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A neuronal ryanodine receptor mediates light-induced phase delays of the circadian clock

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Circadian clocks are complex biochemical systems that cycle with a period of approximately 24 hours. They integrate temporal information regarding phasing of the solar cycle, and adjust their phase so as to synchronize an organism's internal state to the local environmental day and night[1,2]. Nocturnal light is the dominant regulator of this entrainment. In mammals, information about nocturnal light is transmitted by glutamate released from retinal projections to the circadian clock in the suprachiasmatic nucleus of the hypothalamus. Clock resetting requires the activation of ionotropic glutamate receptors, which mediate Ca^{2+} influx[3]. The response induced by such activation depends on the clock's temporal state: during early night it delays the clock phase, whereas in late night the clock phase is advanced. To investigate this differential response, we sought signalling elements that contribute solely to phase delay. We analysed intracellular calcium-channel ryanodine receptors, which mediate coupled Ca^{2+} signalling. Depletion of intracellular Ca^{2+} stores during early night blocked the effects of glutamate. Activators of ryanodine receptors induced phase resetting only in early night; inhibitors selectively blocked delays induced by light and glutamate. These findings implicate the release of intracellular Ca^{2+} through ryanodine receptors in the light-induced phase delay of the circadian clock restricted to the early night.

In order to understand the mechanisms underlying directionality in the biphasic light response, we studied the rat suprachiasmatic nucleus (SCN) in a hypothalamic brain slice. The circadian clock persists stably for several days in this preparation[4]. The mean firing frequency of the ensemble of SCN neurons exhibits a spontaneous 24-h oscillation *in vitro* (Fig. 1a). Clock phase was determined from the time-of-peak of this neuronal circadian rhythm. We have previously shown that direct application of Glu to this SCN brain slice induces phase-dependent, light-like clock resetting[3]. We used

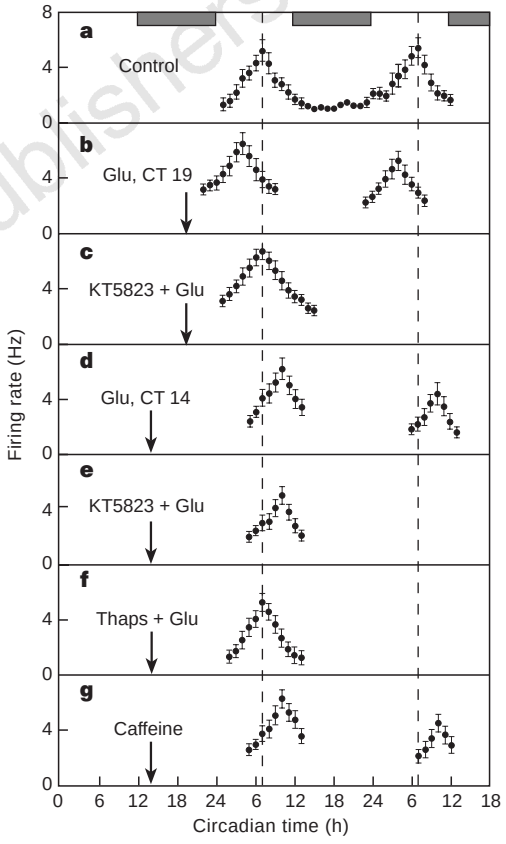

Figure 1 Glu-induced phase advance of SCN neuronal activity rhythm is dependent on PKG, whereas phase delay is not. **a**, The unperturbed SCN neuronal activity over 36 h. The firing rate of this circadian rhythm peaked in mid-subjective day at CT 7, on both day 2 and day 3 *in vitro* in a continuous recording from two SCN. **b**, A microdrop of 10 mM Glu applied at CT 20 advanced the peak of the SCN activity by 3 h ($n = 5$). Neuronal activity was recorded over 10–11 h on each of the next two cycles to define the time-of-peak activity. **c**, Preincubation in 0.1 μM KT5823, a specific PKG inhibitor, blocked the Glu-induced phase advance at CT 20 ($n = 4, P < 0.0001$). **d**, At CT 14, Glu delayed the peak of the SCN activity rhythm by 3 h ($n = 6$). **e**, KT5823 did not affect Glu-induced phase delay at CT 14 ($n = 4, P > 0.2$). **f**, Preincubation with 0.5 μM thapsigargin, a specific inhibitor of the intracellular Ca^{2+}-ATPase, fully blocked Glu-induced phase delay at CT 14 ($n = 4, P < 0.0001$). **g**, A microdrop of 1 mM caffeine to the SCN at CT 14 produced a 3-h phase delay ($n = 4$). Shaded horizontal bars indicate the subjective night of the circadian cycle. The broken vertical lines mark the time of the normal peak of the circadian rhythm of the neuronal activity in unperturbed and EBSS-treated controls. Arrows indicate the time of drug treatment. Statistical analyses applied to this and other microdrop experiments were analyses of variance (ANOVA) with Tukey *post hoc* test.

