Ryanodine-Sensitive Intracellular Ca2+ Channels in Rat Suprachiasmatic Nuclei Are Required for Circadian Clock Control of Behavior

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Abstract Electrophysiological and calcium mobilization experiments have suggested that the intracellular calcium release channel ryanodine receptors (RyRs) are involved in the circadian rhythmicity of the suprachiasmatic nucleus (SCN). In the present report the authors provide behavioral evidence that RyRs play a specific and major role in the output of the molecular circadian clock in SCN neurons. They measured the circadian rhythm of drinking and locomotor behaviors in dim red light before, during, and after administration of an activator (ryanodine 0.1 μM) or an inhibitor (ryanodine 100 μM) of the RyRs. Drugs were delivered directly into the SCN by cannulas connected to osmotic minipumps. Control treatments included administration of artificial cerebrospinal fluid, KCl (20 mM), tetrodotoxin (1 μM), and anysomicin (5 μg/μl). Activation of RyRs induced a significant shortening of the endogenous period, whereas inhibition of these Ca^{2+} release channels disrupted the circadian rhythmicity. After the pharmacological treatments the period of rhythmicity returned to basal values and the phase of activity onset was predicted from a line projected from the activity onset of basal recordings. These results indicate that changes in overt rhythms induced by both doses of ryanodine did not involve an alteration in the clock mechanism. The authors conclude that circadian modulation of RyRs is a key element of the output pathway from the molecular circadian clock in SCN neurons in rats.

Key words circadian timing system, suprachiasmatic nucleus, ryanodine receptor, intracellular calcium, circadian output, behavioral regulation

In mammals, endogenous circadian rhythms are generated within the suprachiasmatic nucleus (SCN) localized in the ventral hypothalamus (Moore, 1983). Functional studies have demonstrated that SCN neurons are able to generate 24-h rhythms of metabolic and electrical activities when they are isolated or in cultured conditions (Inouye and Kawamura, 1982; Welsh et al., 1995). The timing system in each SCN neuron consists of 3 components (Reppert and Weaver, 2002): 1) a synchronization input pathway that entrains the clock to environmental signals, 2) a pacemaker (molecular clock) underlying feedback

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regulation of clock genes that generate circadian rhythmicity, and 3) an output pathway that coordinates the metabolic, endocrine, and behavioral expression of the circadian rhythms. The mechanisms involved in the SCN synchronization by light are well documented. Specialized retinal ganglion cells form the retinohypothalamic tract that projects to the ventral region of the SCN (Hannibal et al., 2000; Hannibal, 2002). Upon photic stimulation glutamate is released to initiate 2nd messenger cascades that lead to molecular regulation of clock genes (Gillette and Mitchell, 2002).

In contrast, the information about the output of the circadian clock is scarce. One of the few established facts is that the SCN electrophysiological activity plays a major role in the output of the circadian pacemaker (Schwartz et al., 1987; Meredith et al., 2006; Aguilar-Roblero et al., 2007). The neuronal excitability is then linked to the rhythmic variations of metabolic activity, hormones levels, physiological processes, and behavioral responses, including the circadian rhythm of drinking and eating. Schwartz et al. (1987) demonstrated that blocking of the electrical properties of the SCN by tetrodotoxin (TTX) administration prevented the circadian expression of drinking behavior. Upon removal of TTX, the rhythm of drinking water reappeared with the same phase and period displayed prior to toxin administration. This experiment suggested that the clock continues running despite that its electrical output is silenced; therefore, the rhythmic electrical activity of SCN neurons is an output of the clock and not a component of the pacemaker machinery itself.

Calcium-binding proteins and intracellular calcium are involved in most of the relevant aspects of SCN physiology. Ca^{2+} entry by *N*-methyl D-aspartate (NMDA) receptor is necessary for light entrainment (Meijer and Schwartz, 2003), and calbindin-containing neurons form a core region that lack rhythmic properties (Kriegsfeld et al., 2004; Jobst et al., 2004; Hamada et al., 2003). Ca^{2+}-activated K^+ channels of large conductance (BK) have been suggested as important for the pacemaker output (Colwell, 2006; Meredith et al., 2006). We have shown that Ca^{2+} mobilization through the intracellular calcium channel activated by ryanodine (RyR) in the SCN is involved in the output pathway between the molecular circadian clock and the electrical output of the SCN. Previous work by our laboratory reported that the RyRs showed a circadian rhythm in [^{3}H]ryanodine binding in membranes of SCN cells (Díaz-Muñoz et al., 1999) and pharmacological manipulation of RyRs modulated the firing frequency in SCN neurons (Aguilar-Roblero et al., 2007).

