Circadian rhythm in intracellular Cl\(^{-}\) activity of acutely dissociated neurons of suprachiasmatic nucleus

MASAHIKO SHIMURA, NORIO AKAIKE, and NOBUTOSHI HARATA

Department of Ophthalmology, Tohoku University School of Medicine, Sendai 980-8574, Japan; Cellular and System Physiology, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan; and Department of Molecular and Cellular Physiology, Beckman Center, Stanford University School of Medicine, Stanford, California 94305-5345

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Shimura, Masahtiko, Norio Akaire, and Nobutoshi Harata. Circadian rhythm in intracellular Cl\(^{-}\) activity of acutely dissociated neurons of suprachiasmatic nucleus. Am J Physiol Cell Physiol 282: C366–C373, 2002; 10.1152/ajpcell.00187.2000.—A link between the circadian rhythm and the function of Cl\(^{-}\)-permeable γ-aminobutyric acid (GABA) type A (GABA\(_A\)) receptors on suprachiasmatic nucleus (SCN) neurons was studied by measuring intracellular activity of Cl\(^{-}\) (\(a_{Cl^-}\)) at different times during a circadian cycle in SCN neurons acutely dissociated from rat brains. To measure \(a_{Cl^-}\), the voltage-clamp mode of the gramicidin-perforated patch-clamp technique was used, and reversal potential of GABA-induced currents (\(E_{GABA}\)) was converted to \(a_{Cl^-}\). Measured \(a_{Cl^-}\) was significantly higher at around noon (20.1 ± 1.4 mM) than at three other time zones of a circadian cycle (means ranging from 11.6 to 14.3 mM). Chord conductance of GABA-induced currents showed no circadian changes, indicating a lack of circadian changes in the number or single-channel conductance of GABA\(_A\) receptors. These results suggest that \(a_{Cl^-}\) participates in modulating GABA\(_A\) receptor functions on SCN neurons during the circadian rhythm.

γ-aminobutyric acid; γ-aminobutyric acid type A receptor; gramicidin-perforated patch clamp; rat

RECENT DEVELOPMENT OF MOLECULAR biological techniques has identified numerous genes involved in controlling circadian rhythms. An emerging view involves an intracellular transcriptional/translational feedback loop. Two clock proteins CLOCK and BMAL1 bind to DNA to enhance transcription of period (per) and timeless (tim) genes, increasing the amount of PER and TIM proteins. After a lag, PER and TIM feed back to negate the activation of CLOCK and BMAL1, reducing the amount of per and tim mRNA. Eventually the level of PER and TIM is reduced, and the loop is completed (6, 9). Some of the clock-controlled genes were also shown to be regulated by this feedback loop (15). Clock genes and proteins are expressed in suprachiasmatic nucleus (SCN), and they control the firing rate of individual SCN neurons (13), suggesting that the circadian mechanism is operational at the individual cellular level. However, outputs of this loop and, therefore, the functional correlates of changes in clock genes and proteins remain unknown.

Physiologically, evidence has been building in support of γ-aminobutyric acid (GABA) as a neurotransmitter that controls circadian rhythms in mammals by its action on Cl\(^{-}\)-permeable GABA type A (GABA\(_A\)) receptors. Administration of GABA or its modulators effectively changes the phases of circadian rhythms. For example, application of muscimol, a specific GABA\(_A\) receptor agonist, in SCN in vivo induces phase shifts in locomotor activity of rodents, and the effect was blocked by a competitive GABA\(_A\) receptor antagonist, bicuculline, or a Cl\(^{-}\) channel blocker, picrotoxin (36). The circadian rhythm of locomotor activity was modified by intraperitoneal injection of diazepam, an allosteric GABA\(_A\) receptor modulator (29). SCN neurons in vitro showed circadian rhythm in firing rate (10, 11, 24, 33). Muscimol inhibited the spontaneous firing of SCN neurons in slices (21), leading to a phase-resetting effect (39). Furthermore, picrotoxin produced phase advances in the firing rate of SCN neurons in slices (30). We also showed (34) the presence of GABA\(_A\) receptors on individual SCN neurons in rats by acutely isolating the neurons from surrounding tissue and monitoring the effects of GABA and related modulators by a patch-clamp technique.

