Doctoral thesis (abridged version)

# Optical analysis of organellar circadian rhythms in the master clock neurons

Hiro, Sota

Doctor of Philosophy

The Graduate University for Advanced Studies, SOKENDAI

School of Life Science

Department of Physiological Sciences

National Institute for Physiological Sciences

Division of Biophotonics

## Introduction

Almost all living organisms on Earth are regulated by a circadian clock, which anticipates the environmental changes for their survival. In mammals, the master circadian clock is located in the suprachiasmatic nucleus (SCN) of the hypothalamus in the brain (Moore and Eichler, 1972; Stephan and Zucker, 1972), which controls various physiological functions (e.g., sleep-wake cycles, hormone secretion), and animal behavior (Welsh et al., 2010; Mohawk et al., 2012). The rodent SCN is composed of ca. 20,000 neurons in the bilateral side of the hypothalamus (Van den Pol, 1980). At the cellular level, the molecular machinery of circadian rhythms is thought to be based on the transcriptional-translational feedback loop (TTFL), which is composed of clock genes and their protein products (Mohawk et al., 2012; Takahashi, 2017). In individual SCN neurons, circadian rhythms are observed at the level of firing frequency (Welsh et al., 1995), peptide release (Shinohara et al., 1995), and cytosolic $Ca^{2+}$ concentration (Colwell, 2000; Ikeda et al., 2003; Enoki et al., 2012a). $Ca^{2+}$ is an important component of the TTFL. The PER2 expression rhythms were abolished when intracellular $Ca^{2+}$ was chelated in SCN tissue culture (Lundkvist et al., 2005). Furthermore, the behavioral rhythm in *Drosophila* was prolonged when parvalbumin, a $Ca^{2+}$-binding protein, was specifically expressed in clock neurons (Harrisingh et al., 2007). Since circadian $Ca^{2+}$ rhythms are attenuated by *Bmal1* knockdown (Ikeda and Ikeda, 2014) and *Cry1/2* knockout (Enoki et al., 2017), it is likely that $Ca^{2+}$ and TTFL are mutually coupled. In general, intracellular $Ca^{2+}$ is regulated by internal organelles, such as the endoplasmic reticulum (ER) and mitochondria, and $Ca^{2+}$ signaling regulates various cellular functions, such as clock gene transcription in the nucleus. However, it remains to be elucidated what the $Ca^{2+}$ dynamics in

organelles are and how these organelles regulate the circadian $Ca^{2+}$ rhythms in the SCN neurons.

In this study, I focused on the mechanisms of $Ca^{2+}$ regulation by organelles in the nucleus, ER, and mitochondria. First, I performed dual-color time-lapse imaging of nuclear and cytosolic $Ca^{2+}$, pharmacological experiments, and $Ca^{2+}$ chelation experiments, and discussed the dynamics of nuclear and cytosolic $Ca^{2+}$ and the regulation mechanism of these $Ca^{2+}$ rhythms by the ER. Subsequently, through the assessment of $Ca^{2+}$ dynamics in mitochondria, I unexpectedly detected a pH rhythm. Utilizing the energy derived from the proton electronic gradient within mitochondria, ATP is synthesized from ADP at complex V (Jonckheere et al., 2012). To elucidate the temporal relationship between the pH rhythm of the mitochondrial matrix and ATP production, I visualized the concentration dynamics of ATP on a circadian timescale.

## Materials and Methods

### Animal experiments

Adult female mice with newborn pups were purchased from an animal breeder (Japan SLC, Inc., Hamamatsu, Japan). The animals were housed and provided with food and water ad libitum under controlled conditions (temperature, 22°C ± 2°C; humidity, 40% ± 20%; 12-h light/12-h dark cycle, with lights on from 8:00 AM to 8:00 PM). Light intensity was adjusted to approximately 100–200 lx at the cage surface. The animals were fed commercial chow (Labo MR Standard; Nosan Corporation, Yokohama, Japan) and tap water. All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of the National Institutes of Natural Sciences and performed according to the National Institute for Physiological Sciences guidelines.

