Ryanodine receptor Ca^{2+}-release channels are an output pathway for the circadian clock in the rat suprachiasmatic nuclei

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Abstract

Ryanodine-sensitive intracellular Ca^{2+} channels (RyRs) are present in suprachiasmatic nuclei (SCN) neurons, but the functions served by these channels are not known. Here we addressed whether mobilization of intracellular Ca^{2+} stores through the RyRs may be a link between the molecular clock and the firing rate in SCN neurons. Activation of the RyRs by administration of either 1 mM caffeine or 100 nM ryanodine increased the firing frequency, whereas inhibition of RyRs by 10 μM dantrolene or 80 μM ryanodine decreased firing rate. Similar results were obtained in experiments conducted at either midday or midnight. Furthermore, these effects were not mediated by synaptic transmission as blockade of GABA A, AMPA and NMDA receptors did not prevent the excitatory or inhibitory effects induced by either dose of ryanodine on SCN firing. We conclude that gating of RyRs is a key element of the intricate output pathway from the circadian clock within SCN neurons in rats.

Introduction

Circadian rhythms in mammals are generated by the suprachiasmatic nuclei (SCN) in the anterior hypothalamus (Klein *et al.*, 1991). The molecular circadian oscillator in SCN neurons consists of a self-regulated transcription–translation loop (see Reppert & Weaver, 2002 for a review) among a group of genes known as 'clock genes'. Entrainment of rhythmicity to the light–dark cycle depends on the retinal input to SCN neurons (the retinohypothalamic tract; RHT) from a subset of retinal ganglion neurons expressing melanopsin (Rollag *et al.*, 2003; Morin & Allen, 2006). Both excitatory amino acids and the pituitary adenylate cyclase activating polypeptide seem to be involved in the synaptic transmission in the RHT (Hannibal, 2002; Morin & Allen, 2006). Recently, the role of voltage-gated Ca^{2+} channels (particularly the T-type Ca^{2+} current) associated with the glutamatergic response has been clearly demonstrated (Kim *et al.*, 2005). Although the specific details are still under study, the signalling pathway within SCN neurons in response to light involves a variety of parameters, such as increased cytoplasmic Ca^{2+}, synthesis of nitric oxide, activation of MAPK and CaMK-II, and CREB phosphorylation, which lead to change in the expression of clock genes (Meijer & Schwartz, 2003). Much less is known about the output pathways from the molecular oscillator. However, it is clear that it must regulate neuronal membrane excitability and thus induce the circadian pattern in the firing rate characteristic of the SCN.

Although calcium-dependent synaptic transmission is not essential to sustain the clock mechanism in SCN neurons (Bouskila & Dudek,

1993), intracellular calcium homeostasis in the SCN is under circadian control (Díaz-Muñoz *et al.*, 1999; Colwell, 2000; Ikeda *et al.*, 2003) and its manipulation affects expression of overt circadian rhythms (Prosser *et al.*, 1992; Shibata & Moore, 1994; Biello *et al.*, 1997). Previously, we showed a circadian rhythm in ^{3}[H]-ryanodine binding that was specific to the SCN but not in other brain areas. The peak of the rhythm occurred at circadian time 07.00 and was due to an increase in the protein expression of the neuronal ryanodine receptor (RyR) type 2 in the SCN during the middle of the day (Díaz-Muñoz *et al.*, 1999). More recently, organotypic cultures from the SCN expressed circadian rhythms in cytoplasmic calcium levels ([Ca^{2+}]i) and electrical firing rate, which could be dissociated by tetrodotoxin applied to the medium. In this condition, the blockade of the electrical activity rhythm did not affect the [Ca^{2+}]i rhythm. The cytoplasmic Ca^{2+} rhythm was damped by treatment with negative modulation of the RyR such as ryanodine (5 and 100 μM) and 8-bromo-cyclic ADP ribose (300 μM), but was not affected by nifedipine, an inhibitor of voltage-gated Ca^{2+} channels (Ikeda *et al.*, 2003). Altogether, these data strongly suggest that the [Ca^{2+}]i circadian rhythm is mainly related to the mobilization of intracellular Ca^{2+} stores through the ryanodine-sensitive Ca^{2+} channels, and are also consistent with the hypothesis that the cytoplasmic Ca^{2+} rhythm may be one of the first transmission elements linking the molecular oscillator to the circadian modulation of the firing rate in SCN neurons.

In the present study we addressed the previous hypothesis by testing the effects of pharmacological activation and inhibition of ryanodine-sensitive Ca^{2+} channels on SCN neuronal membrane potential and spontaneous firing frequency as measured by the perforated-patch technique in acute brain slices *in vitro*. If firing rate in SCN neurons is modulated via the intracellular Ca^{2+} mobilized through ryanodine-

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sensitive Ca^{2+} channels, we expect that its pharmacological opening would increase SCN neuronal firing while its closure would decrease the firing rate. Present results confirm this hypothesis, and further support the notion that intracellular Ca^{2+} mobilization through the ryanodine-sensitive Ca^{2+} channels is part of the output pathway linking the molecular oscillator to the expression of circadian rhythms in the SCN.

Materials and methods

Male Wistar rats were housed under a 12 : 12 h light : dark cycle (lights on at 06.00 h; 400 lux) in a sound-attenuated room with regulated temperature (22° ± 1 °C) for at least 1 week before starting the experiment. For electrophysiological recordings made during subjective midnight the animals were maintained in a reversed 12 : 12 h light : dark cycle (lights on at 22.00 h; 400 lux) for at least 3 weeks before the experiment. Animals had continuous access to food and water. Rats used to prepare brain slices were deeply anaesthetized with ether before extraction of the brain. All the procedures were conducted according to the guidelines for use of experimental animals from the Universidad Nacional Autónoma de México in accordance to national laws on the matter (NOM-062-200- 1999).

