



## Immunohistochemical Localization of Hyperpolarization Activated Current Channel Subunits in Rat Suprachiasmatic Nucleus

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**Methods:** Using immunohistochemistry, the distribution of HCN1, HCN2, and HCN4 in the SCN was studied. Both HCN1 and HCN2 subunits were present in the SCN but with different patterns of localization. HCN4 was not detected in the SCN.  $I_h$  was also recorded from SCN neurons using whole cell voltage clamp.

**Results:** The results show that  $I_h$  is functionally well expressed in SCN neurons. 84% of SCN neurons exhibited  $I_h$ .  $I_h$  recorded had the activation constant ( $\tau$ ) of  $236 \pm 2$  ms and amplitude of  $25 \pm 1$  pA at -40 mV step. At -60 mV step,  $\tau$  was significantly reduced to  $167 \pm 3$  ms ( $p < 0.5$ ) while the current amplitude was significantly increased to  $34 \pm 2$  pA ( $p < 0.01$ ).

**GJMR-K Classification:** NLMC Code: WL102



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**Methods:** Using immunohistochemistry, the distribution of HCN1, HCN2, and HCN4 in the SCN was studied. Both HCN1 and HCN2 subunits were present in the SCN but with different patterns of localization. HCN4 was not detected in the SCN.  $I_h$  was also recorded from SCN neurons using whole cell voltage clamp.

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**Conclusion:** Our results show that  $I_h$  plays a major role in the SCN excitability and therefore may regulate its circadian function.

## 1. INTRODUCTION

The suprachiasmatic nucleus (SCN) is the mammalian circadian pacemaker. As a part of the central nervous system (CNS), SCN neurons exhibit different ionic currents e.g. sodium currents, potassium currents, and calcium currents (1, 2). Of particular interest is the hyperpolarization-activated current ( $I_h$ ), as it has been proposed to play a role in the regulation of spontaneous firing as well as the excitability of SCN neurons (3). This current has been identified in different tissues including the brain and the sino-atrial node (SAN) of the heart (4). Depending on the tissue, the current has different names, e.g.  $I_f$  and  $I_q$  (5,

6). In pacemaker cells,  $I_h$  plays an important role in cell depolarization and the generation of rhythmic activity (7).

$I_h$  is a slowly activating inward mixed  $\text{Na}^+/\text{K}^+$  current. It activates at hyperpolarizing steps more negative than the resting membrane potential (-50 to -70 mV) and slowly depolarize the cell membrane towards its equilibrium potential (8). This current may be further identified by its sensitivity to  $\text{Cs}^{++}$ , which is known to substantially block  $I_h$  at concentrations of 1-3 mM (8, 9). It has been suggested that the physiological role of this current is to limit the duration of hyperpolarizing events and maintain the membrane potential close to the firing threshold (3). In addition, McCormick and Pape (10) have shown that  $I_h$  promotes spontaneous firing in thalamic neurons.

The mammalian Hyperpolarization-activated and Cyclic Nucleotide-gated non-selective cation channels (HCN) that generates  $I_h$  is encoded by four members of a gene family (HCN1-4) (11-14), three of which are expressed in rodent hippocampus (HCN1, 2 and 4) (15). Each HCN is composed of six trans-membrane domains with a pore region between S5 and S6 and a cyclic nucleotide-binding domain in the cytoplasmic C-terminal (9, 11, 16). Studies have shown that four HCN subunits can co-assemble to form a homomeric channel with different functional characteristics. For example, homomeric HCN1 activates rapidly (tens of milliseconds) on hyperpolarization and is modulated by c-AMP, while HCN2 is slower to activate (hundreds of milliseconds) with less sensitivity to c-AMP (11, 13). HCN4 homomeric channels show strong modulation by c-AMP and even slower activation (seconds) (12, 14).

There has been inconsistency in the reports regarding the percentage of cells expressing  $I_h$  in the SCN (3, 17). Also, while immunohistochemical studies have reported the localization of HCN channel subunits in the brain in general (7, 18, 19), but to date no one has specifically described the pattern of distribution of these subunits in the SCN. Therefore, this study aimed to determine the distribution of HCN channel subunits in the SCN to have a better understanding of the role of  $I_h$  in the circadian time keeping function in the SCN.