In the present study we further addressed the role of RyRs in the output of SCN rhythmicity by testing in vivo whether its pharmacological manipulation affects the circadian expression of locomotion and drinking behavior. The transient alteration of behavioral rhythms by the pharmacological activation (0.1 µM ryanodine) and inhibition (100 µm ryanodine) of RyR without long-lasting effects on behavioral rhythmicity, and the return to basal values of period and phase of activity onset after cessation of treatment, confirm that this intracellular Ca^{2+}-release channel participates in the output pathways linking the molecular oscillator in the SCN to the overt behavioral expression of circadian rhythmicity.

MATERIAL AND METHODS

Male Wistar rats weighing 250 to 300 g were housed under 12:12 h light/dark cycle (lights-on at 08:00 h, 400 lux) in a sound-attenuated room with regulated temperature (22 ± 1 °C) for at least 1 week before starting the experiment. Animals had continuous access to food and water. All surgical procedures were conducted in antiseptic conditions and under general anesthesia with pentobarbital (30 mg/kg of body weight, intraperitoneal). At the end of the procedure all animals received benzatinic penicillin (800,000 IU). All other procedures were conducted according to the guidelines for use of experimental animals from the Universidad Nacional Autónoma de México in accordance with national laws (NOM-062-200-1999).

Rats were continuously recorded under constant red light (50 lux, DD) for drinking behavior and locomotion as follows. Animals were individually housed in Plexiglas cages equipped with contact sensors in the water spout and infrared beams in the sides of the cage. Each time the sensors were touched or 2 successive light beams were interrupted, a normalized electric square pulse was generated and added to the respective event counter in a microprocessor. The counters were read at 5-min intervals and reset to 0, and the data from all independent sensors and channels were stored in a computer database for post hoc plotting and analysis.

The animals were randomly assigned for treatment to 1 of the following groups: 1) artificial cerebrospinal fluid (aCSF) containing in mM: 140 NaCl, 3.5 KCl, 1.3 $CaCl_2$, 1.0 $MgCl_2$, and 5.0 HEPES (pH

7.25); 2) ryanodine 0.1 μM to activate the RyR and induce Ca^{2+}release from its intracellular stores; and 3) ryanodine 100 μM to inhibit the RyR and prevent Ca^{2+} release from its intracellular deposits. Additional groups used as controls were 4) TTX 1 μM, which prevents generation of action potentials by blocking Na^{+} channels; 5) KCl 20 mM, which leads to membrane depolarization—the osmolarity of the solution was kept constant by decreasing NaCl to 120 mM; and 6) anisomycin (5 μg/μl), an inhibitor of protein synthesis, was used to discriminate whether the effects of blocking Ca^{2+} release involved alteration in protein synthesis. All drugs and KCl were administered in a CSF vehicle at a rate of 0.5 μl/h by means of an activated osmotic minipump (Alzet model 2002; Durect Corporation, Cupertino, CA) implanted subcutaneously between the scapular blades and filled with the corresponding treatment. The minipump was connected by Tygon tubing to a 26-ga injector (Plastic One, Roanoke, VA) stereotaxically implanted in the midline at the upper edge of the SCN (AP 0.4; L 0.0; V –8.5, according to Paxinos and Watson, 1998). The experiment consisted of 7 days of habituation to the environmental conditions, and the next 15 days were recorded as the experimental baseline. At the end of the baseline the animals were anesthetized between CT02 and CT10 under red light (50 lux) and the surgery to implant the injector and the minipump was performed; 15 to 20 min after surgery, animals were returned to their recording chamber until recovery was complete. The recording continued for at least 30 more days. All treatments took place during the first 14 days after implantation of the minipump, which corresponds to the estimated time of ejection from the reservoir according to the manufacturer. The remaining 16 to 24 days of recording were used to determine whether or not the parameters under study returned to baseline levels.

At the end of the recordings the animals were killed with an overdose of pentobarbital and transcardially perfused with 0.9% NaCl solution followed by 10% formalin. The brain from each animal was then extracted and immersed in sucrose solutions of increasing concentration (10%, 20%, and 30%) until it sank. The anterior hypothalamus was sectioned at 40 μm in the coronal plane and sections 200 μm apart were processed for Nissl staining with cresyl violet. The sections were inspected in a microscope to locate the tip of the injector. Only those animals that showed the injector in the SCN were included in this study.