### SCN slice culture

The brains of neonate mice (4–6 days old, both male and female) were rapidly removed and dipped in an ice-cold balanced salt solution comprising (in mM) 87 NaCl, 2.5 KCl, 8 $MgCl_2$, 0.5 $CaCl_2$, 1.2 $NaH_2PO_4$, 26 $NaHCO_3$, 25 glucose, 10 HEPES, and 75 sucrose. A 200-μm coronal brain slice containing the mid-rostro-caudal region of the SCN was prepared using a vibratome (VT 1200; Leica Microsystems GmbH, Wetzlar, Germany). The bilateral SCNs were cut out from the slice using a surgical knife and explanted onto a culture membrane (Millicell CM; pore size, 0.4 μ m; Millipore; Merck KGaA, Darmstadt, Germany) in a 35-mm Petri dish containing 1.0-mL DMEM (Invitrogen; Thermo Fisher Scientific Inc.) and 5% FBS (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany).

**Gene transfer into SCN neurons**

The pAAV-hSyn-NLS-GCaMP6s-WPRE, pAAV-hSyn-NLS-Parvalbumin(PV)-mCherry-WPRE, pAAV-hSyn-NLS-mCherry-WPRE, AAV-hSyn1-CEPIA2mt-WPRE, pAAV-hSyn1-2xMTS-cpEGFP-WPRE, pAAV-2xMTS-superecliptic pHluorin-WPRE, pAAV-2xMTS-Tq-Ca-FLITS-WPRE were constructed by VectorBuilder Inc.. AAV1-Syn1-nes-jRGECO1a was purchased from Addgene (Dana et al., 2016). pAAV-synapsin-cyto-iATPSnFR1.0 was a gift from Baljit Khakh (Addgene plasmid # 102550; http://n2t.net/addgene:102550; RRID: Addgene_102550). The AAV vectors underwent packaging through a previously described procedure (Sano et al., 2020). AAV aliquots (0.8–1.0 μL) were inoculated onto the surface of the SCN slices on days 3–4 of culture. The infected slice was cultured for a further 10–14 days before imaging.

**Dual-color recording**

Time-lapse imaging was performed 10 to 14 days after AAV infection. For confocal recording, 3–4 days before the recordings, membranes with cultured SCN slices were cut out, flipped over, and transferred to glass-based dishes (3971-035 IWAKI; AGC TECHNO GLASS Co., Ltd., Yoshida, Japan), which were coated with collagen (Cellmatrix Type 1-C, Nitta Gelatin Inc., Yao, Japan). For wide-field recording, culture membrane inserts with cultured SCN slices were transferred to glass-based dishes. Then, the dishes were filled with DMEM (180–200 μL for confocal, 1.2 mL for wide-field) containing 5% FBS and sealed with $O_2$-permeable filters (membrane kit, High Sens; YSI Inc.). The specimen was observed using a time-lapse imaging system as previously described (Enoki et al., 2012). The system

consisted of a Nipkow spinning disk confocal unit (X-Light; CrestOptics S.p.A., Roma, Italy), a sCMOS camera Neo (2,560 × 2,160 pixels, Andor Technology, Oxford Instruments, Belfast, UK), an EM-CCD camera Evolve (512 × 512 pixels, Teledyne Photometrics) or an EM-CCD camera iXon3 (1,024 × 1,024 pixels, Andor Technology), a TIXHB box incubator (Tokai Hit., Co, Ltd., Fujinomiya, Japan), and a Ti-E inverted microscope (Nikon Corporation, Tokyo, Japan). All experiments were performed at 36.5 °C and 5% $CO_2$. The intensity of the excitation light and the exposure time of the sCMOS and EM-CCD cameras were adjusted to obtain optimal images of each sample.