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## II. METHODS

### a) Animals

Long Evans rats (Charles River, Wilmington MD) 4-6 weeks old were entrained for at least 2 weeks to a 12/12 light/dark (LD) cycle prior to experiments. Animals were housed in groups of 4 rats per cage and provided with rodent chow and water ad libitum.

For immunohistochemistry, rats were anesthetized at circadian time (CT) 10-14 with Nembutal Sodium Solution (100 mg/kg) (Abbott Laboratories, Chicago, IL, USA) then fixed by cardiac perfusion using heparinized saline followed by 4% paraformaldehyde fixative in phosphate buffer saline (PBS). Brains were removed and kept in fixative for at least one hour at room temperature. Brains were then rinsed several times with PBS and kept at 4° C until used. At the time of the experiment brains were further cut into blocks containing the hypothalamus. Coronal slices (thickness 70  $\mu\text{m}$ ) containing the SCN were prepared from each brain using a vibrating tissue slicer (Vibratome, TCI, St Louis, MO).

All animal care, handling and sacrifice were in accordance with a protocol approved by the Creighton University Institutional Animal Care and Use Committee.

### b) Immunohistochemistry (IHC)

IHC was performed on free-floating sections (70  $\mu\text{m}$ ) using standard avidin-biotin complex methods as previously described (20). Coronal brain sections were incubated at 4°C in PBS in 24-well plates (Corning Inc., Corning, NY). Sections were washed several times with 0.3% Triton X-100 (Sigma, St. Louis, MO) in PBS (PBS-T) before treatment with 0.3% H<sub>2</sub>O<sub>2</sub>/PBS 30 minutes. Non-specific binding sites were blocked using 2% normal goat serum in PBS for 1 hour. After rinsing thoroughly with PBS-T for at least 15 minutes, sections were incubated for about 36 hours at 4°C with rabbit anti HCN1, 2 or 4 polyclonal antibodies (Chemicon International, Temecula, CA) at concentrations of 1:500 or 1:1000 in PBS then washed three times with PBS-T for at least 10 minutes. Sections were then incubated with biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA) for 1 hour. After washing for 10 minutes with PBS-T, sections were incubated for 2 hours in avidin-biotin-peroxidase complex (ABC) solution (1: 100; Vector Laboratories). The reaction product was visualized by incubating the sections with 0.04% 3, 3' diaminobenzidine (DAB) containing 0.01 H<sub>2</sub>O<sub>2</sub> for 4 to 5 minutes with PBS. Specimens were visualized using an Axioskop II microscope (Carl Zeiss Jena, Jena, Germany) equipped with 40x and 100x objectives. Negative controls were performed by omission of the primary antibody. Images were obtained using a Spot RT digital camera (Diagnostic Instruments, Sterling Heights, MI). Images were prepared using Adobe Photoshop (Adobe Systems, San Jose, CA).

### c) Electrophysiological recording

#### i. Hypothalamic slice preparation

Rats were decapitated quickly after a brief exposure to CO<sub>2</sub> and brains were rapidly removed and cut into blocks containing the hypothalamus. Blocks were chilled on ice-cold artificial cerebrospinal fluid (a-CSF). One 400-500  $\mu\text{m}$  thick slice containing the SCN was prepared from each brain using a vibrating tissue slicer (TCI, St Louis, MO). Slices were prepared as described in Hallworth et al. (21) but in a coronal orientation.

Slices were then incubated in warm a-CSF at room temperature under atmosphere of 95% O<sub>2</sub>, 5% CO<sub>2</sub>. The a-CSF contained (in mM) NaCl 122, KCl 3.8, MgSO<sub>4</sub> 1.2, KHPO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25, CaCl<sub>2</sub> 25, Dextrose 10, and bubbled with 95% O<sub>2</sub>, 5% CO<sub>2</sub>. After incubation for at least one hour, the slice was transferred, and secured to the floor of the recording chamber (RC-25 Warner Inst. Hamden, CT). The slices were continuously perfused with a-CSF and maintained at 32°C using a single channel heater controller (TC-324B, Warner Inst., Hamden, CT). The slice was viewed using a Nikon upright microscope (Japan) and the entire preparation was mounted on an air table.

