

## BEHAVIOURAL NEUROSCIENCE

# Histamine resets the circadian clock in the suprachiasmatic nucleus through the H1R-Ca<sub>v</sub>1.3-RyR pathway in the mouse

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## Abstract

Histamine, a neurotransmitter/neuromodulator implicated in the control of arousal state, exerts a potent phase-shifting effect on the circadian clock in the rodent suprachiasmatic nucleus (SCN). In this study, the mechanisms by which histamine resets the circadian clock in the mouse SCN were investigated. As a first step, Ca<sup>2+</sup>-imaging techniques were used to demonstrate that histamine increases intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) in acutely dissociated SCN neurons and that this increase is blocked by the H1 histamine receptor (H1R) antagonist pyrilamine, the removal of extracellular Ca<sup>2+</sup> and the L-type Ca<sup>2+</sup> channel blocker nimodipine. The histamine-induced Ca<sup>2+</sup> transient is reduced, but not blocked, by application of the ryanodine receptor (RyR) blocker dantrolene. Immunohistochemical techniques indicated that Ca<sub>v</sub>1.3 L-type Ca<sup>2+</sup> channels are expressed mainly in the somata of SCN cells along with the H1R, whereas Ca<sub>v</sub>1.2 channels are located primarily in the processes. Finally, extracellular single-unit recordings demonstrated that the histamine-elicited phase delay of the circadian neural activity rhythm recorded from SCN slices is blocked by pyrilamine, nimodipine and the knockout of Ca<sub>v</sub>1.3 channel. Again, application of dantrolene reduced but did not block the histamine-induced phase delays. Collectively, these results indicate that, to reset the circadian clock, histamine increases [Ca<sup>2+</sup>]<sub>i</sub> in SCN neurons by activating Ca<sub>v</sub>1.3 channels through H1R, and secondarily by causing Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release from RyR-mediated internal stores.

## Introduction

The master circadian clock of the mammal located in the hypothalamic suprachiasmatic nucleus (SCN; Antle & Silver, 2005; Herzog, 2007; Golombek & Rosenstein, 2010; Welsh *et al.*, 2010), which drives the diverse diurnal rhythms of physiological and behavioral variables, is regulated by various neural factors (Gillette & Mitchell, 2002; Reghunandan & Reghunandan, 2006; Challet, 2007; Golombek & Rosenstein, 2010). One example is histamine, a neurotransmitter/neuromodulator that is produced by neurons in the tuberomammillary nucleus (TMN; Watanabe *et al.*, 1984) and plays a key role in the regulation of brain arousal (Haas & Panula, 2003). Studies have shown that the SCN is innervated by histaminergic fibers from the TMN (Watanabe *et al.*, 1984) and that some SCN

neurons in the mouse and rat contain histamine, presumably because they take up histamine released from histaminergic nerve terminals (Michelsen *et al.*, 2005). The application of histamine onto SCN neurons in rodent hypothalamic slice preparations alters the firing rates of these cells (Liou *et al.*, 1983; Stehle, 1991; Scott *et al.*, 1998), while bath application of histamine phase-shifts the circadian rhythms in neural activity recorded in hamster and mouse SCN slices (Cote & Harrington, 1993; Meyer *et al.*, 1998; Biello, 2009). *In vivo*, the intracerebroventricular injection of histamine in the rat phase-shifts the circadian activity rhythms (Itowi *et al.*, 1990) and reduces the time required to entrain activity rhythms to an abruptly advanced light–dark cycle (Itowi *et al.*, 1991). The inhibition of histamine synthesis by  $\alpha$ -fluoromethylhistidine or knockout (KO) of histidine decarboxylase gene disrupts the circadian activity rhythm in rats (Itowi *et al.*, 1990), attenuates the light-induced phase shifts of circadian activity rhythms in hamsters (Eaton *et al.*, 1995) and lowers the levels of wheel-running and spontaneous locomotor activities in mice in both light–dark and dark–dark conditions (Abe

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*et al.*, 2004). Finally, H1 histamine receptor (HIR) KO mice exhibit disrupted diurnal feeding rhythm (Yoshimatsu, 2008). Collectively, these data indicate that histamine is a potent regulator of the circadian system in mammals, and raise questions about the underlying mechanisms.

In the present study, it was sought to determine the molecular mechanism by which histamine resets the circadian clock in the mouse SCN.  $\text{Ca}^{2+}$ -imaging techniques were first used to measure the  $\text{Ca}^{2+}$  transients evoked by application of histamine to isolated SCN neurons and to identify the signaling pathway by which histamine regulates this key intracellular signaling molecule, as circadian clock resetting requires an intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) increase in SCN neurons (Ding *et al.*, 1994, 1998; Hamada *et al.*, 1999; Kim *et al.*, 2005). Next, immunohistochemistry (IHC) was used to examine the co-localization of HIR with  $\text{Ca}_v1.3$  L-type voltage-gated  $\text{Ca}^{2+}$  channels (VGCCs). Finally, extracellular single-unit recordings were used to determine the signaling pathway by which histamine phase-shifts the circadian rhythm in neural activity recorded from the SCN.

## Materials and methods

### Study approval

The experimental procedures described below were approved by the Korea University College of Medicine Animal Research Policies Committee and Korea Institute of Science and Technology, while conforming to National Institutes of Health guidelines.

### Animals and housing

Male C57BL/6 mice (B6 mice, 3–6 weeks old) and  $\text{Ca}_v1.3$  KO mice (3–6 weeks old) with the genetic background of C57BL/6 were used for the current study. The double-KO mouse  $\text{Ca}_v1.3/\text{Ca}_v3.1$  animal was received from Dr Matteo Mangoni at CNRS, UMR-5203, Institut de Génomique Fonctionnelle, Département de Physiologie, LabEx ICST, Montpellier, F-34094, France in August 2008. The targeting of  $\text{Ca}_v1.3$  allele was achieved by thymidine kinase gene, a 1.4-kb genomic fragment 5' to exon 2, the neomycin resistance (neo) gene driven by the pgk promoter inserted in the reverse direction into the ClaI site of exon 2, a 5-kb genomic fragment 3' to exon 2, and the plasmid pKO V910 (Lexicon Genetics). After receiving this mouse line, each allele was isolated by crossing with wild-type mice in several generations to obtain pure  $\text{Ca}_v1.3$  KO line. Animals were housed in group cages (four-six/cage) in a temperature-controlled room (22–24 °C) and adapted to a 12-h light–dark cycle for at least 1 week before being used for experiments. Zeitgeber time (ZT) 0:00 h was designated as the time of lights-on and ZT 12:00 h as the time of lights-off in the colony.

### Brain slice preparation

All brain slices were prepared between ZT 10:00 h and 12:00 h. Animals were anesthetized with urethane (1.25 g/kg, i.p.), and the brain was quickly excised from the skull and submerged in 95%  $\text{CO}_2/5\%$   $\text{O}_2$  saturated ice-cold artificial cerebrospinal fluid (ACSF), composed of (in mM): NaCl, 124;  $\text{MgSO}_4$ , 1.3; KCl, 3;  $\text{NaH}_2\text{PO}_4$ , 1.25;  $\text{NaHCO}_3$ , 26;  $\text{CaCl}_2$ , 2.4; glucose, 10. After being chilled in ice-cold ACSF for 1–2 min, the brain was trimmed to a tissue block containing the hypothalamus. Using a vibroslicer (World Precision Instruments or Leica VT100S), coronal slices (300–400  $\mu\text{m}$  thickness) containing the SCN were cut from the tissue block in ice-cold

ACSF. The slices were kept at room temperature (R/T: 22–24 °C, 30–60 min) in aerated (95%  $\text{O}_2/5\%$   $\text{CO}_2$ ) ACSF before being transferred to a gas interface-type electrophysiological recording chamber that was continuously superfused with warm (35 °C) 95%  $\text{O}_2/5\%$   $\text{CO}_2$ -saturated ACSF.