In control and all experimental groups the data of drinking and locomotor recordings were similar with only slight differences in onset or intensity; therefore, we decided to add the values of both parameters at 15-min time intervals. Actograms from experimental data were double plotted. Complete recordings were analyzed by reiterative χ^2 periodogram analysis of 8 day segments at 3-day intervals. That is, for a recording lasting 45 days the 1st periodogram was performed from the 1st to 8th days of recording, the next one from the 3rd to the 10th day, and so on to the 38th to 45th days. Each successive periodogram was plotted displaced from the previous one to simulate a 3-dimensional array. This procedure is similar to the compress spectral array (Bickford et al., 1973), but instead of the fast Fourier analysis we used the χ^2 analysis. The procedure reveals the time course of the change in amplitude of periodic components throughout the analyzed time span. The effect of the different treatments on the period of rhythmicity was estimated from periodograms obtained from the last 8 days of each segment of the experiment (baseline, treatment, and recovery); the period of rhythmicity was read from the peak in the range from 20 to 28 h that reach an α level of 0.0001; arrythmicity was considered to occur when the amplitude of the peaks did not reach an α level of 0.01.

The activity onset was considered as the point of inflexion in the slope of the cumulative plot of activity for each 24-h segment; the activity onset of each segment of the experiment was identified by visual inspection of the actogram and a line was fitted to successive activity onsets. The value of linear regression fitting of the activity onsets during recovery to the projected line generated from the activity onsets during the basal recording was used to estimate whether the phase was the same before and after the treatments. We also estimated the phase differences in activity onset occurring in the transitions from the baseline to the treatment, and from the treatment to the recovery. For animals in which treatment prevented the expression of overt rhythmicity, we estimated the phase difference in activity onset between the baseline projected to the end of the treatment and the corresponding recovery (Schwartz et al., 1987).

The parameters from each experimental segment were compared by Friedman ANOVA followed by the Dunnett T3 post hoc test when necessary. Due to the absence of rhythmicity induced by some treatments we use the Wilcoxon sign rank test to compare before and after drug administration when rhythmicity returned, and the Cochran Q test to compare between groups showing circadian rhythmicity (score

Table 1. Effect of different treatments on circadian parameters.

	τ (*h:min ± min*)			ϕ (*r*)		
Group	*Basal Mean ± SEM*	*Treatment Mean ± SEM*	*Recovery Mean ± SEM*	*Basal Mean ± SEM*	*Treatment Mean ± SEM*	*Recovery Mean ± SEM*
aCSF	24:10 ± 00:02	24:10 ± 00:03	24:14 ± 00.03	0.95 ± 0.02	0.92 ± 0.05	0.92 ± 0.02
Rya 0.1	24:14 ± 00:02	**23:59 ± 00:12**[a]	24:16 ± 00:02	0.94 ± 0.02	**0.63 ± 0.06**[b]	0.89 ± 0.05
KCl	24:18 ± 00:09	**24:07 ± 00:03**[a]	24:15 ± 00:02	0.96 ± 0.02	**0.73 ± 0.03**[b]	0.89 ± 0.02
Rya 100	24:09 ± 00:06	—	24:09 ± 00:03	0.95 ± 0.03	—	0.87 ± 0.05
TTX	24:13 ± 00:03	—	24:09 ± 00:06	0.95 ± 0.01	—	0.88 ± 0.04
Any	24:15 ± 00:04	—	—	0.94 ± 0.01	—	—

τ = free running period estimated from the χ^2 periodogram; ϕ = phase of the activity onset; aCSF = artificial cerebrospinal fluid; Rya = ryanodine; KCl = 20 mM KCl; TTX = tetrodotoxin. The *r* value refers to the fitting of activity onsets during each experimental phase to the line adjusted from the basal recording. Post hoc comparisons were made with Dunnett T3 test; superscript letters indicate significant differences with respect to the control ([a] $p < 0.05$; [b] $p < 0.01$). Bold face text indicates data with significant differences with the control.

1) versus those arrhythmic groups (score 0). In all analyses, the α level was set at 0.05.