this finding as a means of studying signalling elements downstream of Glu.

Light-induced clock resetting involves the sequential activation of Glu receptors, Ca^{2+} influx, nitric oxide synthase and intercellular movement of nitric oxide. Nitric oxide can activate soluble guanylyl cyclase, which increases cGMP and activates cGMP-dependent protein kinase (PKG). Activation of cGMP-dependent pathways of SCN in brain slices has been shown to stimulate phase advance of the clock at night, but not during the day[5,6]. Furthermore, intracerebroventricular (i.c.v.) injection of KT5823, a specific PKG inhibitor, blocks light-induced advances of wheel-running rhythms in hamsters during the late night[7,8]. To determine whether PKG activation is associated with the Glu-induced phase shifts, SCN slices were bathed in KT5823 before treatment with Glu or media. A 30-min incubation in 0.1 μM KT5823 fully inhibited PKG phosphotransferase activity in the SCN during stimulation by Glu at circadian time (CT; time after entrained lights on) 14 and 20. KT5823 itself had no effect on clock phase in late or early night, but it completely blocked phase advances of the neuronal activity rhythm induced by Glu in the late night (Fig. 1b, c). However, in the early night, KT5823 had no effect on Glu-induced phase delays (Fig. 1d, e). Therefore, the NO–GC–cGMP–PKG pathway does not mediate the Glu-induced phase delay.

To investigate alternative pathways, we evaluated intracellular Ca^{2+} (Ca_i^{2+}) signalling. Thapsigargin depletes Ca_i^{2+} by blocking the Ca^{2+}-ATPase that replenishes Ca_i^{2+} stores in the endoplasmic reticulum[9]. When applied at either at CT 20 or 14, thapsigargin itself did not alter the phasing of the neuronal activity rhythm. Thapsigargin treatment had only a small inhibitory effect on the phase advance induced by Glu (−1.15 h, CT 20, $n = 4$, $P < 0.001$), but it completely blocked the Glu-induced phase delay (Fig. 1f). This finding indicates that the Glu-induced phase delay requires Ca_i^{2+} release.

To examine the Ca_i^{2+} signalling mechanism in early night, we probed the Ca_i^{2+}-channel ryanodine receptor (RyR). RyRs are integral membrane proteins associated with cellular organelles, such as the endoplasmic reticulum, that sequester Ca^{2+}. They are expressed in neurons where they can mediate release of stored Ca_i^{2+} (ref. 10). Ca_i^{2+} mobilization by modulators of RyRs can reset circadian rhythms of melatonin production in avian pinealocytes[11]. A preliminary report[12] suggests that the rat SCN exhibits circadian variation in the binding characteristics of RyRs, whereas inositol-1,4,5-trisphosphate (IP_3) receptor binding remains constant across the 24-h cycle.