### SCN cell dissociation

Hypothalamic slices containing the SCN (300–350  $\mu\text{m}$  thickness; coronal orientation) were prepared between ZT 10:00 h and 12:00 h from B6 mice as above. After being incubated in aerated (95%  $\text{O}_2/5\%$   $\text{CO}_2$ ) ACSF at R/T (30–60 min), the slices were transferred to a HEPES-buffered high-sucrose solution ('high-sucrose' solution) [composed of (in mM):  $\text{Na}_2\text{SO}_4$ , 30;  $\text{K}_2\text{SO}_4$ , 2; sucrose, 185; glucose, 10; HEPES, 10;  $\text{CaCl}_2$ , 0.5;  $\text{MgCl}_2$ , 6; pH 7.4] containing 2 mg/mL protease XXIII (Sigma-Aldrich) and incubated for 15 min at 34 °C. After this enzyme treatment, the slices were rinsed with the high-sucrose solution at R/T and incubated again in the high-sucrose solution containing 1 mg/mL trypsin inhibitor and 1 mg/mL bovine serum albumin (Sigma-Aldrich) for 15 min at R/T. Subsequently, the slices were rinsed with the high-sucrose solution, and the SCN was 'punched out' with blunt 21-G needle for mechanical dissociation of cells. The cells dissociated with the use of fire-polished glass pipettes were plated on poly-D-lysine (Sigma-Aldrich)-coated glass coverslips. The cells were allowed to settle down on the coverslips for ~30 min at R/T and then were washed with the HEPES-buffered saline, before fura-2 loading (below).

### $\text{Ca}^{2+}$ imaging

Dissociated SCN cells on the coverslips were loaded with fura-2 AM (Invitrogen) for  $\text{Ca}^{2+}$  imaging. For this, the coverslips were incubated for 30 min in fura-2 AM (5  $\mu\text{M}$ )-containing HEPES-buffered saline [composed of (in mM): NaCl, 150; KCl, 5;  $\text{CaCl}_2$ , 2;  $\text{MgCl}_2$ , 1; HEPES, 10; glucose, 10; pH 7.4] at R/T, and then rinsed with HEPES-buffered saline before being transferred to the imaging chamber (RC-25; Warner Instrument, Hamden, CT, USA) that was superfused continuously (1–1.5 mL/min) with  $\text{Mg}^{2+}$ -free HEPES-buffered saline (22–24 °C).  $\text{Ca}^{2+}$  responses to a mixture of 100  $\mu\text{M}$  glycine and 100  $\mu\text{M}$  *N*-methyl-D-aspartate (NMDA) were used as a positive control in  $\text{Ca}^{2+}$  imaging tests; glycine acts as a co-agonist of NMDA receptors (Johnson & Ascher, 1987). Background-subtracted intensity images at two excitation wavelengths (340 and 380 nm) were acquired using an EMCCD camera (Andor Technology, UK) attached to an inverted microscope (IX71; Olympus, Tokyo, Japan) and Axon Imaging Work Bench 5.1 image processing program (Indec Systems, CA, USA).  $[\text{Ca}^{2+}]_i$  was expressed as the ratio of the fura-2 emission fluorescence intensities of 510 nm, excited at 340 and 380 nm. All the  $\text{Ca}^{2+}$ -imaging experiments were performed between ZT 13:00 h and ZT 18:00 h.

### IHC

B6 mice were anesthetized with avertin (2,2,2-tribromoethanol; 0.4 g/kg i.p.) or urethane (1.25 g/kg, i.p.) and perfused with ice-cold 0.9% saline, and then with 4% paraformaldehyde (prepared in 0.1 M phosphate buffer). Brains were excised from the skull and post-fixed in ice-cold 4% paraformaldehyde overnight. Then, the brains were cryoprotected in 30% (w/v) sucrose-containing 0.1 M phosphate-buffered saline (PBS) for 24 h and frozen in powdered dry ice. Coronal sections (30  $\mu\text{m}$ ) containing the SCN were cut from the frozen brain tissue with a cryostat and transferred to

0.1 mM PBS for washing. Subsequently, they were transferred to a blocking solution, the 0.1 mM PBS containing 0.3% Triton-X 100 and 4% normal donkey serum. After 1 h in the blocking solution, the tissue sections were incubated overnight at 4 °C with a mixture of primary antibodies: goat anti-H1R (1 : 50, sc-19770; Santa-Cruz) antibody plus rabbit anti-Ca<sub>v</sub>1.2 (1 : 200, ACC-003; Alomone LAB, Jerusalem, Israel) antibody or goat anti-H1R antibody (1 : 50; Santa-Cruz) plus rabbit anti-Ca<sub>v</sub>1.3 antibody (1 : 50; Shen *et al.*, 2006). The sections for double-IHC were washed with 0.1 mM PBS and incubated with Alexa Fluor-488 and Alexa Fluor-555 (each 1 : 200; Invitrogen)-conjugated secondary antibodies diluted in blocking solution for 2 h at R/T. After being washed in 0.1 mM PBS, the sections were stained with 4',6-diamidino-2-phenylindole (DAPI), a fluorescent stain that binds strongly to DNA and thus delineates cellular nuclei. The floating sections were transferred to Superfrost Plus slides (Fisher) and dried. All slides were coverslipped in fluorescence mounting medium (Dako, Glostrup, Denmark) and examined with confocal microscopy. Confocal microscope images were captured with a Fluoview-1000 microscope (Olympus) using standard excitation and emission filters for visualizing Alexa Fluor-488 and Alexa Fluor-555. For images, micrographs were made using 40–100× objective lenses in the Fluoview confocal microscope. For image analysis, FV10-ASW program (Olympus) and Image-J (NIH, MD, USA) were used.

#### Experimental treatment and extracellular single-unit recording

On the first day *in vitro*, the slice was superfused in the recording chamber with a solution containing histamine, 2-pyridylethylamine (H1R agonist) or amthamine [H2 histamine receptor (H2R) agonist]. In some experiments, histamine was superfused together with pyrilamine (H1R blocker), nimodipine (L-type VGCC blocker), dantrolene [ryanodine receptor (RyR) blocker] or a cocktail of D,L-2-amino-5-phosphonopentanoic acid (AP5; NMDA receptor antagonist) and 6,7-dinitroquinoxaline-2,3-dione (DNQX; non-NMDA receptor antagonist). Control slices were not treated with any of the drugs above. The application of histamine or H1R/H2R agonist began at ZT 14:00 h and lasted for 30 min. The application of blockers started at ZT 13:50 h and lasted for 50 min.

On the next day (i.e. second day *in vitro*), extracellular single-unit recordings were obtained from the SCN between ZT 1:00 h and ZT 16:00 h as described previously (Kim *et al.*, 2005). Micropipettes (4–6 MΩ) pulled from borosilicate glass capillary and filled with ACSF were used as recording electrodes. Unit recordings were conducted without restricting the electrode tip to any particular region of the SCN. Usually 6–15 units were sampled every hour, each unit being recorded for a 1-min period. The voltage signals from recording electrodes were fed serially into an Axoclamp-2B amplifier (Molecular Devices, CA, USA), and a differential amplifier (model AM 502; Tektronix) for amplification, AC coupling and band-pass filtering (200 Hz–1 kHz). The processed signals were digitized and sampled at 50-μs intervals (Digidata1320A, Axoscope 8.1; Molecular Devices). The criteria used to identify single units were consistent waveform and spike amplitude. To detect the time-of-peak of circadian firing activity rhythm of SCN neurons, which is a reliable marker of the phase of circadian pacemaker (Chen *et al.*, 1999), the mean firing rates of single units sampled for sequential 2-h periods with 1-h lags were plotted against ZT: i.e. the mean firing rate of units recorded from ZT 0:00 h to 2:00 h at ZT 1:00 h were plotted, the mean firing rate of units encountered from ZT 1:00 h to 3:00 h at ZT 2:00 h were plotted, etc. In order to quantify the phase shift induced by a drug treatment, the time-of-peak detected in the

drug-treated slice was compared with the average time-of-peak of control slices. The difference was taken as the amount of drug-induced phase shift.