One possible link between the circadian rhythm and the function of GABA\(_A\) receptor on SCN neurons is changes in intracellular Cl\(^{-}\) activity (\(a_{Cl^-}\)). The GABA\(_A\) receptor forms a Cl\(^{-}\) channel (14). Therefore, the amplitude of the inhibitory postsynaptic current or the inhibitory postsynaptic potential mediated by GABA\(_A\) receptors is directly modified by changes in Cl\(^{-}\) equilibrium potential (\(E_{Cl^-}\)), which is determined by the ratio of Cl\(^{-}\) activities inside and outside of neurons (\(a_{Cl^-}\) and \(a_{Cl^-}\)). Recently, \(a_{Cl^-}\) has been shown to be a variable parameter, not a fixed constant, during neuronal development (17, 27, 38). There is also an indication that \(a_{Cl^-}\) differs between day and night in...
SCN (43). Circadian change in \( \alpha Cl^- \) of SCN neurons would modify GABA\(_A\) receptor activity by changing \( E_{Cl^-} \) and could contribute to generating and/or modifying circadian rhythm in vivo.

To test this hypothesis in a quantitative manner, we measured \( \alpha Cl^- \) in SCN neurons acutely dissociated from the rat brain at different times during the circadian cycle. To measure \( \alpha Cl^- \), we used the voltage-clamp mode of the gramicidin-perforated patch-clamp technique, which allows recording of \( Cl^- \) current through GABA\(_A\) receptors without disrupting \( \alpha Cl^- \) (7).

**MATERIALS AND METHODS**

Preparations. The isolation technique used here was similar to those previously described (34). Briefly, 11- and 12-day-old Wistar rats of either sex were maintained under a 12:12-h light-dark cycle (lights on 0700–1900). They were decapitated, and the brains were quickly removed from the skull and sliced coronally at 400-µm thickness. The slices were preincubated in an incubation solution well saturated with 95% O\(_2\)-5% CO\(_2\) for 30–60 min at room temperature (23–25°C). Thereafter, the slices were treated in an incubation solution containing 1 mg pronase/6 ml at 37°C for 25 min. The SCN, ~400 µm in diameter, was punched out by a needle (150 µm in diameter) and transferred into a 35-mm culture dish (Primaria 3801-Falcon, Becton Dickinson, Franklin Lakes, NJ) filled with a standard external solution. The SCN neurons were mechanically isolated with fire-polished Pasteur pipettes with tip diameters of 100–450 µm.

Electrophysiological study. The electrophysiological measurements were performed using either nystatin- or gramicidin-perforated patch recording under voltage-clamp conditions (1, 7). The patch pipettes were pulled from glass capillaries (outer diameter 1.5 mm). The resistance of the patch pipette was 3–7 MΩ. A liquid junction potential of 4 mV was used to calibrate the holding potential. Ionic currents were measured with a patch-clamp amplifier (Axopatch-1C, Axon Instruments, Foster City, CA). After formation of a gigahm seal, GABA (3 × 10^{-5} M) was repetitively applied at 5-min intervals until two consecutive stable currents were obtained. The number of applications was minimized by delaying the first application by ~10 min. Once a stable response was obtained, ramp voltage commands were given before and during application of GABA, to examine the current-voltage (I-V) relationship. After this measurement, GABA was applied again to confirm the stability of the response amplitude. Series resistance was <30 MΩ. All experiments were carried out at room temperature (23–25°C).

For temporal analysis of circadian changes, the following three time parameters were used: 1) time of death of the animals, 2) “recording time” (when the I-V relationship was examined in a neuron), and 3) “lapse time” (time between death and recording). Midnight was defined as 0000. Time of death and recording time were grouped into four time zones of equal duration (6 h).

Fluorescent Nissl staining. Localization of SCN was confirmed in our slices by fluorescent Nissl staining. Rat pups were anesthetized with ether and transcardially perfused with cold standard external solution. Brain slices through SCN were obtained with 400-µm thickness and immediately fixed in 4% paraformaldehyde in Dulbecco’s modified phosphate-buffered saline (PBS; D-5652, Sigma, St. Louis, MO) at 4°C for 1 h. The slices were rinsed in PBS containing 100 mM glycine for 3 h, rinsed in PBS containing 0.1% sodium borohydride for 10 min, rinsed in PBS for 1 h, permeabilized by 0.1% Triton X-100 in PBS for 20 min, and rinsed in PBS for 20 min. They were then treated with 5% Neurored red fluorescence Nissl stain (Molecular Probes, Eugene, OR) in PBS for 40 min. They were treated with 0.1% Triton X-100 in PBS for 10 min, rinsed in PBS for 6 h, and mounted in Citifluor (Ted Pella, Redding, CA). Except where otherwise stated, every treatment was done at room temperature. Fluorescence was detected with a Texas red filter set (excitation 560/55, dichroic 595, emission 645/75 nm).