To evaluate the contribution of RyRs to phase delays, we studied a range of reagents that have different mechanisms of action but all release Ca_i^{2+} through RyRs. Caffeine can directly activate RyRs[10,13], and FK506 and rapamycin bind to immunophilins, a class of proteins that regulate the ion-channel functions of RyRs[13–15]. Although FK506 and rapamycin exert their immunosuppressive actions through different effectors, both bind to FKBP12, a well-studied immunophilin stabilizer of RyRs[13–15]. Application of caffeine, FK506 or rapamycin to the SCN at CT 14 induced Glu-like

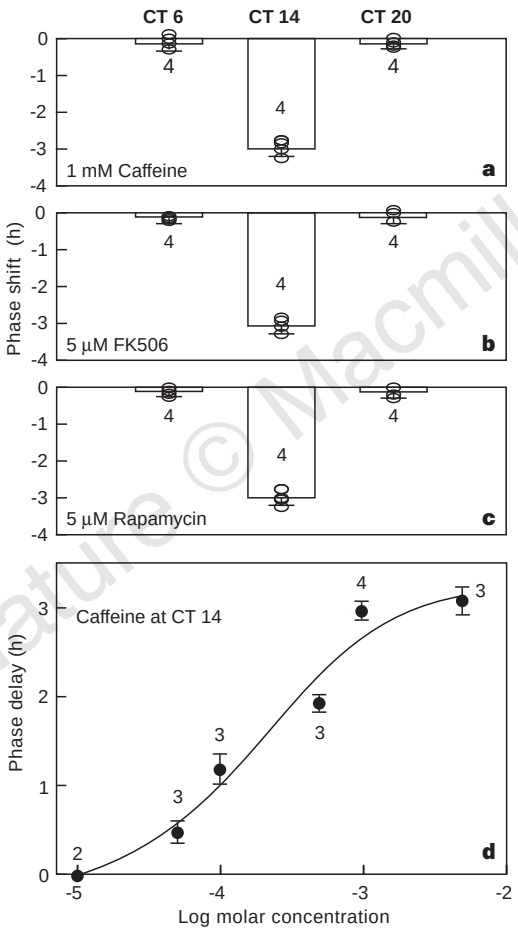

Figure 2 SCN sensitivity to caffeine and to the immunophilin ligands FK506 and rapamycin is restricted to early night. **a**–**c**, Temporal sensitivity to caffeine (**a**), FK506 (**b**) or rapamycin (**c**) was assessed at mid-day (CT 6), early night (CT 14) and late night (CT 20). These reagents did not affect phasing of the circadian rhythm when applied at CT 6 or CT 20. However, at CT 14, each drug induced a 3-h phase delay. Graph shows data points, mean, s.e.m. and *n* (number). The circadian time effect was significant for each drug ($P < 0.0001$). The phase shifts at CT 14 were significantly different from those at CT 6 and at CT 20 ($P < 0.0001$, each comparison, each drug). **d**, Dose–response curve of caffeine at CT 14. Half-maximum response occurred with a 0.1 mM microdrop. Each data point represents mean ± s.e.m. The regression curve was fit to the data points using the Hill equation.

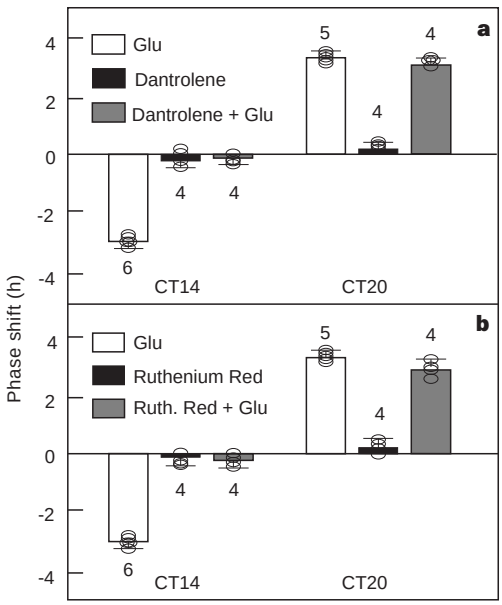

Figure 3 RyR inhibitors selectively block Glu-induced phase delays in the early night. **a**, **b**, Bath application of 20 μM dantrolene (**a**) or 10 μM ruthenium red (**b**) to the brain slice before stimulation by Glu blocked the phase delay normally induced by Glu at CT 14, but not the advance at CT 20. At CT 14, for both inhibitors, the phase delay induced by Glu was significantly different ($P < 0.0001$) from that for the inhibitor alone or the inhibitor plus Glu ($P < 0.0001$). The shifts for inhibitor and inhibitor plus Glu did not differ significantly from one another. At CT 20, for both inhibitors, the phase advances for Glu and Glu plus inhibitor did not differ significantly from each other, but they each did differ significantly ($P < 0.0001$) from the shifts seen with inhibitor alone. The Glu controls at CT 14 and CT 20 in **a** are replotted in **b**.

phase delays of ~3 h (Fig. 1g); these treatments did not affect clock phase at CT 6 or 20 (Fig. 2a–c). Microdrops containing 10^{-6} to 10^{-2} M caffeine elicited a dose-dependent phase delay, with a half-maximal response near 5×10^{-4} M (Fig. 2d).