#### Drugs

All drugs and chemicals used in the current study were purchased from Sigma-Aldrich and Tocris Bioscience (Bristol, UK). The solutions of histamine, 2-pyridylethylamine, amthamine, pyrilamine, AP5 and quinacrine were prepared by dissolving these drugs in ACSF or HEPES-buffered saline at appropriate concentrations. The solutions of DNQX, nimodipine, dantrolene, chelerythrine, bisindolymaleimide-1 and BTP-2 were prepared by diluting their stock solution with ACSF or HEPES-buffered saline [stock solution solvent – dimethyl sulfoxide (DMSO) for DNQX, nimodipine and dantrolene; final concentration of DMSO = 0.003–0.01%]. These drug solutions were applied to SCN slices or dissociated SCN cells by gravity-fed bath-perfusion system or peristaltic pump. Drug solutions were kept in syringe reservoirs connected to a manifold with multiple inputs (Warner Instrument). The opening and closing of each reservoir was controlled by an electronically operated solenoid valve. The output of the manifold was connected to the recording chamber.

#### Statistics

Numerical data are expressed as the mean ± SEM. All statistical tests were conducted using SigmaStat 3.5 (Systat Software). Unpaired *t*-test was used for the comparison of two independent data sets with normal distribution. Paired samples with and without normal distribution were compared with paired *t*-test and Wilcoxon signed rank test, respectively. Kruskal–Wallis one-way analysis of variance (ANOVA) on ranks and pair-wise comparison with Newman–Keuls tests were performed to compare multiple data sets without normal distributions. A value of *P* < 0.05 was considered to be significant. For tests involving 1 degree of freedom, a non-directional test was conducted.

## Results

### H1R mediates histamine-induced [Ca<sup>2+</sup>]<sub>i</sub> increase in SCN neurons

First, it was examined whether histamine increased [Ca<sup>2+</sup>]<sub>i</sub> in acutely dissociated mouse SCN neurons (Fig. 1A) using Fura-2 as a Ca<sup>2+</sup> indicator. Cells showing Ca<sup>2+</sup> responses to NMDA were taken as neurons (Fig. 1B) because cultured mouse glia do not respond to NMDA with changes in [Ca<sup>2+</sup>]<sub>i</sub> (Y.S. Kim and C.J. Lee, unpublished data, 2015). The main reason the use of dissociated cells was chosen over brain slices for Ca<sup>2+</sup> imaging was that the dissociated cell preparation allows to precisely predict the cellular mechanism in an isolated setting; in a brain slice experimental setting, various transmitters can be released from neighboring neurons or glial cells upon activation and give rise to secondary or indirect responses. Bath application of histamine (100 μM; 30 s) produced a reliable increase in [Ca<sup>2+</sup>]<sub>i</sub> in a subset of SCN neurons (323 out of 683 cells from 39 mice; Fig. 1B); the average peak amplitude of histamine-induced Ca<sup>2+</sup> responses (expressed as the change in the ratio of emissions at 340 nm and 380 nm of light) was 0.36 ± 0.02 (*n* = 323). In the remaining neurons, histamine produced either a decrease (*n* = 9 cells, −0.48 ± 0.09) or no change in [Ca<sup>2+</sup>]<sub>i</sub> (*n* = 351 cells).

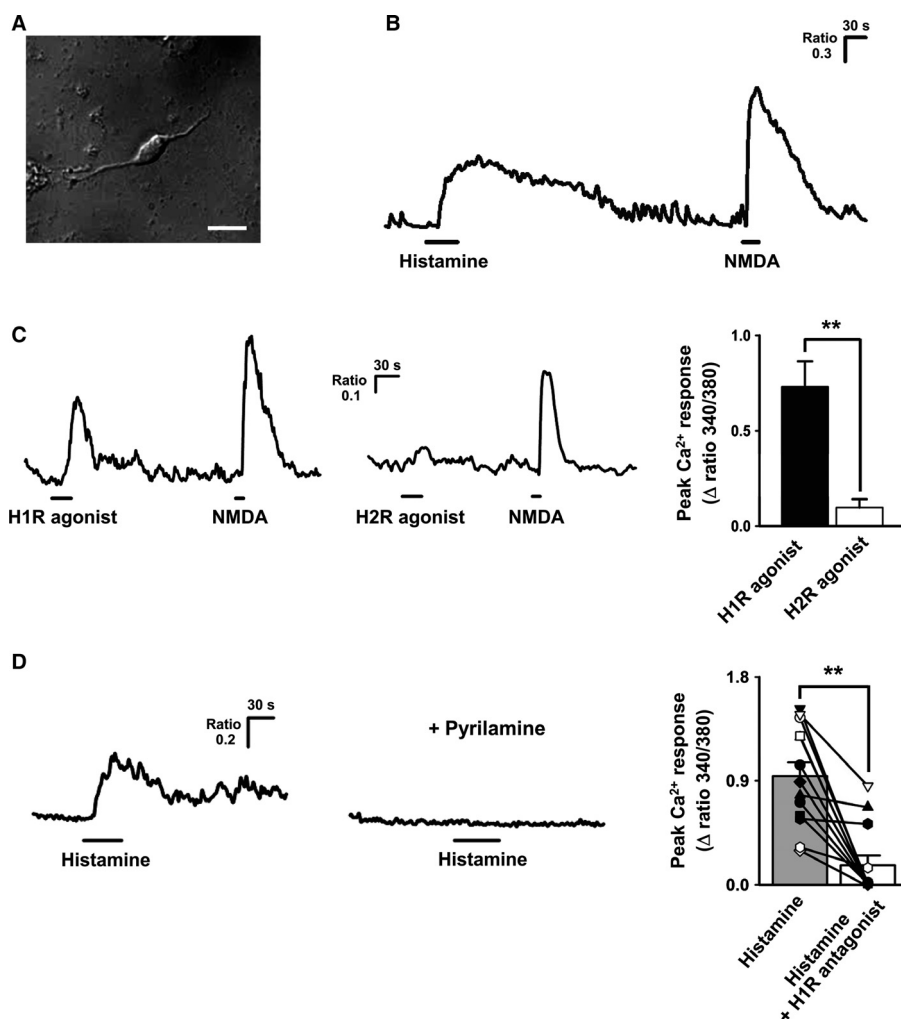


FIG. 1. H1 histamine receptor (H1R) mediates a histamine-induced increase in intracellular  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ) in suprachiasmatic nucleus (SCN) neurons. (A) Brightfield DIC image of an acutely dissociated SCN neuron. Scale bar: 20  $\mu$ m. (B) Histamine-elicited increase in  $[Ca^{2+}]_i$  in an acutely dissociated SCN neuron. *N*-methyl-D-aspartate (NMDA; 100  $\mu$ M, 15 s)-induced  $Ca^{2+}$  response was used as a positive control. (C)  $Ca^{2+}$  responses elicited by agonists of H1R (2-pyridylethylamine, 100  $\mu$ M,  $n = 15$ ) and H2 histamine receptor (H2R; amthamine, 20  $\mu$ M,  $n = 20$ ). Peak  $Ca^{2+}$  response ( $\Delta$  ratio 340/380): maximal change following experimental treatment of the ratio of the fura-2 fluorescence emissions at 340 and 380 nm of light.  $**P < 0.001$ , unpaired *t*-test. (D) Blockade of histamine-elicited  $Ca^{2+}$  response by 10  $\mu$ M pyrilamine (H1R antagonist,  $n = 13$ ). In this and all subsequent experiments, the same cells were tested before and during application of the blocker. The bar charts indicate the mean ( $\pm$ SEM) peak  $Ca^{2+}$  responses.  $**P < 0.001$ , paired *t*-test.