**RESULTS**

SCN neurons in slices and after acute dissociation. For acute dissociation of SCN neurons in electrophysiological studies, we punched out the paramedian region that lies above the optic chiasm. Localization of SCN was confirmed in our preparation with fluorescent Nissl staining. Although SCN was not clearly identifiable under phase-contrast optics (data not shown), it was visualized by fluorescence microscope as a paired ovoid structure lying dorsal to the optic chiasm (Fig. 1A). SCN demonstrated the highest density of neurons in the hypothalamus, as reported previously in studies with conventional Nissl staining (25, 41). Within SCN, neuronal density was highest in the dorsomedial region (Fig. 1A), as reported previously (41).

Dissociated SCN neurons are shown in four images in Fig. 1B. They were monopolar (Fig. 1Ba), bipolar (Fig. 1Bb and Bc), or tripolar (Fig. 1Bd). These morphologies were similar to those of monopolar, bipolar, and radial multipolar neurons found in SCN in situ.
(41), indicating that the acute dissociation was performed properly.

**IGABA recorded with nystatin-perforated patch method.** When the nystatin-perforated patch-clamp technique was used, application of $3 \times 10^{-5}$ M GABA to acutely dissociated SCN neurons induced an inward current at a holding potential ($V_H$) of $-40$ mV (Fig. 2A, left). The current was completely blocked by pretreating the cell with $10^{-4}$ M bicuculline (Fig. 2A, right), a competitive antagonist of GABA$_A$ receptor. This indicates that the $I_{GABA}$ was mediated by GABA$_A$ receptor. To measure the reversal potentials, a ramp voltage command was applied before and during the application of $3 \times 10^{-5}$ M GABA (Fig. 2A, left). The ramp command was linearly changed from a $V_H$ of $-40$ to $+20$ mV, then to $-100$ mV, and back to $-40$ mV during a 1-s period (Fig. 2B, inset). The reversal potential of $I_{GABA}$ ($E_{GABA}$) was measured as the voltage at which $I$-$V$ curves before and during $I_{GABA}$ intersect (Fig. 2, B and D). $E_{GABA}$ measured with the nystatin method was $-3.8 \pm 0.5$ mV ($n = 20$). This was close to the theoretical $E_{Cl}$ of $-4.1$ mV calculated with the Nernst equation. These results confirm that GABA$_A$ receptor mainly allows permeation of Cl$^-$ and give credence to the assumption that $E_{GABA} = E_{Cl}$ in acutely dissociated SCN neurons under the current experimental conditions.

**IGABA recorded with gramicidin-perforated patch method.** Gramicidin forms Cl$^-$-impermeable cation channels that allow ionic current measurement without disrupting $aCl^-$ (7). When the gramicidin-perforated patch method was used, the application of $3 \times 10^{-5}$ M GABA induced an outward current at the same $V_H$ (Fig. 2C, left). The current was blocked by $10^{-4}$ M bicuculline (Fig. 2C, right), as with the nystatin-perforated patch method (Fig. 2A). These results indicate that $I_{GABA}$ was still mediated by GABA$_A$ receptor and that $E_{GABA}$ had shifted to a potential more hyperpolarized than $V_H$. This result was confirmed by analyzing the $I$-$V$ relationship (Fig. 2D). With the same ramp voltage command as in Fig. 2B, $E_{GABA}$ with the gramicidin method was measured as $-54.3 \pm 1.1$ mV ($n = 44$).

**Conversion of $E_{GABA}$ to $aCl^-$.** With the measured $E_{GABA}$ (gramicidin method) and the given $aCl^-$ of 114.5 mM in our solution, it is possible to calculate $aCl^-$, using a Nernst equation

$$aCl^- = aCl^+ \times e^{(E_{GABA} - RT)/F} = 114.5 \times 10^{E_{GABA}/58}$$

where $R$ is the gas constant, $T$ is temperature, $Z$ is valence, and $F$ is the Faraday constant.
tion assumes that the GABA<sub>A</sub> receptor is exclusively permeable to Cl<sup>-</sup> in our nominally HCO<sub>3</sub>-free external solution. The measured \( E_{GABA} \) ranged between –37 and –64 mV, and the calculated \( aCl^- \) ranged between 9.0 and 26.4 mM (n = 44).