To examine further the pathway mediating Glu-induced phase delay, we used inhibitors of RyRs. Dantrolene and ruthenium red each effectively block activation of RyRs, although they act at different sites on the RyR molecule[13,16]. Preincubating the SCN in either dantrolene or ruthenium red before applying a microdrop of Glu at CT 14 fully blocked phase delays. These inhibitors had no effect on Glu-induced phase advances at CT 20 (Fig. 3). To determine whether RyR inhibition affects light-stimulated phase shifts, we tested dantrolene *in vivo*. When photic stimuli were evaluated after i.c.v. injection of dantrolene, the phase delays of the wheel-running rhythm of hamsters under constant darkness were significantly attenuated (Fig. 4).

Because caffeine and FK506 have diverse cellular actions[17,18], we sought to identify the pathway mediating the effects of FK506 and caffeine on the phase-delaying process. The efficacy of these reagents at CT 14 was tested against the RyR antagonist dantrolene. In each case, dantrolene fully blocked the agonist's effect ($n = 4$, $P < 0.0001$, each condition; data not shown). Western blot analysis with affinity-purified anti-neuronal RyR antibody[19,20] demonstrated that the SCN expresses neuronal RyR (relative molecular mass 325K, CT 14, $n = 3$; data not shown). Because dantrolene is highly specific for RyRs and antagonizes the effects of these modulators, FK506 and caffeine are probably affecting phase change within the SCN through their RyR-binding properties.

Our data show that SCN signalling elements diverge downstream from Glu. In the late night, the light/Glu signal causes a 3-h phase advance of the timekeeping mechanism by means of a cGMP-dependent pathway (Fig. 1b, c)[7,8,21]. In the early night, however, signal transduction downstream of Glu follows a distinct pathway leading to activation of RyRs and release of Ca_i^{2+}, resulting in a phase delay of 3 h. Although the steps linking Glu and RyRs are unknown, nitric oxide is a likely intermediary that could stimulate the ADP ribosyl cyclase pathway[22,23] and/or directly activate RyRs by means of nitric oxide-mediated polynitrosylation[24]. Further, our findings demonstrate that RyR activation contributes a pivotal directional signal in the context of dynamic clock state. Although their roles in neurons are not fully understood, RyRs are thought to modulate the duration and/or amplitude of the Ca_i^{2+} signal[25,26]. Potential mechanisms by which an increase in Ca_i^{2+} could contribute to the phase-delaying process include activation of Ca^{2+}-dependent kinases, phosphatases and/or proteases leading to the inactivation or degradation of clock regulatory elements. Indeed, light-induced protein degradation has been reported in the regulation of clock-related genes, such as *tim* in *Drosophila*[27]. □