#### ii. Whole cell voltage clamp

The blind whole cell patch clamp method (22) was used to obtain voltage clamp recordings from SCN neurons. Patch pipettes were pulled from borosilicate glass (Dagan Corp., Minneapolis, MN) using Flaming/Brown electrode puller (Sutter P-97, Novato, CA). Pipettes were polished using a Narshige microforge (MF-830) (Japan) and filled with an intracellular solution containing in mM: EGTA 5, HEPES 10, MgCl<sub>2</sub> 1, K-gluconate 130, NaCl 1, CaCl<sub>2</sub> 1, K<sub>2</sub>ATP 2, osmolarity adjusted to 280 mOsm and pH 7.2. The polished and filled electrode was advanced slowly into the SCN, using an MP-285 (Sutter Inst., Novato, CA) micromanipulator. To keep the tip clean positive pressure was maintained on the electrode interior. Pipette resistance was measured from the current response to a 5 mV step voltage command. Once in contact with the cell, the positive pressure was relieved and gentle suction was applied intermittently. Sealing was indicated by the increase of pipette resistance to at least 1 G $\Omega$ . A holding potential of -70 mV was applied to the pipette, then the interior of the cell was accessed by a brief pulse of suction to the pipette and the cell was maintained under voltage clamp for recording. Data acquisition was performed using a Warner Instruments (Warner Inc., Hamden, CT) patch clamp amplifier and recorded using a PC equipped with a Keithley data acquisition board (Keithley Inst. Inc., Cleveland, OH) and software written in TestPoint (CEC, Corp., Bedford, NH). Series resistance was compensated for as much as possible (about 60-80%). Series resistance was monitored from the height of the initial transient response to the same 5

mV step voltage command that was used to measure seal resistance.

iii. *Data analysis*

Currents waveforms, in voltage clamp mode, were fitted to exponential function using Microcal Origin software 6.0 (Northampton, MA). Averaged data are presented as means  $\pm$  SE.

### III. RESULTS

a) *HCN1 immunoreactivity in the brain*

To demonstrate the specificity of the antibodies, the distribution of the immunoreactivity in the rat brain with HCN1, HCN2 or HCN4 was compared to previous studies (7, 18, 19). Labeling, which consisted of dark brown reaction product, was found in the cerebral cortex as well as the hippocampus and exhibited a distribution in these areas consistent with the results of those studies. In the cerebral cortex, there was strong immunoreactivity for HCN1 in layers 1, 2, 3 and 4. The labeling was more intense towards the apical dendrites (Fig.1A). Also the cell bodies did not show any labeling. In the hippocampus, intense labeling was observed in both CA1 and CA2 mainly in the apical dendrites as with cortical neurons the cell bodies were also immune negative (Fig.1B). In the SCN, intense HCN1 labeling was observed within the SCN as well as toward the optic chiasm (Fig.1C).

b) *HCN2 immunoreactivity in the brain*

HCN2-immunopositive cell bodies were scattered throughout cortical layers however labeling was less intense than HCN1 (Fig.2A). In the hippocampus, labeling was observed in a similar pattern but with less intensity (Fig.2B). In the SCN, immunopositive cell bodies were also observed but more towards the third ventricle and the center of the SCN rather than the optic chiasm. The labeling appeared to be more localized to cell bodies and was scattered throughout the SCN (Fig.2C).

c) *HCN4 immunoreactivity in the brain*

HCN4-immunopositive cell bodies were observed in layers 2, 3 and 4 (Fig. 3A) while in the hippocampus we observed less intense label, mainly in CA1 region (Fig.3B) in the SCN. However, no labeling with HCN4 was observed (Fig.3C).

d) *I<sub>h</sub> in the SCN*

Using the whole cell voltage clamp method, I<sub>h</sub> was recorded from 113 cells within the SCN. 84% of the SCN neurons exhibited I<sub>h</sub> (Fig. 4A). I<sub>h</sub> was identified as being activated at hyperpolarizing potentials more the -70 mV as a slow inward current (Fig. 4B). The activation time constant in these cells exhibited voltage dependence, as it was on average 236 $\pm$ 2 ms at -40 mV step and 167 $\pm$ 3 ms at -60 mV step. This difference in voltage dependence was statistically significant ( $p < 0.5$ ). The current amplitude was on average 25 $\pm$ 2 pA at

-40 mV, while at -60 mV the current recorded was 34 $\pm$ 2 pA this voltage dependence was also statistically significant ( $p < 0.01$ ) (Fig.5).