**Relationship between \( E_{GABA} \), \( aCl^- \), and time of death.** To examine a circadian change in \( aCl^- \), the measured \( E_{GABA} \) and calculated \( aCl^- \) were plotted against time of death (Fig. 3). With division of 24 h into four time zones (Fig. 3B), the 1200 zone showed \( aCl^- \) of 20.1 ± 1.4 mM (n = 13). The values were significantly higher than in the three other zones. \( aCl^- \) was 11.9 ± 0.8 mM (n = 12) for the 0000 zone (\( P < 0.0001 \)), 11.6 ± 0.5 mM (n = 9) for the 0600 zone (\( P < 0.0001 \)), and 14.3 ± 1.3 mM (n = 10) for the 1800 zone (\( P < 0.01 \)). These results clearly indicate that \( aCl^- \) showed a circadian change.

**Lack of relationship between \( aCl^- \), recording time, and lapse time.** In addition to time of death, we analyzed the effects of different recording times on \( aCl^- \). The plot of \( aCl^- \) against the recording time of individual neurons did not show a significant difference among the four time zones (\( P > 0.1 \); Fig. 4A).

Another temporal parameter is lapse time. If dissociated neurons showed time-dependent deterioration, it is possible that neurons with longer lapse time could not sustain a constant level of \( aCl^- \) lower than passively acquired \( aCl^- \). In this case, \( aCl^- \) would become higher as lapse time prolonged. However, \( aCl^- \) did not show positive correlation with lapse time (\( r = 0.071, P > 0.1, n = 44 \) (Fig. 4B)). This finding excludes the possibility that the observed change in \( aCl^- \) was induced by artifactual deterioration of neurons.

**Chord conductance of GABA<sub>A</sub> receptor-Cl<sup>-</sup> channel complex.** We used another set of analyses to address whether there was a circadian change in the number or single-channel conductance of GABA<sub>A</sub> receptors. The effect of open GABA<sub>A</sub> receptors is determined by a simple equation

\[
I = N \times g \times (E_{GABA} - E_{rest})
\]

where \( I \) is the amplitude of \( I_{GABA} \) from the whole neuronal surface, \( N \) is the number of channels on the neuron, \( g \) is the single-channel conductance, and \( E_{rest} \) is the resting membrane potential. The first two factors on the right-hand side of Eq. 2 can be lumped into a single parameter, chord conductance (G)

\[
G = N \times g
\]

changing Eq. 2 to

\[
I = G \times (E_{GABA} - E_{rest})
\]
Under the voltage-clamp condition used in this study
\[ E_{\text{rest}} = V_H \]  
Therefore, Eq. 4 is rewritten as
\[ I = G \times (E_{\text{GABA}} - V_H) \]  
We measured the transient peak amplitude of \( I_{\text{GABA}} \) as \( I \) and used Eq. 6 to calculate \( G \) for each recorded neuron. Figure 5 shows the plots of \( G \) against time of death (Fig. 5A), recording time (Fig. 5B), and lapse time (Fig. 5C). The three plots showed no time-zone-dependent changes (\( P > 0.1, n = 44 \)). This result demonstrates that there were no time-dependent changes in the lumped parameter representing a number and/or a single-channel conductance of GABA_A receptors.

Lack of correlation of chord conductance with measured \( aCl^- \). One general drawback of the patch-clamp technique is that high series resistance results in more depolarized reversal potentials (closer to 0 mV). This is especially true for perforated patch methods if ionophores (gramicidin channels) are not functioning well. This artifact can be excluded by examining \( G \) because \( G \) takes a smaller value when ionophores are closed. However, the plot of \( G \) against \( aCl^- \) showed a lack of correlation (\( r = 0.175, P > 0.1, n = 44 \); Fig. 6). These results ensure that the circadian change in \( aCl^- \) was accurately measured.