H1R and H2R mediate the excitatory actions of histamine in the brain (Haas & Panula, 2003). Thus, it was tested whether H1R or H2R mediates the histamine-induced  $Ca^{2+}$  increase in SCN neurons using selective histamine receptor agonists and antagonists. The H1R agonist 2-pyridylethylamine (100  $\mu$ M; 30 s) evoked a large increase in  $[Ca^{2+}]_i$ , whereas the H2R agonist amthamine (20  $\mu$ M; 30 s) showed only a marginal effect [peak  $Ca^{2+}$  responses, H1R agonist:  $0.7 \pm 0.3$ ,  $n = 15$  neurons (from three mice); H2R agonist:  $0.1 \pm 0.0$ ,  $n = 20$  neurons (from three mice); unpaired *t*-test,  $t_{33} = 5.09$ ,  $P < 0.001$ ; Fig. 1C]. In addition, the H1R antagonist pyrilamine (10  $\mu$ M) mostly blocked the histamine-elicited  $Ca^{2+}$  response [% block of  $Ca^{2+}$  peak amplitude:  $86.6 \pm 5.5\%$ ,  $n = 13$  neurons (from three mice); paired *t*-test,  $t_{12} = 5.32$ ,  $P < 0.001$ ; Fig. 1D], indicating that H1R mediates the histamine-evoked  $Ca^{2+}$  increase in SCN neurons. Pylramine and all other blockers except the protein kinase C (PKC) inhibitor chelerythrine, which were tested for their possible antagonistic effects on histamine-elicited  $Ca^{2+}$  responses (see below), did not alter the baseline  $Ca^{2+}$  level (data not shown). Also, the drug vehicle (i.e. 0.01% DMSO) had no significant effect on the baseline  $[Ca^{2+}]_i$  (data not shown).

#### *Histamine-induced $[Ca^{2+}]_i$ increase relies on L-type $Ca^{2+}$ channel activation and subsequent $Ca^{2+}$ -induced $Ca^{2+}$ release through RyRs*

To investigate the possible mechanisms underlying the histamine-induced  $[Ca^{2+}]_i$  increase in SCN neurons, first it was determined whether histamine can evoke a response in a  $Ca^{2+}$ -free bath solution containing 100  $\mu$ M EGTA. EGTA was included to remove possible remaining free  $Ca^{2+}$  in nominally  $Ca^{2+}$ -free bath solution. After switching to the  $Ca^{2+}$ -free medium, the baseline ratio (340/380) gradually decreased by 0.3–0.7 until a new baseline ratio was established within 5–10 min. In this condition, histamine application failed to increase  $[Ca^{2+}]_i$  (Fig. 2A), indicating that the histamine-induced increases in  $[Ca^{2+}]_i$  in SCN neurons crucially depend on the influx of  $Ca^{2+}$ . Next, because L-type VGCCs are a major route for  $Ca^{2+}$  influx and are highly expressed in the SCN (Nahm *et al.*, 2005), the effect of the L-type VGCC blocker nimodipine on histamine-induced  $Ca^{2+}$  response was examined. Nimodipine (2  $\mu$ M) blocked the  $Ca^{2+}$  response ( $t_{18} = 6.11$ ,  $P \leq 0.001$ ; Fig. 2B). These results indicate that L-type VGCC is responsible for the  $Ca^{2+}$  entry.

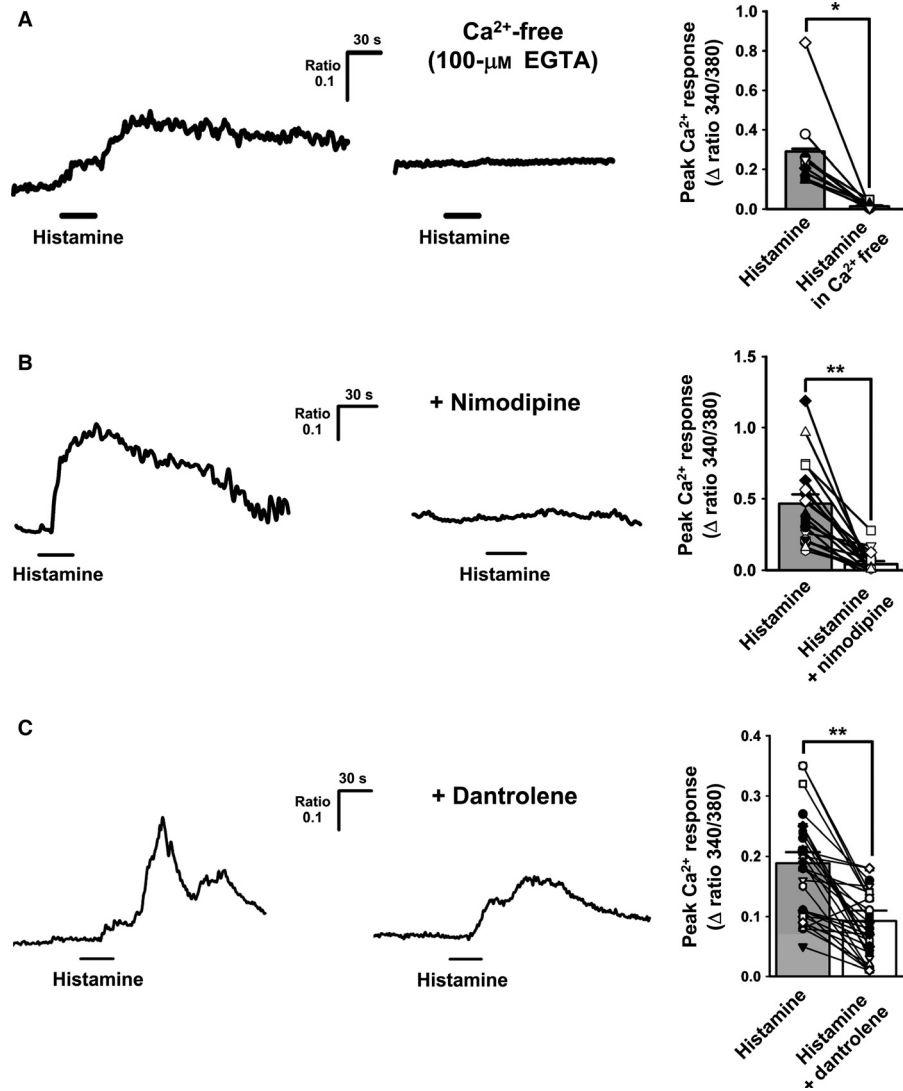


FIG. 2. Influx of Ca<sup>2+</sup> through L-type voltage-gated Ca<sup>2+</sup> channels (VGCCs) and subsequent Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release from ryanodine receptor (RyR)-mediated internal stores are responsible for the histamine-induced increase in intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) in suprachiasmatic nucleus (SCN) neurons. (A) Histamine-induced Ca<sup>2+</sup> response in the presence (left panel) and absence (middle panel) of Ca<sup>2+</sup> in the cell-bathing medium. \**P* < 0.01 (paired *t*-test; *n* = 10 neurons from two mice), Wilcoxon signed rank test for paired samples. (B) Histamine-induced Ca<sup>2+</sup> response in the absence (left panel) and presence (middle panel) of nimodipine (2 μM). \*\**P* ≤ 0.001 (paired *t*-test, *n* = 19 neurons from three mice). (C) Histamine-induced Ca<sup>2+</sup> response in the absence (left panel) and presence (middle panel) of dantrolene (10 μM). \*\**P* ≤ 0.001 (paired *t*-test, *n* = 33 neurons from three mice). The bar charts in (A–C) indicate the mean (±SEM) peak Ca<sup>2+</sup> responses.