### IV. DISCUSSION

Our results show that HCN1 and HCN2 channel subunits are present in the rat SCN and have different localization patterns. HCN1 is present in the ventral aspects of the SCN toward the optic chiasm with a diffuse pattern probably due to labeling of the neuronal processes. In contrast, HCN2 has a more scattered pattern of distribution labeling throughout the SCN, but was mainly observed in somas. These data agree with Notomi and Shigemoto (7) who found labeling of both subunits in the SCN but they did not describe any labeling pattern.

I<sub>h</sub> was present in the majority of SCN neurons (84%). Its activation time constant ( $\tau$ ) was 236 $\pm$ 2 ms and the current amplitude was 25 $\pm$ 2 pA for a -40 mV step. For a -60 mV the current amplitude was significantly increased to 34 $\pm$ 2 pA while  $\tau$  was significantly reduced to 167 $\pm$ 3 ms. These voltage dependence data agrees with what has been reported about I<sub>h</sub> (for reviews (8, 9)). This is also in agreement with other electrophysiological data (3, 9, 17, 23). For example, De Jeu and Pennartz (3) reported that the activation time constant for I<sub>h</sub> ranged from 107 to 467 ms. Jiang et al. (17) also reported that I<sub>h</sub> in the majority of SCN neurons had an amplitude of 5-35 pA.

The activation kinetics of I<sub>h</sub> recorded here are closer to those for HCN2 homomeric channels that have a slow activation properties (11). In vivo, the activation kinetics of I<sub>h</sub> channels does not always correspond to those of homomeric ion channels. For instance, Stevens and co-workers (23) characterized I<sub>h</sub> in the taste buds that exhibited  $\tau$  in the range of 103 to 478 ms that did not conform with either of the subunits identified (HCN1 and 4) using immunohistochemistry. Another possibility is that both HCN1 and HCN2 homomeric channels co-exist in neurons and that the recording reflects the sum of the properties of the two channels. Finally, it is also possible that both HCN1 and HCN2 may co-assemble to form a heteromer within the SCN with the resultant I<sub>h</sub> channel having different properties different from those of the homomer (24, 25). This, however, does not exclude the possibility that the results presented here could be attributed to the blind approach used in this study and the technical constraints on recording from the areas of the SCN that express HCN1.

On the other hand, no labeling for HCN4 channel subunit was observed within the SCN in this study. This result supports electrophysiological results that showed that I<sub>h</sub> channel in the SCN are of the relatively fast-activating type, compared to the very slow HCN4 homomeric channels (12, 14). Although Notomi and Shigemoto (7) found HCN4 labeling in the SCN, this



could be attributed to the fact they used different strain of rats (Sprague Dawley, versus Long Evans used in this study).

In conclusion,  $I_h$  is functionally well expressed in the SCN. Both HCN1 and HCN2 channel subunits are present within the rat SCN however; they have a different pattern of distribution. Together with the fact that melatonin can inhibit  $I_h$  at dusk (our unpublished results) this suggests that  $I_h$  may play an important role in modulation of SCN circadian function. These results support the model we proposed describing the role of melatonin in the regulation of SCN circadian function(26). In this model melatonin, via MT1 receptors activation, inhibits  $I_h$ . This effect may reduce SCN neurons excitability and decrease its spontaneous firing, which alters SCN rhythm at dusk and dawn. This model is further supported by the fact that MT1 receptors exhibited circadian variation in their expression while there is no evidence of such a variation in  $I_h$  (3, 26, 27).