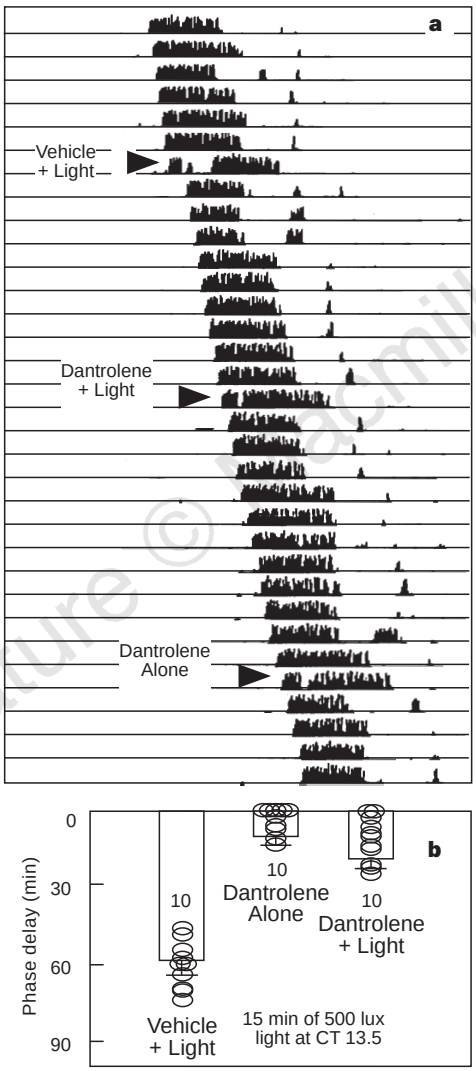

Figure 4 Dantrolene blocks light-induced phase delays of wheel-running activity rhythm of free-running hamsters. **a**, The actogram of one hamster throughout the various treatments. Each horizontal line represents 24 h. Light exposure at CT 13.5 after vehicle (1 μl) administered i.c.v. 20 min earlier induced a 60-min phase delay. Dantrolene (50 μM, 1 μl, i.c.v.) administered before light exposure significantly attenuated the light-induced phase delay. Dantrolene without light did not affect the phase. **b**, The phase delay for the vehicle plus light group was significantly different from that for either of the dantrolene-treated groups ($P < 0.0001$, Kruskal-Wallis rank ANOVA), but the dantrolene-treated groups did not differ significantly from each other.

Methods

Electrophysiology and pharmacology. Inbred Long–Evans rats 6–10 weeks old were used in these experiments. Procedures for brain slice preparation and electrophysiology have been described previously[3,4]. Briefly, spontaneous activity of single SCN neurons was sampled extracellularly for 4-min periods. From sequential unit activities, 2-h running means of the neuronal ensemble were calculated. The phase of the underlying circadian clock was determined by the time of the peak in the oscillation[3,4]. Under constant conditions *in vitro*, the unperturbed sinusoidal pattern of neuronal activity is predictably high during the subjective day and low during the subjective night. Activity peaks mid-subjective day, at circadian time 7 (CT 7, 7 h after lights on in the rat colony; 12:12 LD). To evaluate experimental stimuli, the perfusion pump was stopped, a droplet of 0.2 or 0.5 μl of test substance was applied bilaterally to the SCN for 10 min, and the SCN was then rinsed with glucose- and bicarbonate-supplemented Earle's balanced salt solution (EBSS). To evaluate potential inhibitors, perfusion medium was replaced with medium containing the antagonist for 20 min before microdrop application of the phase-shifting stimulus. To determine the phase after drug treatments, the time-of-peak neuronal activity was assessed for 1–2 days.

Stereotactic surgery for i.c.v. cannulation and drug injection. Male Syrian hamsters (Harlan, 100 g) were used to assess pharmacological effects on behavioural rhythms[7,8]. A 23-gauge guide cannula was implanted to the lateral ventricle of each (0.4 mm rostral to bregma, 3.0 mm ventral to dura, 2.4 mm lateral to midline, with bregma and lambda in a levelled plain). A 30-gauge stylet was placed in the guide cannula to maintain patency. For i.c.v. injections the stylet was removed and a 30-gauge injector attached to a microsyringe was inserted. At 20 min before light exposure, the animals were lightly anaesthetized with methoxyflurane during i.c.v. injection. Each animal received a 1-μl injection under dim red light (<1 lux) over 30 s. Generally, animals began to wake up before completion of the injection and were completely awake before light exposure (500 lux, 15 min). The animals were then returned to their cages and maintained in darkness for 10–14 days before the next treatment.