Slice preparation

Male Wistar rats weighing between 100 and 120 g were deeply anaesthetized with ether between Zeitgeber time (ZT) 03 and 05 (ZT 0 is lights on) and the brain was quickly removed and placed in ice-cold extraction solution (low-Ca^{2+} aCSF) containing (in mM): NaCl, 126; KCl, 2.5; NaH_2PO_4, 1.2; $MgCl_2$, 4; $CaCl_2$, 0.5; $NaHCO_3$, 26; and glucose, 10; pH 7.38, 330 mOsm/L, oxygenated with 95% O_2 and 5% CO_2 gas mixture. To avoid phase shifts induced by light, the brain slices used during subjective night were prepared between ZT 11 and 12 (before lights off). Coronal sections of 250 μm were obtained using a vibratome (Pelco) and the slices containing the SCN were transferred to fresh low-Ca^{2+} aCSF under continuous oxygenation at room temperature and kept in this condition until use. One slice was then placed in the recording chamber and continuously superfused with oxygenated aCSF at room temperature. The bath solution was identical to the extraction solution except that $CaCl_2$ was increased to 2.4 mM, $MgCl_2$ reduced to 1.3 mM and the pH adjusted to 7.38 at room temperature. SCN neurons were visualized and the recording electrodes were positioned by infrared Nomarski microscopy at 600× using a Nikon Eclipse 600 with Dage MTI video camera and monitor.

Patch-clamp recording

Recordings were made at room temperature (20–25 °C) using the whole-cell or perforated-patch technique at different moments of the circadian cycle, either between ZT 05 and ZT 10 (subjective day) or between ZT 17 and ZT 22 (subjective night). For whole-cell recordings electrodes were filled with a solution containing (in mM): KH_2PO_4, 115; $MgCl_2$, 2; HEPES, 10; EGTA, 0.5; Na_2ATP, 2; Na_2GTP, 0.2; pH 7.2, 275 mOsm/L, as previously reported by Vergara *et al.* (2003). In the case of whole-cell recordings, the membrane was disrupted by gentle suction. For perforated-patch recordings amphotericin was dissolved in dimethyl sulfoxide, 10 mg/mL, and diluted before recording to a final concentration of 80–150 μg/mL in an electrode-filling solution consisting of 150 mM KCl, pH 7.2, 300 mOsm/L. Once a good seal (>2 GΩ) was obtained between the electrode and the neuron, we waited

between 2 and 8 min in order to obtain a perforated patch. Recordings were made in a current-clamp configuration with an Axoclamp 200A amplifier (Axon Instruments, Foster City, CA, USA). On-line data collection was performed using a PC compatible with a digital acquisition board (DAQ; National Instruments) using a custom-made program in the LabView environment.

After at least 5 min of basal recording of spontaneous activity, drugs were applied by changing the extracellular solution to one containing the drug for testing, and the neuronal activity was recorded again. Each recorded neuron received only one of the following treatments: controls with aCSF; opening of the ryanodine-sensitive Ca^{2+} channels with 100 nM ryanodine or 1 mM caffeine; closure of these channels with 80 μM ryanodine or 10 μM dantrolene. Only one cell from each hypothalamic slice was recorded after administration of the drugs. Statistical comparison before and after treatment was made with the Wilcoxon test and the α level was set at 0.05. All drugs were purchased from Sigma (St Louis, MO, USA). Digitized data were stored on disk as ASCII or binary files, and were imported for analysis into commercial graphing (Origin, Microcal) and analysis (MiniAnalysis, Synaptosoft) software. Spike frequency was estimated from the inverse of the median interspike interval. Regular (rhythmic) firing was considered to occur in those neurons showing a narrow Gaussian distribution in the interspike interval histogram, while irregular (arrhythmic) firing was considered to occur in those neurons with a skewed distribution (Groos & Hendricks, 1979; Shibata *et al.*, 1984; Thomson *et al.*, 1984). In order to measure the membrane potential and the duration of the action potential, 20 consecutive segments starting 20 ms before an action potential and ending 20 ms before the next one were aligned, and the average profile was plotted to measure the membrane potential at the following instants: 20 ms before the spike (Vm); at the threshold of the spike (Vthr); at the peak of the spike (Vspk); and at the lowest point of the afterhyperpolarization potential (Vahp). The duration of the action potential was measured at the level of Vthr; the rising time of the action potential (τ_{spk}) was measured from Vthr to Vspk, and the decay time of the action potential (τ_{ahp}) was measured from Vspk to the 66% decay from Vspk to Vahp. Statistical comparison before and after the treatment was made with a paired t-test; the α level was set at 0.05.

In order to determine whether effects of ryanodine treatment on firing rate are a consequence of its effects on synaptic activity, in some experiments during subjective day we also blocked GABA A, AMPA and NMDA receptors. After recording the basal firing pattern, neurons received ryanodine (either 100 nM or 80 μM) and a cocktail containing DL-2-amino-5-phosphonopentanoic acid (AP-V), 50 μM, to block NMDA glutamate receptors; 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX), 10 μM, to block AMPA glutamate receptors; and bicuculline, 10 μM, to block GABA A receptors. AP-V, DNQX and bicuculline were always applied in sequence, but they were applied in a counterbalanced design with respect to the dose of ryanodine. Recording procedures and drug administration were performed between ZT 05 and ZT 10 as described in the previous paragraphs.