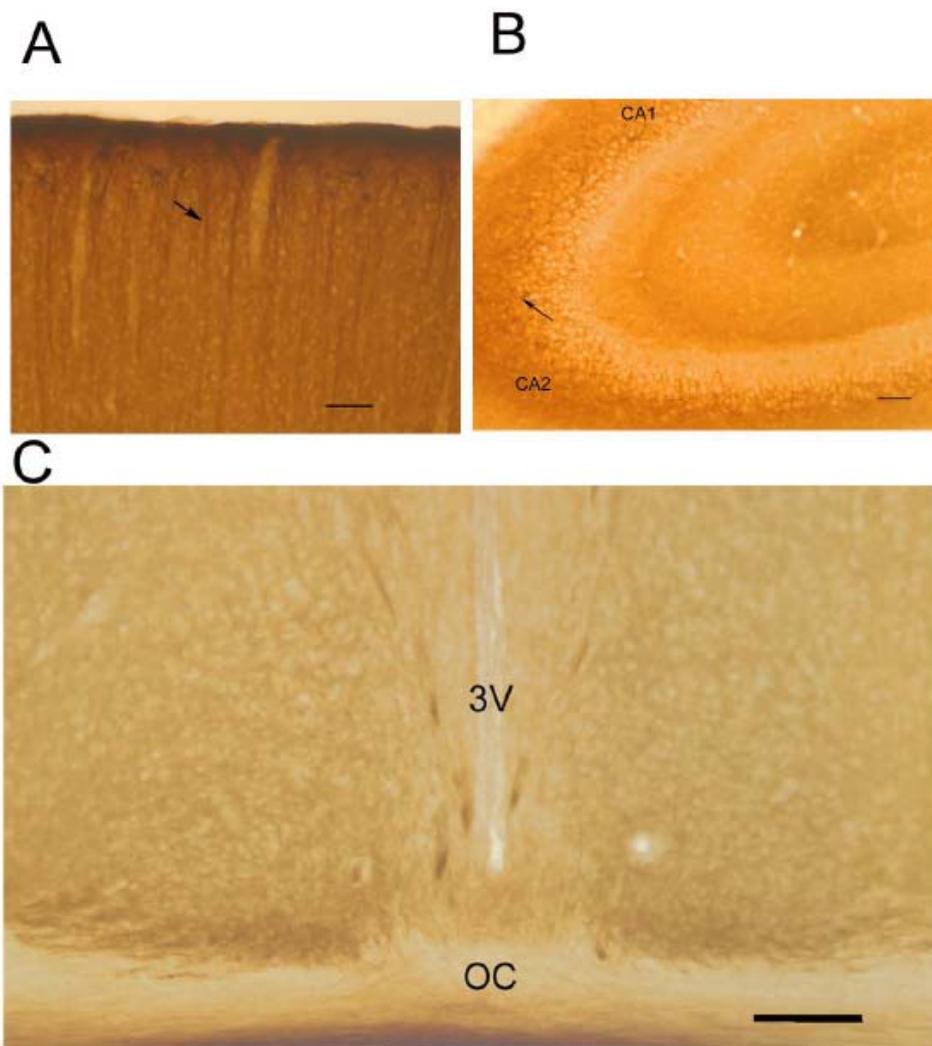
*List of abbreviation:*

- $I_h$  The hyperpolarization-activated current
- CNS Central nervous system
- SCN Suprachiasmatic nucleus
- HCN Hyperpolarization-activated and Cyclic Nucleotide-gated non-selective cation channel
- CT Circadian time
- SAN Sino-atrial node
- PBS Phosphate buffer saline
- ABC Avidin-biotin-peroxidase complex
- DAB 3, 3' diaminobenzidine
- a-CSF Artificial cerebrospinal fluid

*Availability of data and material:* All data are presented in the main paper.

*Competing interest:* The authors declare no competing interest.

FIGURE LEGENDS



**Figure 1 :** HCN1 immunostaining in the brain. A) Shown is HCN1 staining in the cortex, B) in the hippocampus and C) in the SCN. 3V is the third ventricle, OC is the optic chiasm. Scale bar is 20  $\mu$ m.