## Results

First, I examined the $Ca^{2+}$ dynamics and its regulatory mechanism in the nucleus of the SCN neuron on the circadian timescale. To visualize the $Ca^{2+}$ dynamics, I performed dual-color time-lapse $Ca^{2+}$ imaging in the nucleus and cytosol using highly sensitive genetically encoded $Ca^{2+}$ indicators, GCaMP6s and jRGECO1a. I found robust nuclear circadian $Ca^{2+}$ rhythms in phase with the cytosolic rhythms in single SCN neurons and the entire SCN regions. The nucleus is covered by a nuclear envelope with nuclear pores, and ryanodine and inositol 1,4,5-trisphosphate ($IP_3$) receptors, which are mainly expressed in the endoplasmic reticulum (ER), are also expressed on the inner membrane. To investigate their involvement in the regulation of nuclear circadian $Ca^{2+}$ rhythms, I applied drugs that inhibit $Ca^{2+}$ influx via ryanodine receptors, $IP_3$ receptors, or neuronal firing. Inhibiting action potentials reduced the amplitude of both nuclear and cytosolic $Ca^{2+}$ rhythms, whereas blocking $Ca^{2+}$ release from the ER, either via ryanodine or $IP_3$ receptors, had a minor effect on nuclear and cytosolic $Ca^{2+}$ rhythms. I conclude that the in-phasic circadian $Ca^{2+}$ rhythms in the cytosol and nucleus are primarily driven by $Ca^{2+}$ influx from the extracellular space, likely through the nuclear pores. It also raises the possibility that nuclear $Ca^{2+}$ rhythms directly regulate transcription *in situ.*

Second, I examined the $Ca^{2+}$ dynamics in the mitochondrial matrices of the SCN neurons, because the mitochondria are known to function as $Ca^{2+}$ stores. To directly visualize $Ca^{2+}$ dynamics in the mitochondria, I expressed a genetically encoded $Ca^{2+}$ indicator, CEPIA2mt, in the mitochondrial metrics of SCN neurons. I found circadian rhythms of the CEPIA2mt signals, which were nearly antiphase to the cytosolic $Ca^{2+}$ rhythms. Since CEPIA2mt is

known to be affected by pH, I expressed the circularly permuted EGFP (cpEGFP), which excludes the $Ca^{2+}$ binding site of CEPIA2mt. Unexpectedly, I detected the circadian rhythms of the cpEGFP signals, suggesting the existence of $Ca^{2+}$-independent rhythms. I further evaluated these signals using a high-sensitive pH indicator, superecliptic pHluorin. I observed the circadian pH rhythm in the mitochondrial matrix, where the level was high during the subjective night and low during the subjective day. In mitochondria, adenosine triphosphate (ATP) is produced when $H^+$ flows into the matrix utilizing the electrochemical proton gradient. I hypothesized that mitochondrial pH rhythm might be coupled with ATP synthesis. To test this, I expressed a genetically encoded ATP indicator, iATPSnFR, in the cytosol of SCN neurons. I found circadian ATP rhythms in the SCN neurons, where the level was high during the subjective night and low during the subjective day. During the subjective night, the pH in the mitochondrial matrix is kept high, which may increase the concentration gradient and enhance the ATP production capacity.

## Discussion

Circadian $Ca^{2+}$ rhythms were identified within the nucleus, and the rhythms were synchronized with the cytosolic $Ca^{2+}$ rhythms. The nuclear $Ca^{2+}$ oscillations may be primarily driven by the influx and efflux through the nuclear pore complex. The nuclear $Ca^{2+}$ rhythm is likely implicated in the direct modulation of nuclear $Ca^{2+}$-dependent protein activities and may play a role in the transcriptional regulation of clock genes.

Additionally, I detected circadian rhythms in mitochondrial pH, with the peak phase occurring during the subjective night. And cytosolic ATP concentrations in SCN neurons exhibited circadian rhythm, with their phase aligning in phase with the pH rhythm. This synchrony suggests a potential regulatory relationship between pH rhythms and ATP production through oxidative phosphorylation. The circadian modulation of pH within mitochondria may contribute to the temporal regulation of ATP production in SCN neurons, representing a crucial aspect of their circadian functionality.