Results

Basal recordings

A total of 76 neurons were recorded within the SCN boundaries between ZT 5 and ZT 10; 22 neurons were recorded in whole-cell, 52 were recorded in perforated-patch and two were recorded in cell-attached mode. Twenty-nine neurons (38.2%) showed regular (rhythmic) firing at a rate of 5.0 ± 0.6 Hz. Most rhythmic neurons (25) were recorded in the perforated-patch, two were recorded in cell-attached

and the remaining two in whole-cell mode. In these regular firing neurons the pacemaker membrane potential ranged from -52.1 ± 2.0 mV at Vahp to -39.0 ± 1.0 mV at the spike threshold potential. The remaining 47 (61.8%) neurons fired with an irregular (arrhythmic) pattern. Arrhythmic neurons recorded in whole-cell mode ($n = 20$) fired at a rate of 1.3 ± 0.3 Hz, the resting potential was -54.8 ± 1.8 and the firing threshold was -46.2 ± 1.3 mV. Arrhythmic neurons recorded in the perforated-patch mode ($n = 27$) fired at a rate of 2.0 ± 0.2 Hz. These neurons showed a Vm of -46.3 ± 1.4 mV and Vthr was -40.6 ± 2.1 mV. In 24 neurons which did not received any treatment the spontaneous firing remained stable for at least 40 min, which was the average duration of the pharmacological experiments. Firing rates of these neurons were 3.8 ± 0.6 Hz between the 5th and 10th min of recording and 4.5 ± 0.9 Hz between the 35th and 40th min of recording (Table 1).

The effects of drugs affecting the gating of the intracellular Ca^{2+} channel sensitive to ryanodine were studied in 52 neurons as follows: caffeine, $n = 12$; dantrolene, $n = 21$; 100 nM ryanodine, $n = 12$; and 80 μM ryanodine, $n = 7$. The main results are summarized in Table 1. The effects of the different drugs started ~ 3 min after their addition to the extracellular solution and were stable after 6 min. Drug effects were not washed out even 15 min after replacement of the extracellular solution with fresh aCSF.

Effect of activating the RyRs

Ryanodine at 100 nM induced an increase in the spontaneous firing rate in seven of 12 neurons (Figs 1A and 2A), from 1.5 ± 0.7 to 4.0 ± 1.0 Hz ($P < 0.05$, Wilcoxon test). All responding neurons had an arrhythmic firing pattern prior to the ryanodine administration. Other effects of this activatory dose of ryanodine included (Table 1) a decrease in Vahp from -52.8 ± 1.7 to -44.0 ± 3.4 mV ($t = 2.98$, $P < 0.05$) and an increase in the duration of the action potential from 5.3 ± 1.1 to 11.0 ± 2.3 ms ($t = -3$, $P < 0.05$), which was due to an increase in both the τ_{spk} and τ_{ahp}. In the remaining five rhythmically firing neurons no significant changes in firing frequency were found from before (3.2 ± 0.7 Hz) to after (3.3 ± 0.8 Hz) the treatment (Fig. 2B). From the 12 neurons treated with 1 mM caffeine we found increased spontaneous firing rates in seven neurons (Fig. 2C), from 1.1 ± 0.3 to 3.0 ± 0.9 Hz ($P > 0.05$, Wilcoxon test); no other parameter was changed by the treatment. In the remaining five neurons we did found no significant change in the firing rate (Fig. 2D) from 3.5 ± 1.4 before to 3.0 ± 1.4 Hz after the treatment. Most neurons responding to caffeine ($n = 6$) showed an arrhythmic firing pattern; the remaining neuron was rhythmic.
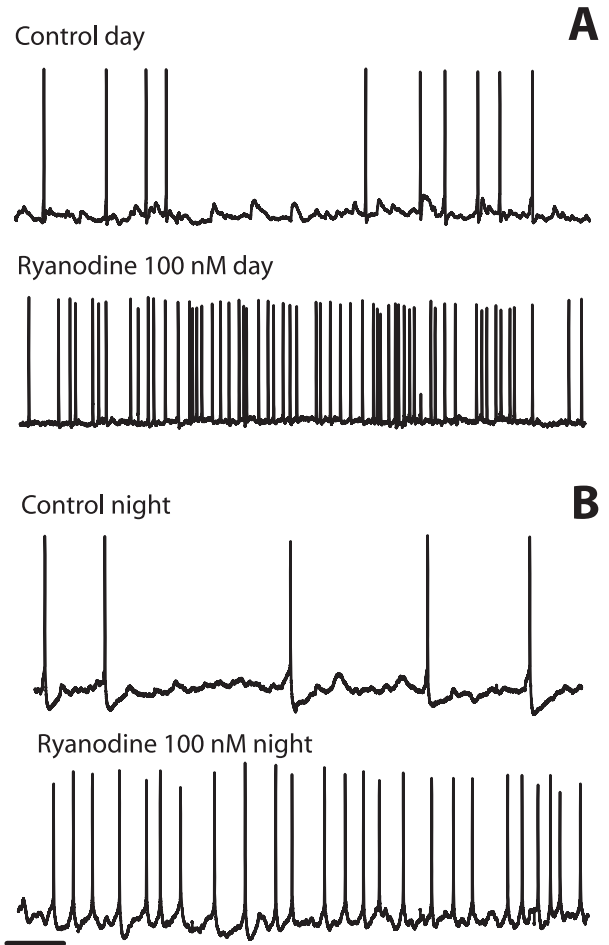

FIG. 1. Firing rate from two SCN neurons treated with 100 nM ryanodine at either (A) subjective day or (B) subjective night; at both times pharmacologically opening the RyRs led to an increase in spontaneous firing rate. Calibration bars, 10 mV and 1.0 s.

Effect of inhibiting the RyRs

Ryanodine, 80 μM, induced a decrease in the firing rate in all the seven (rhythmic) neurons tested (Figs 3A and 4C), from 5.6 ± 1.5 to 0.9 ± 0.4 Hz ($P < 0.05$, Wilcoxon test). Other effects of this inhibitory dose of ryanodine (Fig. 5A, Table 1) were a decrease in the value of Vspk from 6.3 ± 4.1 to -5.8 ± 4.3 mV ($t = 2.8$, $P < 0.05$), a

TABLE 1. Effect of different drugs acting on the RyRs on the electrical activity from SCN neurons