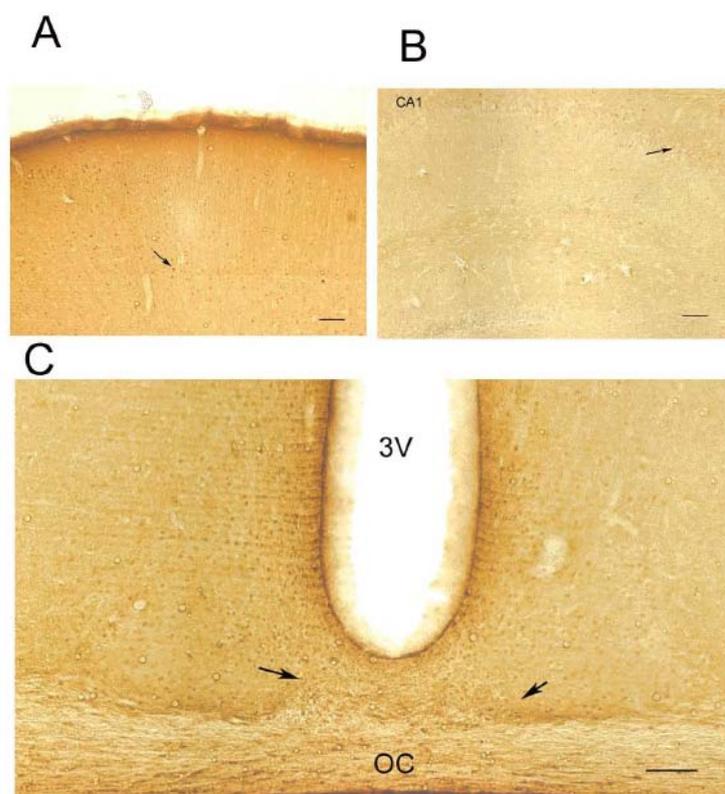


Figure 2 : HCN2 immunostaining in the brain. A) Shown is HCN2 labeling the cortex, B) hippocampus and C) SCN. The arrows points at somas. 3V is the third ventricle, OC is the optic chiasm. Scale bar is 20  $\mu$ m.

