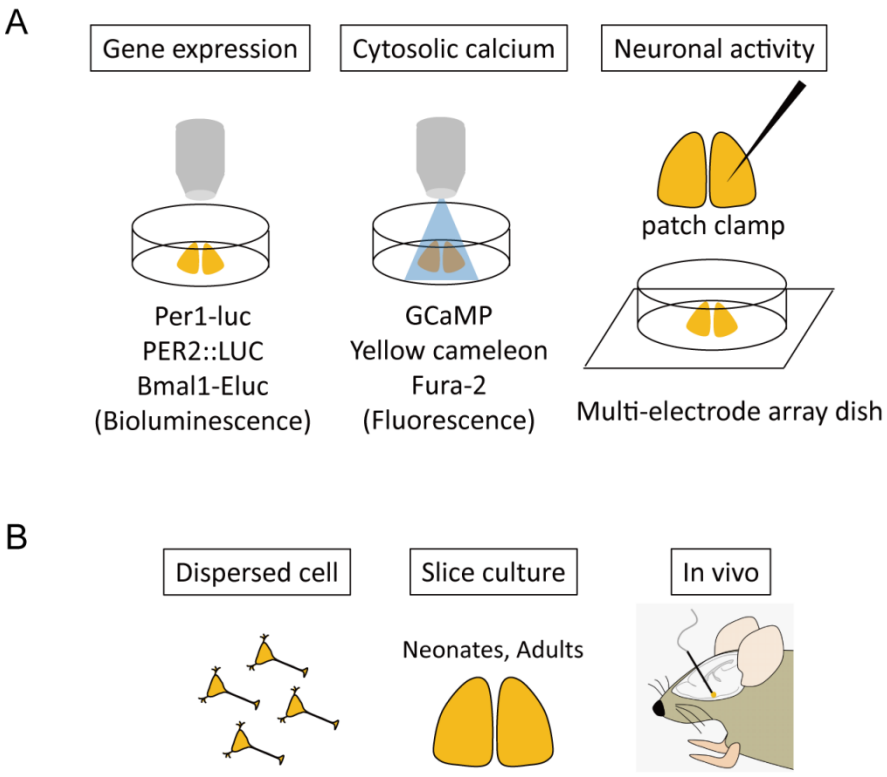

Figure 4: Variety of circadian rhythm recording techniques

(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 fluorescence 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 coupling strength and age of mice.

Compliance with Ethical Standards

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