Treatment	Vm (mV) Before	After (↑/↓)	Firing rate (Hz) Before	After (↑/↓)	Spike duration (ms) Before	After (↑/↓)	AHP (mV) Before	After (↑/↓)
Control	-45.4 ± 1.3	-46.4 ± 1.7	3.8 ± 0.6	4.5 ± 0.9	4.5 ± 0.6	4.9 ± 0.8	-53.6 ± 1.0	-55.0 ± 1.4
Caffeine 1 mM	-48.5 ± 2.3	-49.0 ± 1.9	1.1 ± 0.3	3.0 ± 0.9*↑	5.1 ± 1.5	4.8 ± 0.9	-54.0 ± 2.4	-51.0 ± 1.9
Ryanodine 100 nM								
Day	-46.3 ± 2.9	-41.3 ± 3.2	1.5 ± 0.7	4.0 ± 1.0*↑	5.3 ± 1.1	11.0 ± 2.3*↑	-52.8 ± 1.7	-44.0 ± 3.4*↓
Night	-46.1 ± 0.5	-47.6 ± 0.6	1.9 ± 0.4	3.9 ± 0.4*↑	4.5 ± 0.1	5.4 ± 0.1*↑	-55.0 ± 0.3	-52.2 ± 0.2*↓
Dantrolene 10 μM	-47.3 ± 1.0	-54.3 ± 0.8*↑	2.4 ± 0.6	0.7 ± 0.2*↓	3.8 ± 0.8	3.6 ± 1.4	-52.0 ± 4.4	-61.7 ± 3.1*↑
Ryanodine 80 μM								
Day	-42.0 ± 1.9	-41.7 ± 1.8	5.6 ± 1.5	0.9 ± 0.4*↓	3.9 ± 1.2	4.2 ± 0.9	-54.6 ± 2.2	-43.8 ± 2.7*↓
Night	-44.0 ± 0.5	-45.0 ± 0.4	3.9 ± 1.4	2.4 ± 0.8*↓	4.0 ± 0.1	4.58 ± 0.1*↑	-56.1 ± 0.4	-48.9 ± 0.6*↓

Values are mean ± SEM. *$P < 0.05$, t-test, compared to baseline before treatment (↑, increase; and ↓, decrease).
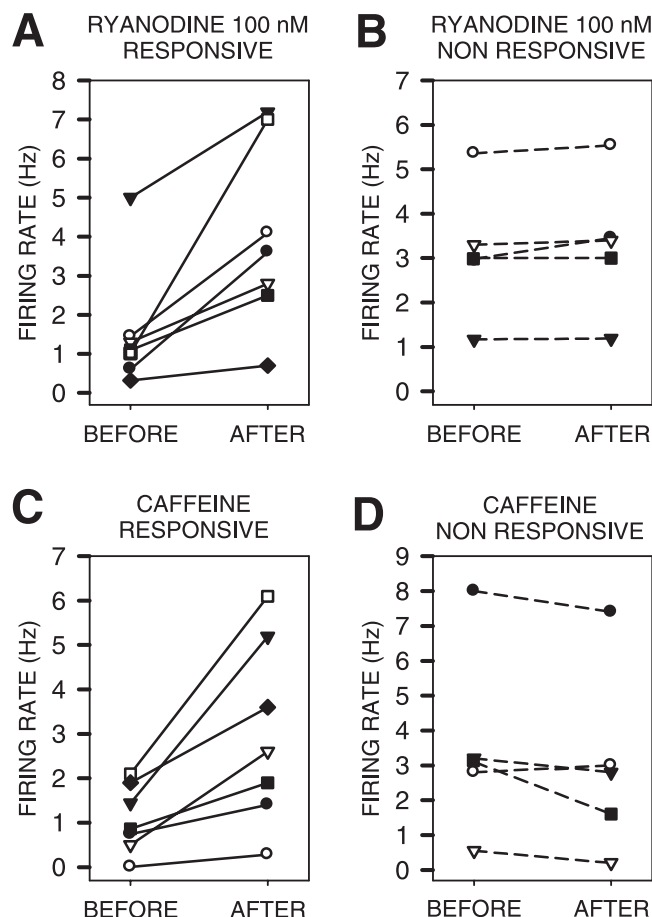
FIG. 2. Pharmacological opening the RyRs with (A) 1 mM caffeine or (C) 100 nM ryanodine increased basal firing rate in 58% of neurons tested; (B and D, respectively) the remaining neurons did not respond. The basal firing frequency partially predicted the responsiveness to either treatment.
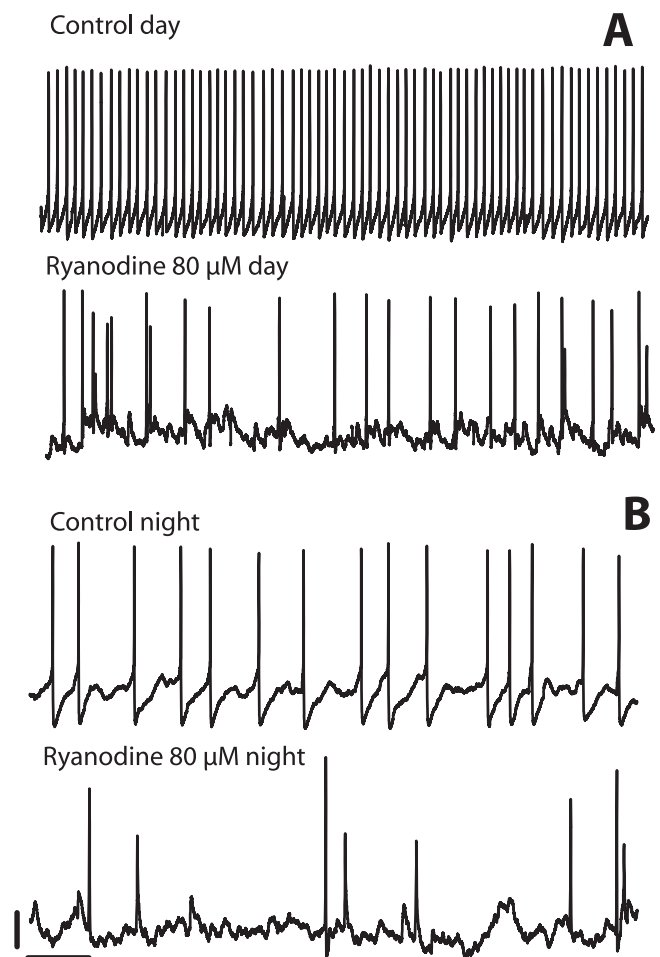