Then, the possible contribution of Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release from internal stores (Chavis *et al.*, 1996; Dulhunty *et al.*, 2002) with the use of the RyR blocker dantrolene was examined. The application of this agent (10 μM) reduced the histamine-induced Ca<sup>2+</sup> response by 50.3 ± 6.1% (0.19 ± 0.03 to 0.09 ± 0.02, *t*<sub>32</sub> = 7.20, *P* ≤ 0.001, *n* = 33 from three mice; paired *t*-test; Fig. 2C). In contrast, the histamine-induced Ca<sup>2+</sup> responses were not impacted by the application of the transient receptor potential cation channel (TRPC) blocker BTP-2 (3 μM; He *et al.*, 2005), the PKC inhibitors (chelerythrine, 2 μM; bisindolymaleimide-1, 1 μM), or the phospholipase A2 (PLA2) inhibitor quinacrine (10 μM; Kim *et al.*, 2004; Shim *et al.*, 2007; Fig. 3). Collectively, these results indicate that histamine-induced [Ca<sup>2+</sup>]<sub>i</sub> increases in mouse SCN neurons are largely mediated by L-type VGCCs with some contribution from Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release from internal stores, whereas downstream effectors of phospholipase C (PLC) and PLA2 are not involved.

### Ca<sub>v</sub>1.3 mediates the actions of histamine

While there are four subtypes of L-type VGCCs (Ca<sub>v</sub>1.1–1.4), only Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3 subtypes are expressed in the CNS (Moosmang *et al.*, 2005; Calin-Jageman & Lee, 2008). To see which subtype is expressed in mouse SCN cells, double-IHC with antibodies against HIR and Ca<sub>v</sub>1.2 or Ca<sub>v</sub>1.3 was performed. The control staining conditions for Ca<sub>v</sub>1.2, Ca<sub>v</sub>1.3 and HIR include the absence of staining for Ca<sub>v</sub>1.2 in Ca<sub>v</sub>1.2 conditional KO mouse, absence of staining for Ca<sub>v</sub>1.3 in Ca<sub>v</sub>1.3 KO mouse, and absence of staining for HIR with the control peptide-preabsorbed antibody, respectively (Fig. 4A). It was found that Ca<sub>v</sub>1.3 and HIR are mainly expressed in the somata of SCN cells, whereas Ca<sub>v</sub>1.2 is located primarily in the processes (Fig. 4B). More importantly, almost all of HIR-positive somata were also positive for Ca<sub>v</sub>1.3, but rarely for Ca<sub>v</sub>1.2 (% co-localization, HIR and Ca<sub>v</sub>1.3: 98.8 ± 0.4%, *n* = 3 sections from

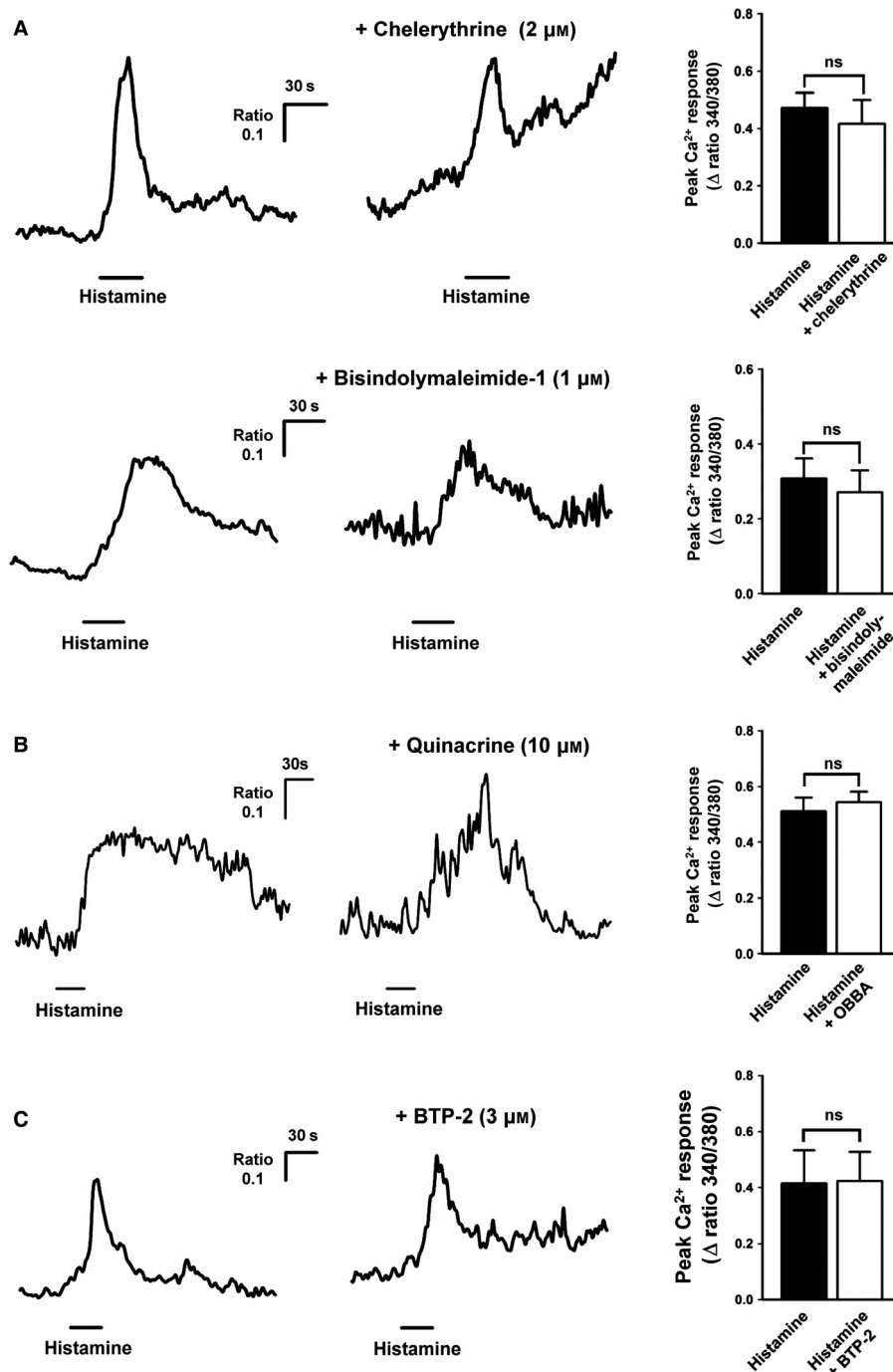


FIG. 3. Phospholipase C (PLC) and phospholipase A2 (PLA2) pathways do not mediate histamine-induced Ca<sup>2+</sup> responses in suprachiasmatic nucleus (SCN) neurons. Effects of protein kinase C (PKC) inhibitors [chelerythrine,  $n = 37$ ; bisindolymaleimide-1,  $n = 39$ ; (A)], PLA2 inhibitor [quinacrine,  $n = 26$ ; (B)] and transient receptor potential cation channel (TRPC) blocker [BTP-2,  $n = 26$ ; (C)] on histamine-elicited Ca<sup>2+</sup> responses in SCN neurons. The effect of bisindolymaleimide-1 was examined in addition to the effect of chelerythrine, because the latter induced the upward sloping of the baseline ratio that may indicate dying cell. ns, statistically not significant, paired  $t$ -test. The bar charts in (A–C) indicate the mean ( $\pm$ SEM) values.

three mice; H1R and Ca<sub>v</sub>1.2:  $5.5 \pm 1.5\%$ ,  $n = 6$  sections from six mice; unpaired  $t$ -test,  $t_7 = -22.68$ ,  $P < 0.001$ ).