RESULTS

From 56 implanted rats at the beginning of the experiment, only 32 had the microinjector tip within the SCN boundaries. From the remaining 24 animals the microinjector tip was found rostral ($n = 8$) or caudal ($n = 10$) to the SCN; in the other 6 animals the microinjector was found within or below the SCN and substantial damage to at least one of the nuclei was evident. In most of these animals the treatments had no noticeable effects; in the animals bearing an SCN lesion there were changes of period, phase shifts after implantation of the microinjector, and in 1 subject receiving TTX the arrhythmic pattern persisted through the remainder of the recording. In 5 animals in which the microinjector was placed caudal to the SCN we observed changes in period and transient loss of rhythmicity in 2 TTX-treated subjects. All other results are summarized in Table 1.

All subjects showed clear free-running circadian rhythmicity during the baseline recording. Continuous infusion of aCSF into the SCN vicinity did not induced noticeable changes in period or phase during and after the treatment (Fig. 1A). Dynamical χ^2 periodogram (Fig. 1B) confirmed no effects on the period during or after the aCSF infusion with respect to the baseline 24:10 ± 2 (mean [h:min] ± SEM [min]) and 24:10 ± 2.6 and 24:14 ± 2.6, respectively (Friedman $\chi^2 = 3.8$, NS; Table 1). Furthermore, the *r* value of activity onset after the aCFS fitted to the lines projected from the basal recordings was 0.92 ± 0.02 (Friedman $\chi^2 = 4.8$. NS; Table 1).

Animals treated with the activator dose of ryanodine (100 nM) maintained clear circadian rhythms, but the period was slightly shortened and the interval of activity apparently was compressed during and after the treatment with respect to the baseline (Fig. 1C). The effect on the period was clearly seen in the χ^2 periodogram: 24:14 ± 2.40 (mean [h:min] ± SEM [min]) during baseline; 23:59 ± 12.2 during treatment, and 24:16 ± 2.10 during recovery (Fig. 1D; Friedman $\chi^2 = 8.6$, $p = 0.014$). The *r* value of activity onset after 100 nM ryanodine fitted to the lines projected from the basal recordings was 0.89 ± 0.05 (Table 1; Friedman $\chi^2 = 8.4$, $p = 0.015$). Administration of 20 mM KCl produced a very similar effect to the one induced by a 0.1 μM ryanodine, that is, shortening of period and compression of the activity interval during and after the treatment. The period estimated by the χ^2 periodogram was 24:18 ± 9.0 during the baseline, 24:07 ± 3.00 during the treatment, and 24:15 ± 1.6 during recovery (Friedman $\chi^2 = 8.7$, $p = 0.013$). The *r* value of activity onset after high potassium treatment fitted to the lines projected from the basal recordings was 0.89 ± 0.02 (Table 1; Friedman $\chi^2 = 10$, $p = 0.007$).

Rats treated with 100 μM ryanodine showed disruption of behavioral rhythms by disorganization of the daily pattern of activity during the period of administration of the drug (Fig. 1E). After withdrawal of ryanodine the rhythm reappeared and the activity onset adjusted to a line projected from the activity onsets from the baseline recording. The effects on the period of the rhythm were evident in the χ^2 periodogram (Fig. 1F): baseline 24:09 ± 5.60, recovery 24:09 ± 3.40. Because of the absence of rhythmicity induced by ryanodine 100 μM we use the Wilcoxon sign rank test to compare before and after drug administration ($Z = -1.3$, NS); the Cochran test was used to compare all 3 groups coding 1 for circadian rhythm and 0 for arrhythmcity ($Q = 10$, $p = 0.007$). The *r* value of activity onsets after the treatment fitted to the line projection from the basal recordings was 0.87 ± 0.05 (Table 1; Wilcoxon sign rank test $Z = -2.02$, $p = 0.053$). Similar alterations were