FIG. 3. Firing rate from two SCN neurons treated with 80 μM ryanodine at either (A) subjective day or (B) subjective night; at both times pharmacologically closing the RyRs led to a decrease in spontaneous firing rate. Calibration bars, 10 mV and 1.0 s.

decrease in the value of Vahp from -54.6 ± 2.2 to -43.8 ± 2.7 mV ($t = -3.7$, $P < 0.05$) and a change in the firing pattern from rhythmic to arrhythmic. On the other hand, in 14 (six arrhythmic and eight rhythmic) of 22 neurons receiving 10 μM dantrolene we found a decrease in the spontaneous firing rate (Fig. 4A), from 2.4 ± 0.6 to 0.7 ± 0.2 Hz. Other effects of dantrolene were (Fig. 5A, Table 1) to induce increases in Vm from -47.3 ± 1.0 to -54.3 ± 0.8 mV ($t = 3.3$, $P < 0.05$), Vthr from -36.0 ± 0.9 to -46.3 ± 1.3 mV ($t = 4.3$, $P < 0.05$) and Vahp from -52.0 ± 4.4 to -61.7 ± 3.1 mV ($t = 5.4$, $P < 0.05$), as well as a decrease in the V_{spk} from -5.7 ± 9.5 mV to -17.5 ± 10.5 mV ($t = 5.9$, $P < 0.05$). In the remaining eight (arrhythmic) neurons no significant effect was observed in firing frequency (Fig. 4B), which was 3.4 ± 0.8 before and 4.1 ± 1.4 Hz after treatment.

Effects of ryanodine during subjective night

In order to assess whether the effects of ryanodine on the firing frequency also occurred during the subjective night, the effects of both doses of ryanodine on SCN firing frequency were tested in 14 neurons during subjective night from ZT 17 to ZT 22 (one from each animal maintained in a shifted light:dark cycle as described in Materials and Methods). Consistent with the results obtained during the subjective day, in seven out of 10 neurons treated with 100 nM ryanodine, the neuronal firing frequency increased (Fig. 1B and 5B) from 1.9 ± 0.4 to 3.9 ± 0.4 Hz ($P < 0.05$, Wilcoxon test). On the other hand, all four

neurons treated with 80 μM ryanodine decreased their firing rate(Fig. 3B and 5B) from 3.9 ± 1.4 to 2.4 ± 0.8 Hz ($P < 0.05$, Wilcoxon test).

Blockade of SCN synaptic activity and the effects of ryanodine

Bicuculline, DNQX and APV were applied to 28 neurons in a counterbalanced design with respect to ryanodine as follows: seven neurons received 100 nM ryanodine before the synaptic blockers, and 14 neurons received 100 nM ryanodine and seven 80 μM ryanodine after the administration of the synaptic blockers. In the presence of the receptor blockers 15 of 21 neurons treated with 100 nM ryanodine increased their firing rate (Fig. 6) from 1.1 ± 0.3 to 3.1 ± 0.6 Hz ($P < 0.05$, Wilcoxon test). Likewise, all seven neurons treated with 80 μM ryanodine in the presence of the blockers decreased their firing rate from 6.3 ± 1.6 to 3.3 ± 1.0 Hz (Fig. 7). Administration of DNQX and APV had no effect on SCN firing frequency, regardless of whether they were applied before or after ryanodine. In contrast, when administered before ryanodine (21 neurons), bicuculline increased the firing rate in nine arrhythmic neurons which became rhythmic, decreased firing rate in six neurons (four arrhythmic and two rhythmic), and did not changed the firing rate in the remaining six rhythmic neurons. It is worth noting that 100 nM ryanodine further increased the firing rate in eight of the nine neurons which had already increased their firing following application of bicuculline.
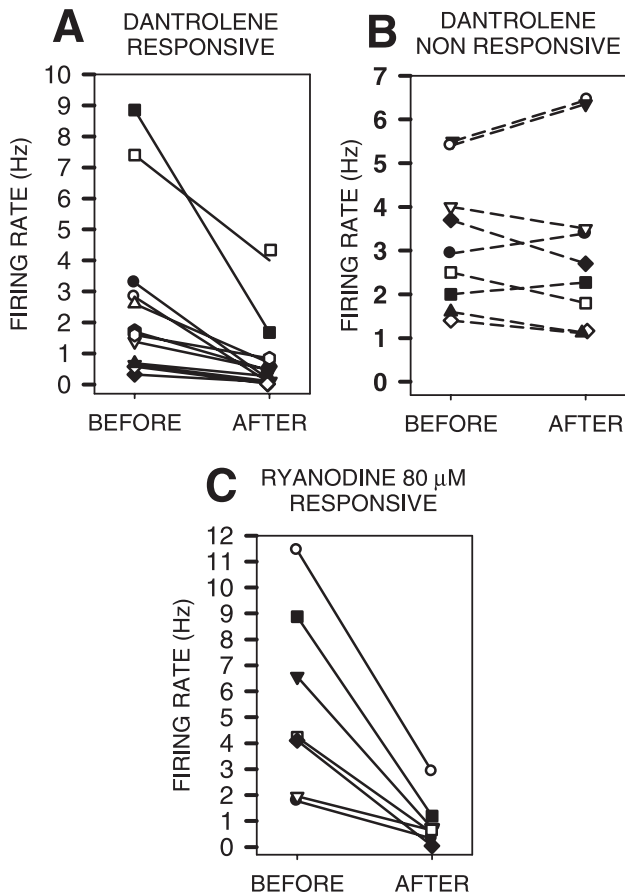
FIG. 4. Pharmacological closing of the RyRs with 10 μM dantrolene (A) decreased basal firing rate in 64% of neurons tested; (B) the remaining neurons did not respond. (C) We selected only rhythmic neurons to be treated with the inhibitory dose of ryanodine (80 μM) and all neurons decreased their firing rate.
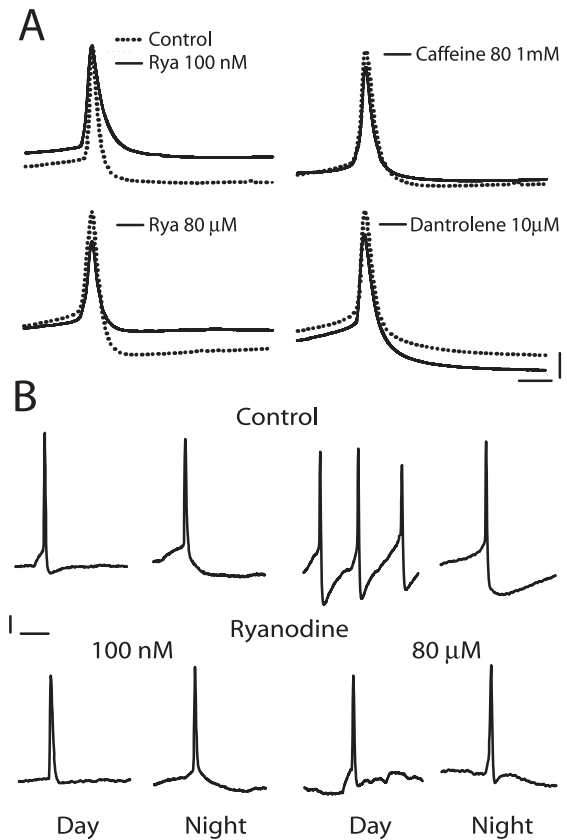