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High-frequency firing helps replenish the readily releasable pool of synaptic vesicles

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Synapses in the central nervous system undergo various short- and long-term changes in their strength[1–3], but it is often difficult to distinguish whether presynaptic or postsynaptic mechanisms are responsible for these changes. Using patch-clamp recording from giant synapses in the mouse auditory brainstem[4–7], we show here that short-term synaptic depression can be largely attributed to rapid depletion of a readily releasable pool of vesicles. Replenishment of this pool is highly dependent on the recent history of synaptic activity. High-frequency stimulation of presynaptic terminals significantly enhances the rate of replenishment. Broadening the presynaptic action potential with the potassium-channel blocker tetraethylammonium, which increases Ca^{2+} entry, further enhances the rate of replenishment. As this increase can be suppressed by the Ca^{2+}-channel blocker Cd^{2+} or by the Ca^{2+} buffer EGTA, we conclude that Ca^{2+} influx through voltage-gated Ca^{2+} channels is the key signal that dynamically regulates the refilling of the releasable pool of synaptic vesicles in response to different patterns of inputs.

Glutamatergic excitatory postsynaptic currents (EPSCs) were recorded from the principal neurons of the medial nucleus of the trapezoid body (MNTB) in an all-or-none fashion, consistent with the fact that each postsynaptic neuron is innervated at the soma by a single presynaptic calyx (the calyx of Held)[8,9]. When the presynaptic axon was stimulated with a 100-ms train at a frequency of 100 to 300 Hz, we observed a frequency-dependent depression in the amplitude of the EPSCs (Fig. 1a, b). This depression was short-lasting and reversed within 15 s.

To investigate the mechanisms causing this short-term depression, we first tested whether desensitization of postsynaptic AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors caused a decline in the amplitude of the EPSCs. When the desensitization was blocked with cyclothiazide (CTZ, 0.1 mM)[10,11], the decay time course of individual EPSCs was prolonged, but the relative amplitude of the last EPSC in the train was about the same as that in the absence of CTZ at all frequencies. A typical recording at 200 Hz is shown in Fig. 1c, d. Because these experiments were carried out under conditions of a high quantal output (in 2 mM

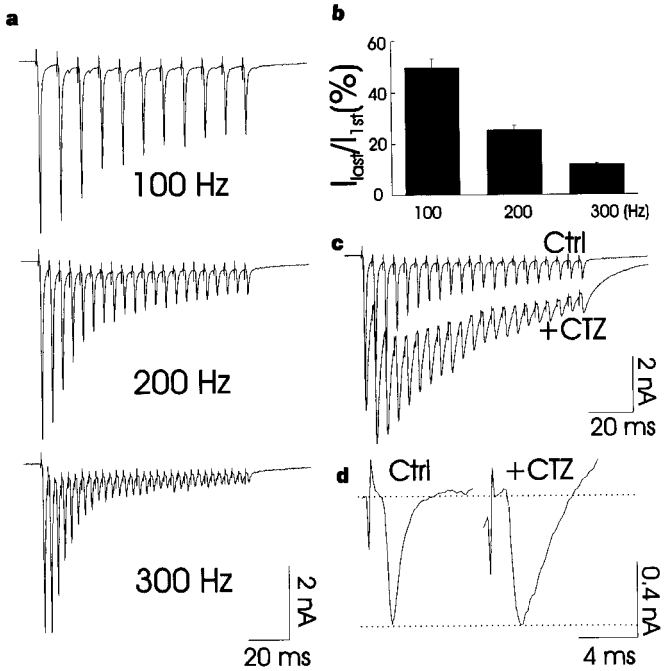

Figure 1 Use-dependent synaptic depression is independent of postsynaptic desensitization. **a**, Typical recording showing frequency-dependent depression in EPSCs in response to a 100-ms train of stimulation at 100, 200 or 300 Hz. Each EPSC is preceded by a stimulation artefact. The holding potential was –60 mV for this and subsequent figures unless otherwise indicated. **b**, Summary of the extent of synaptic depression at three frequencies. On average, the amplitude of the last EPSC in the train, at a frequency of 100, 200 and 300 Hz, was 49.7 ± 3.5%, 25.3 ± 1.8% and 11.8 ± 0.5% of that of the first EPSC (n = 10), respectively. **c**, EPSCs recorded from the same cell as in **a** before and after addition of CTZ (0.1 mM) are superimposed to show that blockade of AMPA-receptor desensitization failed to eliminate synaptic depression. **d**, Comparison of the last EPSC with or without CTZ treatment from **a**, after adjusting the baseline to the same level.