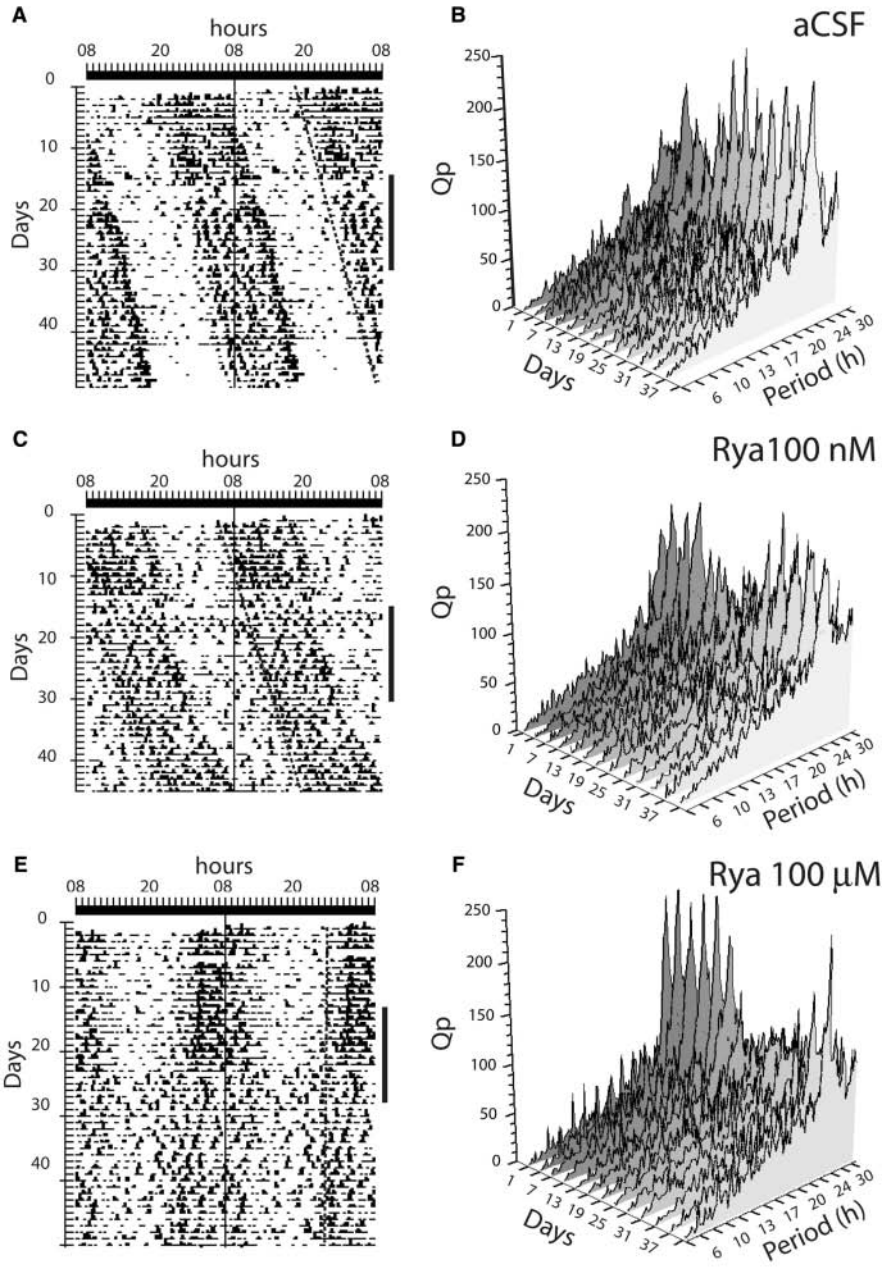

Figure 1. Actogram and the corresponding χ^2 periodogram to exemplify the effect of artificial cerebrospinal fluid (aCSF) and 2 doses of ryanodine on the circadian rhythms of rats. aCSF administration into the SCN (A, B) did not affect the expression of the circadian rhythm. Ryanodine 0.1 µM (C, D) produced shortening of the period of the rhythm, which disappeared shortly after the treatment. Ryanodine 100 µM (E, F) produced disorganization of the daily pattern of activity, which disappears from 7 to 12 days after the end of the treatment. (A, C, and E) Double plotted actograms at 15-min bins. The vertical lines on the right of the actograms indicate the duration of the treatment, from the implant of the injector and the minipump to the nominal duration of activity of the osmotic pump. The dashed lines on the actogram indicate the time of activity onset (CT 12) adjusted during the basal recording and projected to the recordings during and after the treatment. (B, D, and F) Dynamical χ^2 periodograms from 8 consecutive days were obtained at 3-day intervals from the beginning to the end of the recording. Each gray tone indicates a segment of the recordings (basal = dark; treatment = medium; recovery = light). The *y* axis indicates the Qp value.