FIG. 5. Effects of different treatments on the characteristics of the action potentials. (A) Mean value ($n = 5$ neurons) of action potentials from each treatment and its control. (B) Original traces from action potentials before and after treatment with each dose of ryanodine (either 100 nM or 80 μM) administered during the day or night. Calibration bars, 10 mV, 4 ms (A), 10 mV, 25 ms (B).

Discussion

Regulation of Ca^{2+} concentration in the cytoplasm occurs by a fine-tuned set of counterbalanced mechanisms collectively known as intracellular calcium homeostasis. Intracellular calcium stores in the endoplasmic reticulum are a key component in the dynamics of $[Ca^{2+}]i$ and release from these stores depends mainly on high-conductance Ca^{2+} channels from the endoplasmic reticulum, termed RyRs and inositol (1,4,5)-triphosphate receptors (IP$_3$Rs) respectively (Meldolesi & Pozzan, 1998; Verkhratsky, 2005). Intracellular Ca^{2+} is an important element in diverse intracellular signalling pathways including modulation of neuronal excitability (Carafoli *et al.*, 2001; Rizzuto, 2001). In the present work we tested the hypothesis that the pharmacological manipulation of the RyRs is able to modulate the neuronal firing rate in the SCN.

As predicted by our hypothesis, pharmacological manipulation of ryanodine-sensitive intracellular Ca^{2+} channels in SCN neurons *in vitro* modify their firing rate. Thus, opening the RyRs by administration of either 100 nM ryanodine or 1 mM caffeine increased the firing frequency, without effects on the firing threshold, while closing of RyRs by administration of 80 μM ryanodine or 10 μM dantrolene decreased the firing rate. These effects were induced both during daytime (ZT 4–10) and nighttime (ZT 17–22), which clearly indicates that pharmacological opening or closing of the RyRs overrides the control of the SCN neuronal firing rate by the molecular

clock; this suggests that RyRs are functionally located in the pathway from the molecular clock to the membrane excitability.

Blockade of GABA$_A$ receptors with 10 μM bicuculline and activation of RyRs with 100 nM ryanodine in the SCN had similar effects, which were an increase in the spontaneous firing frequency and a change from an arrhythmic to a rhythmic firing pattern (Kononenko & Dudek, 2004). Therefore, we decided to address whether synaptic activity (either GABAergic or glutamatergic) was involved in the effects of the different doses of ryanodine on the SCN. Blockade of synaptic activity did not prevent the increase or the decrease in spontaneous firing rate induced by ryanodine at 100 nM or 80 μM, respectively. This finding indicates that ryanodine modulates the SCN firing rate by acting directly on Ca^{2+} mobilization through RyRs rather than affecting overall synaptic transmission within the SCN. Furthermore, present results confirm previous observations by Kononenko & Dudek (2003) that bicuculline increases the firing rate and changes the firing pattern in some SCN neurons; they also indicate that there is no contribution from glutamatergic synaptic activity to the spontaneous firing rate in SCN neurons.