Furthermore, the percentage of H1R-positive cells in the SCN was 47.4% ( $n = 9$  slices) and this was very similar to the percentage of cells showing a histamine-induced increase in [Ca<sup>2+</sup>]<sub>i</sub> (47%). Previous work has shown that Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3 show differential sensitivity to nimodipine – the IC<sub>50</sub> values of nimodipine for Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3 were 139 nM and 2.7 μM, respectively (Xu & Lipscombe, 2001). To identify the L-type VGCC subtype that is

responsible for the histamine-induced Ca<sup>2+</sup> entry, this differential sensitivity was utilized. It was assumed that 0.3 μM nimodipine would selectively block Ca<sub>v</sub>1.2, while nimodipine at 6 μM would block both Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3. It was found that 0.3 μM nimodipine only modestly reduced the histamine-evoked increase in [Ca<sup>2+</sup>]<sub>i</sub> ( $14.9 \pm 7.1\%$  block,  $n = 8$  neurons from two mice), whereas 6 μM nimodipine blocked almost completely the histamine-induced Ca<sup>2+</sup> response ( $88.0 \pm 8.5\%$  block,  $n = 5$  neurons from two mice). The effects of 0.3 and 6 μM nimodipine were significantly different

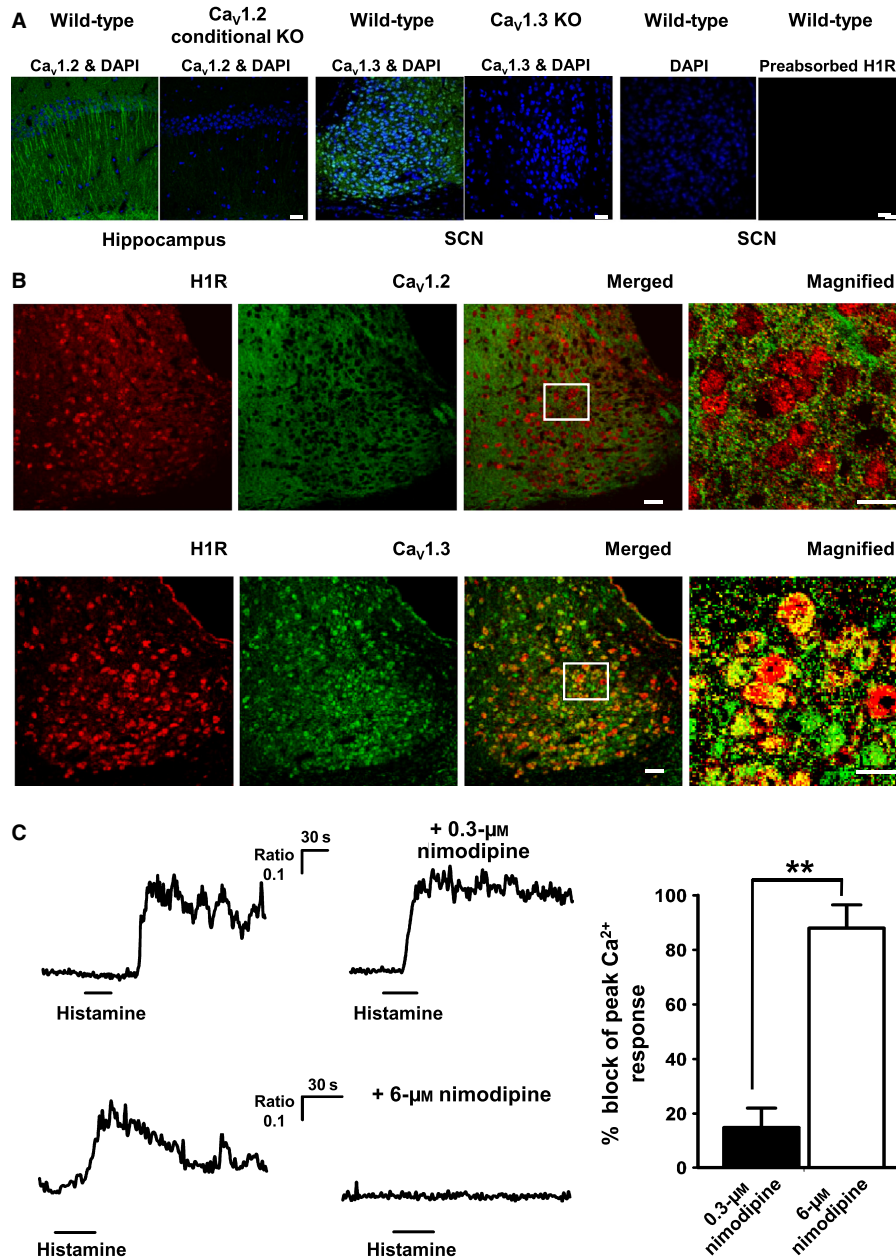


FIG. 4.  $Ca_v1.3$  L-type voltage-gated  $Ca^{2+}$  channels (VGCC) mediates the histamine-induced  $Ca^{2+}$  influx. (A, left two panels) Double-immunohistochemical (IHC) staining for  $Ca_v1.2$  VGCCs and 4',6-diamidino-2-phenylindole (DAPI) staining for the CA1 hippocampal area of wild-type and  $Ca_v1.2$ -conditional knockout (KO) mice. (A, two panels in the middle) Double-IHC staining for  $Ca_v1.3$  VGCCs and DAPI staining for the suprachiasmatic nucleus (SCN) of wild-type and  $Ca_v1.3$  KO mice. (A, right two panels) DAPI staining and IHC with the use of control peptide-preabsorbed H1 histamine receptor (H1R) antibody for the SCN of wild-type mice. Scale bars: 30  $\mu$ m. (B) Double-IHC staining for H1R and  $Ca_v1.2$  VGCCs (upper panels) or  $Ca_v1.3$  VGCCs (lower panels) for the mouse SCN. Scale bars: 20  $\mu$ m. (C) Effects of low (0.3  $\mu$ M,  $n = 8$  neurons) and high (6  $\mu$ M,  $n = 5$  neurons) concentrations of nimodipine on histamine-elicited rise in intracellular  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ) in SCN neurons.  $**P < 0.001$ , unpaired  $t$ -test. The bar charts indicate the mean ( $\pm$ SEM) values.

( $t_{11} = -6.49$ ,  $P < 0.001$ , unpaired  $t$ -test; Fig. 4C). Therefore, the expression data as well as nimodipine sensitivity indicate that  $Ca_v1.3$  L-type VGCC mediates the histamine-induced  $Ca^{2+}$  influx in mouse SCN neurons.