observed during TTX administration, which also induced clear disruption of the circadian rhythm. After the drug was withdrawn the rhythm was restored. These effects were clearly observed in the dynamical χ^2 periodogram (baseline 24:13 ± 2.80, recovery 24:09 ± 5.60; Wilcoxon sign rank test $Z = -1.8$, NS; Cochran $Q = 8$, $p = 0.018$). In all but 1 case, the phase of the restored rhythm was predicted by projecting of the activity onset from the baseline recording to the days after treatment. The r value of activity onsets after the treatment fitted to the line projection from the basal recordings was 0.80 ± 0.08 (Table 1; Wilcoxon sign rank test $Z = -1.83$, $p = 0.068$). Finally, because 100 µM of ryanodine partially prevents the release of Ca^{2+} from the endoplasmic reticulum into the cytoplasm and this may impair protein synthesis, we studied the effect of inhibiting protein synthesis in the SCN area on the expression of behavioral rhythms. Administration of anisomycin 5 µg/µl disrupted the circadian rhythmicity, and this effect apparently persists after 2 weeks of drug withdrawal. Dynamical χ^2 periodogram confirmed the lack of rhythmicity during and after drug treatment (Cochran $Q = 10$, $p > 0.007$).

DISCUSSION

The present data are consistent with the hypothesis that RyRs are an output pathway from the molecular oscillator to the expression of overt circadian rhythms. When applied directly into the SCN both doses of ryanodine transiently change the

expression of behavioral circadian rhythms but did not affect the circadian clock itself; as indicated by the restoration to baseline levels of the period and the phase of the rhythm shortly after withdrawal of ryanodine. We have previously shown that a low dose of ryanodine (0.1 μM), which activates the RyRs, increases SCN spontaneous neuronal firing rate secondary to release of intracellular Ca^{2+} by opening of the RyRs, whereas a higher dose of ryanodine (100 μM), which blocks the RyRs, decreases the SCN spontaneous firing rate as a consequence of a diminishing of intracellular Ca^{2+} release by closing the RyRs, (Chu et al., 1990; Aguilar-Roblero et al., 2007).

As previously reported by Schwartz et al. (1987), we found that manipulating SCN excitability with either 1 μM TTX or 20 mM KCl modifies behavioral circadian rhythms without affecting the clock mechanism. Thus, a nonspecific increase of cellular excitability produced by high K^+ membrane depolarization induces shortening of the endogenous period and compression of the activity interval of behavioral circadian rhythms, whereas depression of excitability by blockade of Na^+ channels by TTX completely abolishes expression of circadian rhythms. These effects were transitory and the period returned to basal values with the phase predicted from the basal recording after ceasing high K^+ or TTX administration. The effects of high K^+ may also involve nonspecific behavioral feedback on the clock. Although TTX does not seem to affect the molecular clock in SCN organotypic cultures from *mPer$_1$-luc* mouse, its effects on behavioral rhythmicity may be due to disruption of the synchrony between individual neuronal oscillators (Yamaguchi et al., 2003). Altogether previous results are consistent with the hypothesis that RyRs are regulated by the molecular circadian oscillator in SCN neurons, and thus Ca^{2+} mobilized from the endoplasmic deposits modulates the neuronal firing frequency and eventually regulates the circadian expression of behavior (Fig. 2).

It is worth noting that the effects of 100 μM ryanodine persisted beyond the administration time in 4 of the 5 subjects, but in all cases the rhythm was restored by the 2nd week after the administration ceased. This long-lasting effect may be related to some other effect on intracellular Ca^{2+} homeostasis, including inhibition of protein synthesis. Nevertheless, when the rhythmic pattern was recovered the phase of the rhythm was the one predicted from the baseline rhythm; this observation is consistent with the hypothesis that the time-keeping mechanism