As shown in Table 1, the neuronal responsiveness to the treatments that induced opening of the RyRs was related to basal firing frequency. Thus increases in firing rate induced by activation of the RyRs were found in neurons with a low mean firing frequency, while the opposite was found in neurons responding to drugs closing the RyRs. Nevertheless, Figs 2 and 4 also show that responsive and nonresponsive neurons showed a wide range of firing rates, suggesting that other
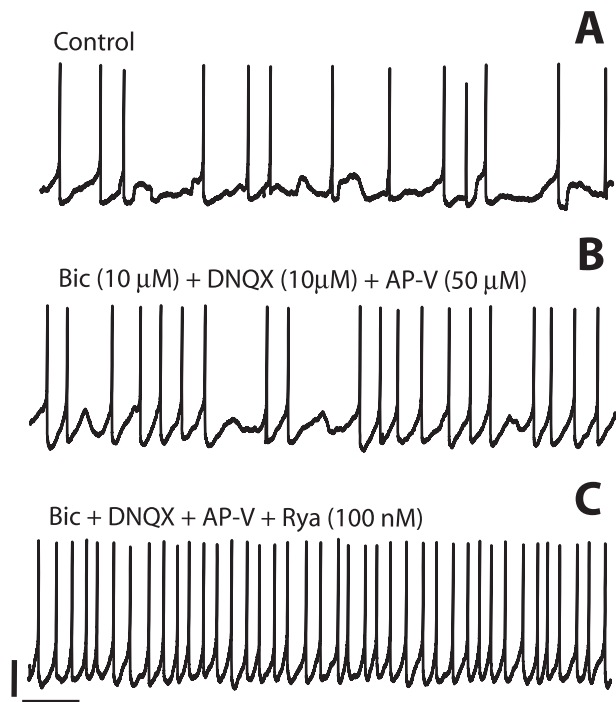
FIG. 6. Firing rate from an SCN neuron (A) before and (B) after treatments with a cocktail containing 10 μM bicuculline (Bic), 10 μM DNQX and 50 μM AP-V, and (C) the previous cocktail with 100 nM ryanodine (Rya) added. Calibration bars, 10 mV and 1.0 s.
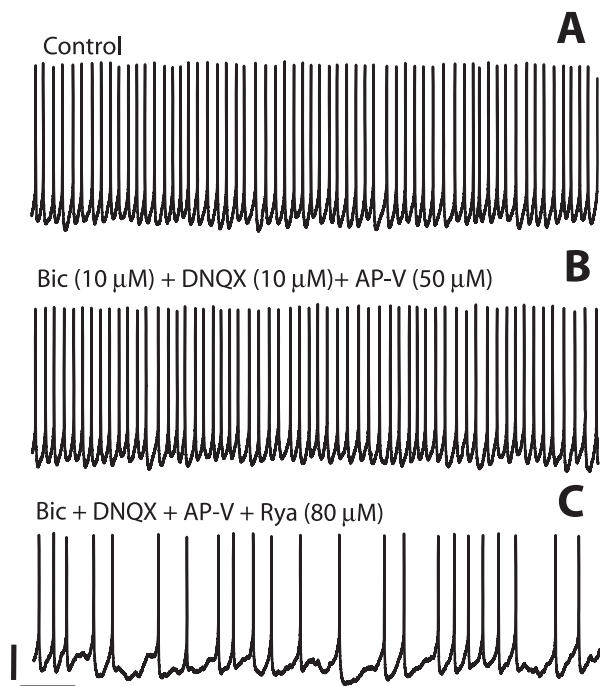

FIG. 7. Firing rate from an SCN neuron (A) before and (B) after treatments with a cocktail containing 10 μM bicuculline (Bic), 10 μM DNQX and 50 μM AP-V, and (C) the previous cocktail with 80 μM ryanodine (Rya) added. Calibration bars, 10 mV and 1.0 s.

factors are involved in neuronal responsiveness to ryanodine. Activation of RyRs by caffeine or 100 nM ryanodine increased firing rates in neurons with irregular firing patterns, whereas neurons which did

not respond already showed a rhythmic pattern before the treatment. On the other hand, inhibition of RyRs with dantrolene decreased firing rate, mostly in neurons showing a regular firing pattern before treatment, whereas those neurons which did not respond to dantrolene already had an irregular pattern. Based on these observations, we applied 80 μM ryanodine only to those neurons showing a regular firing pattern before treatment, and found both a decrease in the firing rate and a change to an irregular firing pattern in 100% of the tested neurons.

Although caffeine at μM doses is also an antagonist of adenosine receptors (Fredholm *et al.*, 1999), which are present in the SCN (Watanabe *et al.*, 1996; Antle *et al.*, 2001; Elliot *et al.*, 2001), *in vitro* studies on the effect of agonists and antagonists to A_1 and A_{2A} adenosine receptors suggest that caffeine will not by itself have any effect on the spontaneous SCN activity, but rather would prevent the effects induced by activation of presynaptic A_1 adenosine receptors (Chen & van den Pol, 1997; Hallworth *et al.*, 2002). The effects of caffeine (1 mM) found in this study are mainly due to its effects on the RyRs, as 100 nM of ryanodine induced a similar increase in firing rate. Nevertheless, the differential effects on the spike duration and the value of the afterhyperpolarization potential (AHP) found between 1 mM caffeine and 100 nM ryanodine indicates that the present dose of caffeine may involve other mechanisms of action.

Further studies are needed to characterize the ionic currents involved in the effects previously described, but those involved in AHP which is characteristic of regular firing neurons are of particular interest. The role of ryanodine-sensitive intracellular Ca^{2+} stores on the generation of the AHP in SCN neurons was discounted in a previous study (Cloues & Sather, 2003). However, the dose of ryanodine used (10 μM) did not inhibit the RyRs, but rather induced a subconductance state characterized by a long-term open state of the channel, which led to an eventual depletion of internal calcium stores (Chu *et al.*, 1990). The use of 80 μM ryanodine to effectively block RyRs used in this study clearly abolished the AHP of rhythmic SCN neurons and indicates AHP modulation by intracellular Ca^{2+} mobilized through RyRs. Similar dependence on RyR activity was observed in the onset of plateau potentials and wind-up in spinal motoneurons (Mejía-Gervacio *et al.*, 2004) On the other hand, the role of Ca^{2+} inward current through L- and R-type voltage gated Ca^{2+} channels on driving the pacemaker firing rate is still controversial: from studies in brain slices it has been suggested that modulation of these channels regulates AHP and thus the firing rate (Pennartz *et al.*, 2002; Cloues & Sather, 2003), whereas studies from dissociated SCN neurons indicate only a minor contribution from inward Ca^{2+} currents in these processes (Jackson *et al.*, 2004). Present data are consistent with a role for intracellular Ca^{2+} mobilization through RyRs, acting in concert with plasmatic membrane Ca^{2+} currents in regulating both the AHP and the firing rate in SCN neurons, probably via large- and small-conductance Ca^{2+}-dependent K^+ (K_{Ca}) channels (i.e. BKCa- and SKCa-type, respectively), and apamine- and iberotoxin-insensitive K_{Ca} channels (Cloues & Sather, 2003; Teshima *et al.*, 2003). However, inactivation of L- and R-type Ca^{2+} channels by intracellular calcium concentration (reviewed in Eckert & Chad, 1984) can also play a role in the firing rate as well as the dynamics of the action potential. Furthermore, the role of intracellular Ca^{2+} in the firing-rate modulation of SCN is not a simple one, as indicated by the increase in the duration of the action potential and the decrease in Vahp induced by 100 nM ryanodine. These results suggest the modulation of other currents besides those involved in the AHP; this could be related to the fact that, in addition to directly modulating the activity of Ca^{2+}-sensitive channels, such as Ca^{2+}-activated K^+ and Cl^- channels (Faber & Sah, 2003 and Frings *et al.*, 2000) and inactivation of L- and R-type Ca^{2+}