**DISCUSSION**

We have shown the circadian change in \( E_{\text{GABA}} \) in acutely dissociated SCN neurons of rats. In our previous work (34), we showed that GABA_A receptors on SCN neurons are mainly permeable to Cl^- under the present experimental conditions, demonstrating that \( E_{\text{GABA}} \) is equivalent to \( E_{\text{Cl}} \). Because \( aCl^- \) is assumed to be constant as a result of rapid perfusion of extracellular solution around dissociated neurons (19), a circadian change in \( E_{\text{GABA}} \) (or \( E_{\text{Cl}} \)) indicates a circadian change in \( aCl^- \). In contrast, conductance of GABA_A receptors in each neuron remained constant. These
findings indicate that $\alpha Cl^-$ alone is responsible for circadian change in GABA$_A$ receptor functions in the present preparation. GABA is not a static inhibitory transmitter. Response to GABA changes under various conditions. GABA is depolarizing in immature neurons, whereas it changes to hyperpolarizing in more mature neurons (3, 5). To support this view, several groups measured the neuronal $\alpha Cl^-$ using gramicidin patch. It was assessed in rat hypoglossal motoneurons with reversal potentials of glycine currents, with a reduction from 33 to 8 mM during the first weeks of postnatal development (35). This corresponded to a shift from depolarizing to hyperpolarizing effects. In rat cerebral cortical neurons, it was 37 mM at embryonic day 16 and was gradually reduced to 12 mM at postnatal day 16 (28). In acutely dissociated Meynert neurons, $\alpha Cl^-$ showed a similar decrease: 33.4, 21.1, and 11.3 mM in 0- to 1-day-old, 2-wk-old, and 6-mo-old rats, respectively (31). In addition to these developmental changes in $\alpha Cl^-$, that span days to weeks, a change within a shorter time was reported. Normal $\alpha Cl^-$ in gerbil inferior colliculus neurons was 8 mM, but it was increased to 22 mM after 1 day of afferent denervation (40). Even faster change in $\alpha Cl^-$ was suggested, although not quantitatively, in cultured hippocampal neurons, in which activity-dependent synaptic plasticity induced a change in $\alpha Cl^-$ over a time scale of minutes (8). Our quantitative analysis showed that average $\alpha Cl^-$ in SCN neurons ranged between 11.6 and 20.1 mM in four time zones and that $\alpha Cl^-$ at noon (20.1 mM) was 1.4- to 1.7-fold higher than in the other time zones (Fig. 3). Thus a circadian change in $\alpha Cl^-$ was within the reported ranges of concentration and time.

The effect of GABA on the activity of SCN neurons has been documented by several groups. They showed inhibitory effects of GABA on spontaneous firing rate during day and night (20, 22, 23). One possible reason why these reports did not observe circadian changes between day and night (enhancement during day and suppression during night) might be the use of non-physiological pipette solutions for extracellular recording. These solutions were either 0.5 M NaCl (22, 23) or 0.5 M Na acetate (20). Leakage of the pipette solutions into extracellular space would change $\alpha Cl^-$, shifting $E_{GABA}$ to a different level. Depending on the variable degrees of leakage and variable distances of pipettes to recorded cells, there could have been cell-to-cell differences in $\alpha Cl^-$, obscuring circadian changes in $E_{GABA}$. Furthermore, the effects of GABA on spontaneous firing rate are influenced by multiple factors, including the driving force determined by $E_{GABA}$ and $E_{rest}$, the properties of Na$^+$ and K$^+$ channels, and shunting inhibition, to name a few, rendering the interpretation of the results more difficult. Another factor is the age of the animals (not given in Refs. 20, 22, and 23). In one study, suppressing effects of GABA on the firing rate were reported in rats over 3 wk of age, using 1) multiple-unit extracellular recording with a platinum-iridium wire electrode, 2) cell-attached recording, and 3) gramicidin-perforated patch recording (12). In light of the developmental change in $\alpha Cl^-$, assessment of circadian changes in $\alpha Cl^-$ would not be straightforward if assessment was done using a crude factor, firing rate, and especially if the reported $E_{rest}$ was depolarized (−47 mV) (Ref. 12; see below).