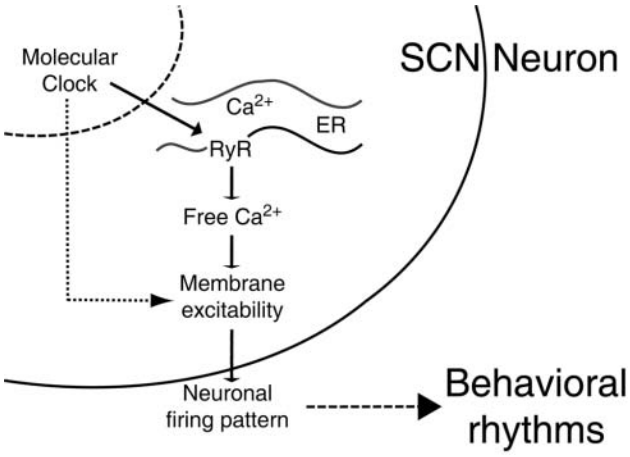

Figure 2. Schematic model of an output pathway from the molecular circadian clock to expression of behavioral rhythms. The core circadian clock drives the sensitivity and conductance of ryanodine receptors (RyR; Diaz-Muñoz et al., 1999), which in turn generate a rhythm in free cytosolic Ca^{2+} (Colwell, 2000; Ikeda et al., 2003), which in turn modulates the rhythm in neuronal excitability and spontaneous firing rate (Aguilar-Roblero et al., 2007). Once the rhythm is encoded into a neuronal firing rate it is transmitted to the neuronal effectors involved in behavioral expression (present data).

was functioning during this treatment. Previous interpretation is further supported by the observation that anisomycin (a general protein synthesis inhibitor) disrupted expression of circadian rhythmicity, but, in contrast to 100 μM ryanodine, its effects persisted for the remainder of the experiment. The persistence of an arrhythmic pattern of behavior during and after the anisomycin may reflect severe impairment on the molecular oscillator involving protein synthesis. In SCN organotypic cultures from the *mPer$_1$-luc* mouse, it has been shown that inhibition of protein synthesis by cycloheximide administration stops the molecular clock, which restarts at the same phase in all neurons after 3 h of washout from the medium (Yamaguchi et al., 2003). In the present study the onset of rhythmicity after 100 μM ryanodine administration showed different phases among the animals and, as we have previously stated, were better predicted by the phase of activity onset previous to the drug administration.

Different Ca^{2+} pools have specific roles in regulating the spatiotemporal neuronal responses to Ca^{2+} signaling (Finkbeiner and Greenberg, 1998; Rizzuto, 2001). In the SCN there are reports on the role of intracellular Ca^{2+} as a signal involved in resetting the clock, maintaining the molecular oscillation, and

transmitting the rhythmicity from the clock itself to the efferents involved in the expression of the overt rhythmicity. Thus, manipulation of calmodulin phase shifts metabolic and electrical rhythms in SCN neurons (Shibata et al., 1987), while RyRs mediate light-induced phase delays during early subjective night (Ding et al., 1998). Cytoplasmic free Ca^{2+} levels display circadian oscillations in the SCN, being higher during the light phase than in the night (Colwell, 2000; Ikeda et al., 2003). It has been suggested that such intracellular Ca^{2+} rhythm may modulate extracellular Ca^{2+} influx in response to light pulses during the night (S. Michel, personal communication, August 26, 2008; Michel et al., 2002). Díaz-Muñoz et al. (1999) demonstrated a circadian rhythm of activity from RyR type 2 in SCN neurons, and pharmacological modulation of the RyR modifies the spontaneous firing rate frequency in SCN neurons (Aguilar-Roblero et al., 2007). Previous evidence indicates that cytosolic Ca^{2+} mobilized through these channels is a direct link between the core oscillator and the SCN electrical activity rhythm, as previously suggested from 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 RyRs blockers (Aguilar-Roblero et al., 2007; Ikeda et al., 2003). Finally, Nitabach et al. (2002) had shown that neuronal electrical activity is necessary for expression of the molecular oscillator and suggested that the coupling of these events could involve Ca^{2+} entry through voltage-dependent calcium channels and some yet unidentified calcium signal pathway. Lundkvist et al. (2005) and Nahm et al. (2005) had extended this observation to the rodent SCN and confirmed the role of voltage-dependent calcium channels in maintaining the molecular clock oscillations; more recently Harrishing et al. (2007) provided evidence on a role for intracellular Ca^{2+} signaling regulating intrinsic cellular oscillations in *Drosophila* in vivo.

Present data extend to the expression of behavioral overt rhythmicity, the evidence supporting the hypothesis that circadian modulation of the RyRs is part of the output pathway from the molecular oscillator. The participation of Ca^{2+} handling proteins and its relation to the RyRs in the output pathway from the molecular oscillator remains to be explored. Further studies are also needed to dissect other roles of Ca^{2+} signaling in the clock mechanism and resetting, as well as studies aimed to unravel the specific targets of Ca^{2+} signaling to modulate neuronal excitability in the SCN.

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