channels (Eckert & Chad, 1984), cytoplasmic calcium can also regulate the gating of plasma membrane ion channels by influencing the equilibrium between phosphorylated and dephosphorylated channels via Ca^{2+}–calmodulin kinases and phosphatases (Kortvely & Gulya, 2004). In addition, cytoplasmic calcium may also alter physical properties of phospholipids which influence membrane fluidity (Simkiss, 1998) and, in the longer term, activate transcriptional programs (Konur & Ghosh, 2005).

The role of intracellular Ca^{2+} in the regulation of circadian rhythms in SCN neurons has been previously suggested by independent groups. Glutamate and serotonin can elicit $[Ca^{2+}]i$ oscillations in SCN neurons and glia even in the absence of extracellular Ca^{2+}, suggesting that the stimulus-induced Ca^{2+} mobilization derives mainly from intracellular stores (Van den Pol et al., 1992). Díaz-Muñoz et al. (1999) demonstrated circadian regulation of type 2 RyRs in SNC neurons, and Ding et al. (1998) showed that light- and glutamate-induced phase delays during early subjective night are dependent on RyR activity. Colwell (2000) suggested that cytoplasmic Ca^{2+} rhythms were driven by voltage-sensitive Ca^{2+} channels. Recently two groups reported a role for transmembrane Ca^{2+} flux to sustain the rhythmic clock gene expression in the SCN (Lundkvist et al., 2005; Sang-Soep et al., 2005). Intracellular calcium dynamics involve both Ca^{2+} influx through plasma membrane Ca^{2+}-dependent channels and Ca^{2+} efflux from internal deposits (Carafoli et al., 2001). Accordingly, Ikeda et al. (2003) blocked or decreased the amplitude in the neuronal activity rhythm without affecting the rhythm of cytoplasmic Ca^{2+} concentration. The independence between voltage-sensitive Na^+ and Ca^{2+} membrane currents and the intracellular Ca^{2+} rhythm, and the decrease in amplitude of both rhythms by RyR blockers, suggests that Ca^{2+} mobilized through these channels might directly link the core oscillator with the electrical activity rhythm. However, Honma & Honma (2003) have suggested that cytosolic Ca^{2+} might only indirectly regulate the firing rate in SCN neurons due to the 4-h phase delay between the intracellular Ca^{2+} and the electrical firing rhythms in the same neuron found by Ikeda et al. (2003). Present data demonstrate that the SCN neuronal firing rate can be regulated by intracellular Ca^{2+} mobilized through RyRs. It remains to be established whether the circadian variation in the number of RyRs present in SCN neurons is the only process under regulation of the clock core, or whether other elements involved the gating of RyRs are also circadian-regulated, such as metabolic signals, regulatory proteins and plasmatic membrane channels interacting with the endoplasmic reticulum (Fill & Copello, 2002; Meissner, 2002; Butanda-Ochoa et al., 2003). Any of these elements is likely to also be under control of the clock genes, and therefore to be a part of their output pathway. On the other hand, although we did not find circadian modulation in inositol (1,4,5)-triphosphate receptors (IP$_3$Rs) in the rat brain (Díaz-Muñoz et al., 1999), coordination between IP$_3$Rs and RyRs has been demonstrated in other systems (Morales-Tlalpan et al., 2005), and it is necessary to evaluate its contribution to the regulation of cytoplasmic Ca^{2+} in SCN neurons. Finally, intracellular Ca^{2+} is able to control neuronal excitability at many levels (Berridge, 1998; Morikawa et al., 2003) and, as pointed out by Honma & Honma (2003), it is not possible to rule out a priori the contribution of these processes on the clock output.

In conclusion, we propose that RyRs are key elements in the transmission of the circadian oscillation from the transcription–translation loop of clock genes to the membrane excitability in the SCN neurons, which in turn would send a circadian modulated firing pattern to other brain areas involved in behavioural rhythm expression. Intracellular Ca^{2+} mobilization through RyRs may affect neuronal excitability, directly through Ca^{2+}-modulated plasma membrane channels and indirectly as a second messenger activating Ca^{2+}-dependent protein kinases and phosphatases regulating a variety of cellular processes converging at the cell membrane. In the long term, intracellular calcium fluctuations may impact on membrane potential by controlling, at the transcriptional level, the synthesis of new ion channels and receptors. Further studies are needed to continue unraveling the role of other elements of cellular Ca^{2+}homeostasis in this process.

Acknowledgements

Abbreviations

$[Ca^{2+}]i$, cytoplasmic calcium levels; AHP, afterhyperpolarization potential; AP-V, DL-2-amino-5-phosphonopentanoic acid; DNQX, 6,7-dinitroquinoxaline-2,3(1H,4H)-dione; RyR, ryanodine receptor; SCN, suprachiasmatic nuclei; τ_{ahp}, decay time of the action potential; τ_{spk}, rise time of the action potential; Vahp, membrane potential at the lowest point of the afterhyperpolarization; Vm, membrane potential 20 ms before the spike; Vspk, membrane potential at the peak of the spike; Vthr, membrane potential at the threshold of the spike; ZT, Zeitgeber time.

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