#### Histamine resets the circadian clock through the H1R- $Ca_v1.3$ -RyR pathway

In the final set of experiments, it was sought to determine if histamine causes phase shifts using the H1R- $Ca_v1.3$ -RyR signaling pathway. Electrophysiological techniques were used to determine

how the blockade or removal of H1R,  $Ca_v1.3$  or RyR by selective pharmacological or genetic manipulations altered the histamine-induced phase delay of circadian neural activity rhythm recorded from the mouse SCN. In this brain slice preparation, spontaneous neural activity is rhythmic with a peak of electrical activity at midday or ZT 6.00  $\pm$  0.26 h ( $n = 6$ ; Fig. 5A and P). In those slices treated with histamine (100  $\mu$ M, for 30 min at ZT 14 h), the peak was phase-delayed almost 6 h to ZT 11.50  $\pm$  0.23 h ( $n = 6$ ; Fig. 5B and P) by the next cycle. This phase-delaying effect of histamine was mimicked by the H1R agonist 2-pyridylethylamine (100  $\mu$ M; Fig. 5C and P), but not the H2R agonist amthamine (20  $\mu$ M;

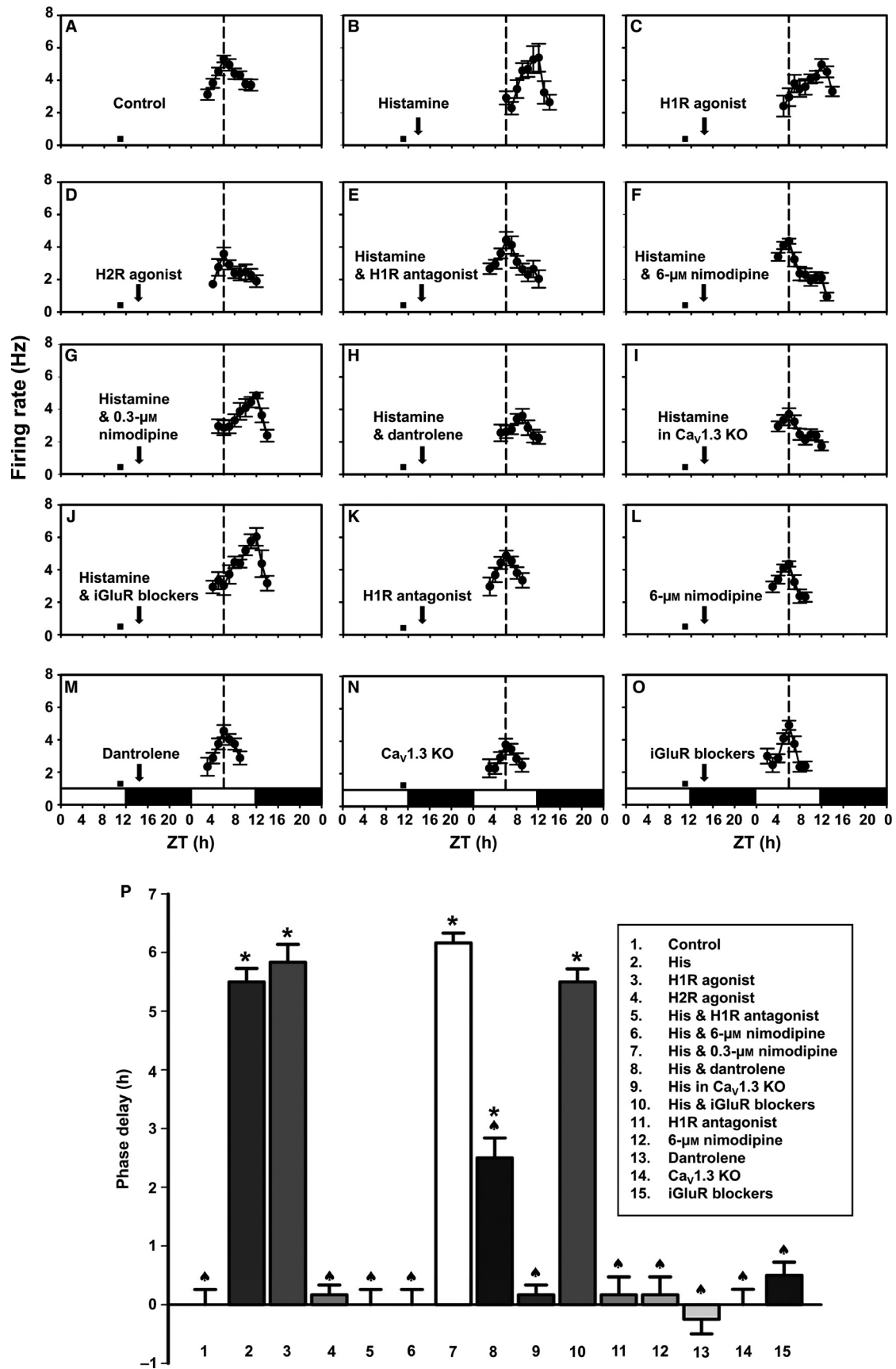




FIG. 5. Effects on the histamine-induced phase delay of circadian firing activity rhythm of suprachiasmatic nucleus (SCN) neurons recorded in the mouse hypothalamic slice, of the blockade or gene knockout (KO) of H1 histamine receptor (H1R), ryanodine receptor (RyR), ionotropic glutamate receptors (iGluRs) and  $Ca_v1.3$ . (A–O) Plots against Zeitgeber time (ZT) of the firing rate of SCN neurons recorded in different experimental conditions. Each plot shows the representative result of six repeated experiments. The projected light and dark phases of the animal room are indicated with open and filled horizontal bars, respectively. The dashed vertical line in each plot indicates the average time of peak firing activity for control slices. The filled square denotes the time of slice preparation, while the arrow indicates the time of drug application. (P) Graph summarizing the effects of various experimental treatments on the time of peak of circadian firing activity rhythm. Newman–Keuls comparison tests were performed after Kruskal–Wallis one-way ANOVA on ranks ( $H_{14} = 68.64$ ,  $P \leq 0.001$ ). The results of pair-wise comparisons of the value of each experimental group with those of control and histamine groups are denoted with asterisk and spade, respectively. \*,  $\spadesuit P < 0.05$ ; His, histamine.

Fig. 5D and P), and almost completely blocked by pyrilamine (H1R antagonist,  $10 \mu\text{M}$ ) and  $6 \mu\text{M}$  nimodipine (L-type VGCC blocker; Fig. 5E, F and P). Nimodipine at  $0.3 \mu\text{M}$ , a concentration that is selective for  $Ca_v1.2$  over  $Ca_v1.3$ , had no effect on the histamine-induced phase delay (Fig. 5G and P). The RyR antagonist dantrolene ( $10 \mu\text{M}$ ) reduced the magnitude of the phase delay by  $\sim 50\%$  (Fig. 5H and P). In addition, the histamine-evoked phase shifts were absent in KO mice lacking  $Ca_v1.3$  (Fig. 5I and P). Further, to investigate the possible involvement of ionotropic glutamate receptors (iGluRs) in histamine-induced phase delay in the mouse, the effect of a cocktail of AP5 ( $100 \mu\text{M}$ ) and DNQX ( $20 \mu\text{M}$ ) on histamine ( $100 \mu\text{M}$ )-induced phase delay was examined. The phase delay induced by histamine was not affected by the AP5 and DNQX cocktail (Fig. 5J and P). The antagonists or gene KO by themselves had no significant effect on the phase of the circadian rhythm (Fig. 5K–P). Thus, taken together, these results from the functional assay indicate that histamine phase-delays the circadian clock through the H1R– $Ca_v1.3$ –RyR pathway.