To reconcile discrepancies among previous reports, we present here a hypothesis on circadian changes in $E_{GABA}$ ($E_{Cl}$) in SCN neurons (Fig. 7). $E_{GABA}$ shows a developmental, hyperpolarizing change described by a single exponential function (3). Circadian change in $E_{GABA}$ overlies this downward trend. Assuming $E_{rest}$ to

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**Fig. 5.** Lack of correlation of chord conductance ($G$) with time of death (A), recording time (B), or lapse time (C). Linear regression line was drawn with $r = 0.116$ ($P > 0.1, n = 44$).
be from −55 to −60 mV (4, 42) and to be constant throughout development, the effect of GABAergic synaptic transmission could be uniformly depolarizing during early development, mixed (depolarizing-hyperpolarizing) during middle stages of development, and uniformly hyperpolarizing late during development. The subtle differences and/or degrees of depolarization or hyperpolarization induced by GABA might have been difficult to assess by firing rate alone, because the effect of GABA on membrane potential is a continuous function of $E_{\text{GABA}}$ and $E_{\text{rest}}$, whereas the firing of Na$^+$ action potentials is a step function with a certain threshold. Differential GABA effects are clearly demonstrated only with voltage-clamp experiments as in this study.

αCl$\text{−}$ is balanced by influx and efflux of Cl$^−$ across neuronal plasma membrane. Several transporters control these processes. Major transporters that increase αCl$\text{−}$ under physiological conditions are the Na$^+$-K$^+$-Cl$^−$ cotransporter and the Cl$^−$-HCO$_3^−$ exchanger. Transporters that decrease αCl$\text{−}$ are the K$^+$-Cl$^−$ cotransporter, Cl$^−$-ATPase, and the Na$^+$-dependent Cl$^−$-HCO$_3^−$ exchanger (16). Of these transporters, the Na$^+$-K$^+$-Cl$^−$ cotransporter and the K$^+$-Cl$^−$ cotransporter merit further consideration. Developmental change in αCl$\text{−}$ of hippocampal pyramidal neurons was mediated by increased expression levels of the Cl$^−$-extruding K$^+$-Cl$^−$ cotransporter KCC2. Blockade of the transporter by antisense RNA led to a dramatic reduction in the hyperpolarizing GABA responses in mature neurons, which normally exhibited hyperpolarization in response to GABA (32). A similar change in lateral superior olive neurons was made possible by an interplay of the K$^+$-Cl$^−$ cotransporter and the Na$^+$-K$^+$-Cl$^−$ cotransporter (17). On the basis of these reports, we propose that the activity of one (or some) of the transporter proteins that controls αCl$\text{−}$ is actively involved in circadian rhythm in SCN neurons.

The present study does not preclude the possibility that αCl$\text{−}$ also shows circadian changes in situ. The microenvironment of a narrow synaptic cleft has been shown to drastically change on synaptic stimulation. For example, extracellular Ca$^{2+}$ concentration in the synaptic cleft was reduced by at least one-third when pre- or postsynaptic neurons were activated and there was a large Ca$^{2+}$ influx into neurons (2, 37). This opens a possibility that αCl$\text{−}$ in the synaptic cleft might also show a circadian change, although small in effect, if neuronal firing rate shows a circadian change and Cl$^−$ influx/efflux is altered due to variable postsynaptic GABA$_A$ receptor activation by released GABA.

We found that circadian rhythm was not evident in recording time or lapse time, whereas it was evident in time of death. These results indicate that αCl$\text{−}$ in neurons was frozen after death of the animals and dissociation of neurons. The lack of changes in αCl$\text{−}$ during lapse time further suggests that there was little deterioration of the preparation, because deterioration would lead to lessening of the difference between αCl$\text{−}$ and αCl$\text{−}$ and therefore a higher αCl$\text{−}$ and more depolarized $E_{\text{GABA}}$. The freezing effect could be brought about by the lack of synaptic inputs in our preparation. The acute dissociation procedure removed most of the synapses that had formed close to maturation level on postnatal days 11–12 (25). Although the circadian change in firing rate of cultured SCN neurons was shown to be independent of the presence of synaptic inputs (44), further investigation is needed to elucidate the effect of synaptic inputs on the circadian change in αCl$\text{−}$.

![Image](http://ajpcell.physiology.org/)

**Fig. 7. Hypothesis on circadian changes in $E_{\text{GABA}}$.** Developmental change in $E_{\text{GABA}}$ ($E_{\text{GABA}}$) has been shown to be exponential (3). $E_{\text{rest}}$, resting membrane potential of SCN neurons.

**REFERENCES**

CIRCADIAN RHYTHM IN $\text{ACl}^-$ OF SCN NEURONS