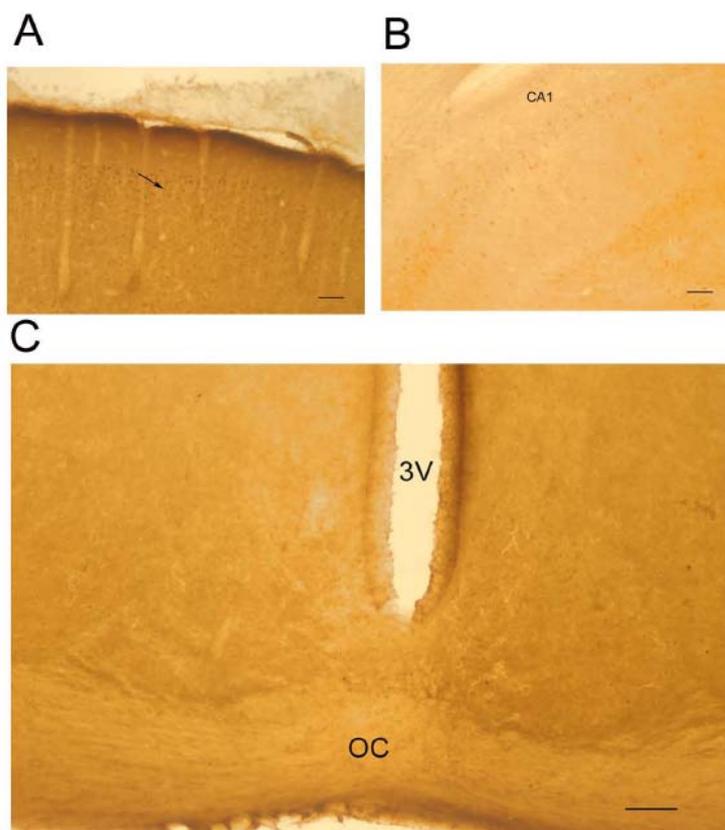
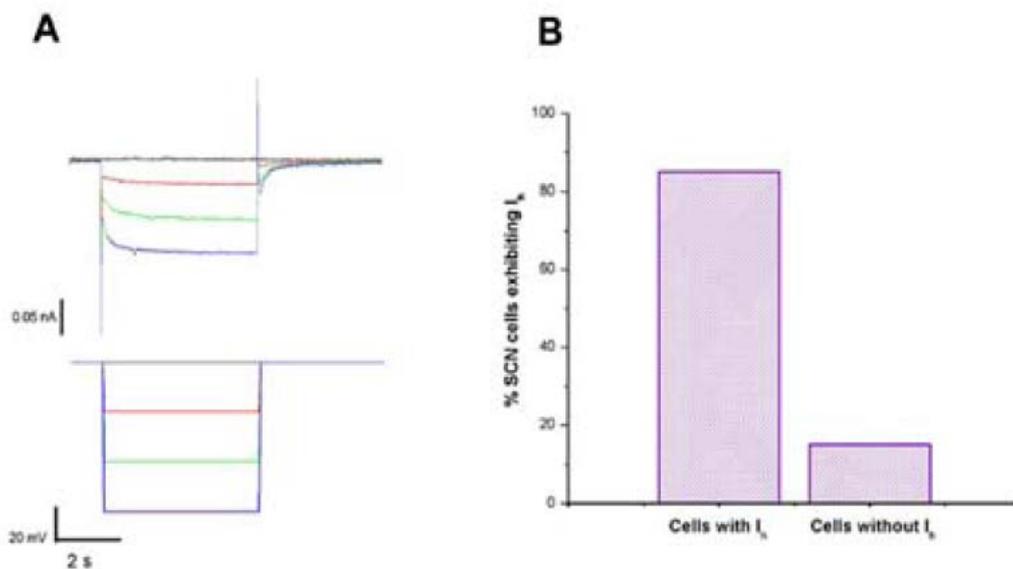
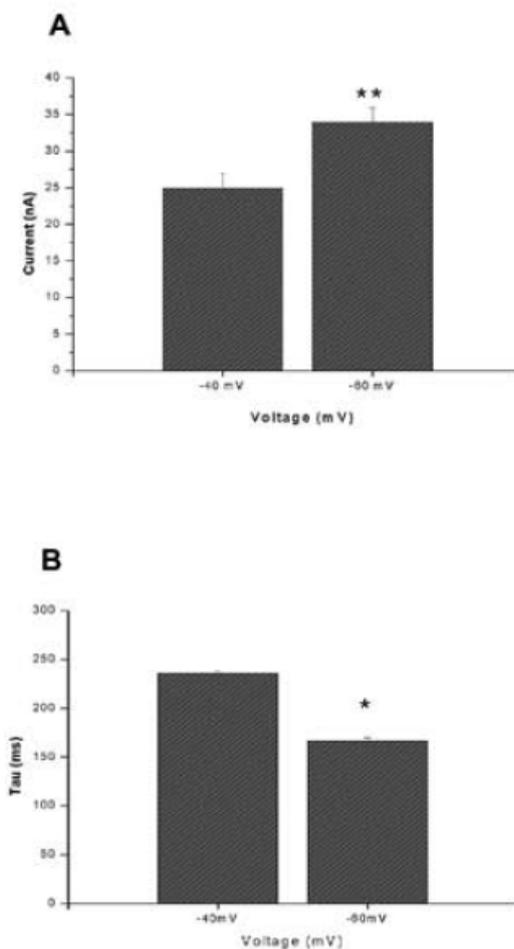


Figure 3 : HCN4 immunostaining in the brain. A) Shown is labeling with HCN4 antibody in the cortex, B) hippocampus and C) SCN. 3V is the third ventricle, OC is the optic chiasm. Scale bar is 20  $\mu$ m.



**Figure 4 :** Hyperpolarization activated current ( $I_h$ ) in the SCN A) Activation of  $I_h$  in the SCN neurons. In voltage command mode, hyperpolarizing steps of 20 mV magnitudes from a holding potential of  $-70$  mV were applied to activate the inward current. The top panel shows a voltage clamp record in a neuron in the suprachiasmatic nucleus. The lower panel shows the protocol used for eliciting  $I_h$ . B) Frequency of  $I_h$  recorded in the SCN at dusk.



**Figure 5 :** Voltage dependence of  $I_h$  magnitude A) and B) activation time constant in SCN neurons. \* indicates a p value less than 0.05.

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