## Discussion

### Mechanism underlying the histamine-elicited $Ca^{2+}$ rise in SCN neurons

It is well known that the H1R is coupled to the  $G_{q/11}$ –PLC pathway, which mediates most of the excitatory histaminergic actions in the brain. The activation of the  $G_{q/11}$ –PLC pathway leads to the formation of inositol 1,4,5-trisphosphate ( $IP_3$ ), diacyl-glycerol (DAG) and PLA2 (Haas & Panula, 2003; Haas *et al.*, 2008).  $IP_3$  can induce  $Ca^{2+}$  release from the intracellular stores by acting on  $IP_3$  receptor, while DAG can cause  $Ca^{2+}$  influx by activating TRPC channels (Hofmann *et al.*, 1999; Haas *et al.*, 2008). Previous work has indicated that  $IP_3$ -mediated  $Ca^{2+}$  release from SCN neurons is critical for the glutamate-induced phase delay of the circadian neural activity rhythm in the SCN (Hamada *et al.*, 1999). The current results do not support the idea that histamine leads to  $IP_3$ -mediated intracellular  $Ca^{2+}$  release in SCN neurons mainly because histamine-induced  $[Ca^{2+}]_i$  rise was eliminated by the removal of external  $Ca^{2+}$  or by the L-type  $Ca^{2+}$  channel blocker nimodipine. In addition, the histamine-induced  $[Ca^{2+}]_i$  rise persisted in the presence of the TRPC channel blocker BTP-2, PKC inhibitors (chelerythrine and bisindolylmaleimide-1) or the PLA2 inhibitor quinacrine. These results indicate that the PLC or PLA2 signaling pathways do not contribute to the histamine-induced  $[Ca^{2+}]_i$  increase in mouse SCN neurons. Instead, it was found that histamine increases  $[Ca^{2+}]_i$  by activating L-type VGCCs through H1R, and secondarily by causing  $Ca^{2+}$ -induced  $Ca^{2+}$  release from RyR-mediated internal stores. Previous work has provided evidence that RyR expression (Pfeffer *et al.*, 2009) and binding (Díaz-Muñoz *et al.*, 1999) are under circadian clock control. Furthermore, RyRs have been implicated to play a crucial role in the light-induced phase delay of the circadian clock (Ding *et al.*, 1998), and are known to be potent regulators of SCN

neural activity (Aguilar-Roblero *et al.*, 2007), intracellular calcium (Ikeda *et al.*, 2003) and circadian behavior (Mercado *et al.*, 2009).

### $Ca_v1.3$ , but not $Ca_v1.2$ , mediates the histamine-induced $Ca^{2+}$ influx

The IHC results demonstrate that both  $Ca_v1.2$  and  $Ca_v1.3$  are present in mouse SCN cells. However, the results from the  $Ca^{2+}$ -imaging study suggest that  $Ca_v1.3$  mediates most of the histamine-induced  $Ca^{2+}$  influx. This could be due to the fact that  $Ca_v1.3$  is tightly coupled to H1R through certain signaling pathway(s), whereas  $Ca_v1.2$  is not or is weakly coupled to this receptor. The current finding that  $Ca_v1.3$ , but not  $Ca_v1.2$ , is co-localized extensively with H1R in the somas of SCN cells is consistent with this hypothesis.

### Histamine phase-delays the circadian clock through the H1R– $Ca_v1.3$ –RyR pathway

The results of the functional assay indicate that histamine activates the H1R– $Ca_v1.3$ –RyR pathway to phase-delay the circadian pace-maker. This conclusion is consistent with the finding of a previous study that the H1R antagonist mepyramine blocked the histamine ( $1 \mu\text{M}$ )-induced phase delays of circadian firing activity rhythms recorded in golden hamster SCN slices (Cote & Harrington, 1993). However, it does not agree with the report from the same laboratory that histamine-induced phase shifts were not blocked by mepyramine but were sensitive to the NMDA receptor antagonist AP5 (Meyer *et al.*, 1998). In the current study, it was found that a cocktail of AP5 and DNQX (non-NMDA receptor antagonist) had no effect on the histamine ( $100 \mu\text{M}$ )-induced phase delay in the mouse SCN slice. Instead, the H1R antagonist pyrilamine ( $10 \mu\text{M}$ ) was very effective in blocking the phase delay. It is unclear why histamine-induced phase shifts were not blocked by H1R antagonists but were sensitive to NMDA receptor antagonist in a previous study (Meyer *et al.*, 1998). The use of a low concentration of mepyramine ( $100 \text{ nM}$ ) might be an explanation. Or, it may be related to the use of the hamster, a species in which the SCN is almost devoid of histaminergic fibers, histamine exerts only a weak phase-shifting effect on the free-running locomotor activity rhythm, and the modest effects of histamine on the firing rates of SCN neurons are blocked by neither H1R nor H2R antagonists (Scott *et al.*, 1998).

At present, it is not known which signaling mechanism(s) couples H1R to  $Ca_v1.3$ . The PLC or PLA2 pathway, however, is unlikely to be upstream of  $Ca_v1.3$  in the light of the results from the current  $Ca^{2+}$ -imaging study.  $Ca_v1.3$  is a VGCC that is activated by membrane depolarization, and it has been reported that histamine induces membrane depolarization in other hypothalamic neurons (Li & Hatton, 1996; Smith & Armstrong, 1996; Zhou *et al.*, 2007) and that H1R activation leads to membrane depolarization through the  $G_{q/11}$  protein-mediated blockade of leak channels (Li & Hatton, 1996; Jafri *et al.*, 1997; Haas & Panula, 2003; Zhou *et al.*, 2007). Thus, it

is possible that the membrane depolarization arising from leak channel blockade by  $G_{q/11}$  is responsible for the activation of  $Ca_v1.3$  in SCN neurons that follows the binding of histamine to H1R.

$Ca_v1.3$  is activated not only by membrane depolarization, but also by phosphorylation (Qu *et al.*, 2005; Ramadan & Boutjdir, 2009). Previous work indicates that H1R is positively coupled to the cAMP signaling pathway through  $G_{\beta\gamma}$  protein (Marley *et al.*, 1991; Maruko *et al.*, 2005), and it is well known that cAMP is a strong activator of protein kinase A (PKA). Thus, it is also conceivable that the  $G_{\beta\gamma}$ -cAMP/PKA signaling pathway is intercalated between H1R and  $Ca_v1.3$ . Whether or not these are indeed the mechanisms linking H1R to  $Ca_v1.3$  awaits future studies.

### Functional significance

The circadian system controls the timing of arousal through the regulation of the electrical activity in a network of neurotransmitter systems including the histamine-expressing cell population in the TMN. Conceptually, blocking the actions of histamine will help induce or maintain sleep, while increasing histamine levels will enhance arousal. At this point, anti-histamines are commonly used as 'over the counter' sleeping aids, while H3 histamine receptor antagonists are in clinical trials as potential treatment for cognitive impairments associated with neurodegenerative disorders (Benarroch, 2010; Lin *et al.*, 2011; NIH clinical trials database – <https://clinicaltrials.gov>). These possible clinical uses raise questions about how the circadian system is influenced by histamine as well as pointing out the need to understand the underlying mechanisms. In this study, a new signaling pathway is uncovered that underlies the histamatergic regulation of the circadian system.

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### Abbreviations

$[Ca^{2+}]_i$ , intracellular  $Ca^{2+}$  concentration; ACSF, artificial cerebrospinal fluid; AP5, D,L-2-amino-5-phosphonopentanoic acid; B6 mouse, C57BL/6 mouse; DAG, diacyl-glycerol; DAPI, 4',6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; DNQX, 6,7-dinitroquinoxaline-2,3-dione; H1R, H1 histamine receptor; H2R, H2 histamine receptor; iGluR, ionotropic glutamate receptor; IHC, immunohistochemistry; IP<sub>3</sub>, 1,4,5-trisphosphate; KO, knockout; NMDA, N-methyl-D-aspartate; PBS, phosphate-buffered saline; PKA, protein kinase A; PKC, protein kinase C; PLA2, phospholipase A2; PLC, phospholipase C; R/T, room temperature; RyR, ryanodine receptor; SCN, suprachiasmatic nucleus; TMN, tuberomammillary nucleus; TRPC, transient receptor potential cation channel; VGCC, voltage-gated  $Ca^{2+}$  channels; ZT, Zeitgeber time.

### Author contributions

Y.S.K., T.-W.S., H.C.H., C.J.L. and Y.I.K. conceived this project. Y.S.K., Y.-B.K., W.B.K., B.E.Y., F.-Y.S. and S.W.L. performed the experiments and analysed the results. Y.S.K., C.S.C., C.J.L. and Y.I.K. wrote the manuscript.

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