Intracellular $\text{Na}^+$ and metabolic modulation of Na/K pump and excitability in the rat suprachiasmatic nucleus neurons

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Wang YC, Yang JJ, Huang RC. Intracellular $\text{Na}^+$ and metabolic modulation of Na/K pump and excitability in the rat suprachiasmatic nucleus neurons. J Neurophysiol 108: 2024–2032, 2012. First published July 5, 2012; doi:10.1152/jn.00361.2012.—Na/K pump activity and metabolic rate are both higher during the day in the suprachiasmatic nucleus (SCN) that houses the circadian clock. Here we investigated the role of intracellular $\text{Na}^+$ and energy metabolism in regulating Na/K pump activity and neuronal excitability. Removal of extracellular K$^+$ to block the Na/K pump excited SCN neurons to fire at higher rates and return to normal K$^+$ to reactivate the pump produced rebound hyperpolarization to inhibit firing. In the presence of tetrodotoxin to block the action potentials, both zero K$^+$-induced depolarization and rebound hyperpolarization were blocked by the cardiac glycoside strophanthidin. Ratiometric Na$^+$ imaging with a Na$^+$-sensitive fluorescent dye indicated saturating accumulation of intracellular Na$^+$ in response to pump blockade with zero K$^+$. The Na$^+$ ionophore monensin also induced Na$^+$ loading and hyperpolarized the membrane potential, with the hyperpolarizing effect of monensin abolished in zero Na$^+$ or by pump blockade. Conversely, Na$^+$ depletion with Na$^+$-free pipette solution depolarized membrane potential but retained residual Na/K pump activity. Cyanide inhibition of oxidative phosphorylation blocked the Na/K pump to depolarize resting potential and increase spontaneous firing in most cells, and to raise intracellular Na$^+$ levels in all cells. Nonetheless, the Na/K pump was incompletely blocked by cyanide but completely blocked by iodoacetate to inhibit glycolysis, indicating the involvement of both oxidative phosphorylation and glycolysis in fueling the Na/K pump. Together, the results indicate the importance of intracellular Na$^+$ and energy metabolism in regulating Na/K pump activity as well as neuronal excitability in the SCN neurons.

intracellular Na$^+$; metabolism; Na/K pump; suprachiasmatic nucleus

THE CENTRAL CLOCK RESIDES in the hypothalamic suprachiasmatic nucleus (SCN) to coordinate the peripheral oscillators in controlling circadian rhythms in mammals (Dibner et al. 2010; Guilding and Piggins 2007; Yoo et al. 2004). These clock neurons vary their activity across the time of day, exhibiting circadian rhythms in spontaneous firing rate (Green and Gillette 1982; Groos and Hendriks 1982; Inouye and Kawamura 1979; Shibata et al. 1982) and in intracellular Ca$^{2+}$ concentrations (Colwell 2000; Ikeda et al. 2003). They also exhibit metabolic rhythms, with higher 2-deoxyglucose uptake (Newman et al. 1992; Schwartz and Gainer 1977), cytochrome oxidase activity (López et al. 1997), and Na/K pump activity (Wang and Huang 2004) during the day and higher ATP contents at night (Yamazaki et al. 1994). Because 2-deoxyglucose uptake and cytochrome oxidase activity reflect, respectively, the glycolytic flux and mitochondrial respiration (Wong-Riley 1989), the observations suggest the rates of both glycolysis and oxidative phosphorylation are higher during the day in the SCN.

As the Na/K pump uses the energy derived from ATP hydrolysis to actively extrude Na$^+$ to maintain transmembrane Na$^+$ gradient to power various secondary active transport processes (Glitsch 2001), the enzyme consumes a large proportion of metabolic energy (Ames 2000). As a result, a higher daytime Na/K pump activity may account for the higher daytime 2-deoxyglucose uptake and cytochrome oxidase activity in the SCN. However, the contribution of glycolysis and oxidative phosphorylation to the regulation of Na/K pump activity remains to be determined. Furthermore, the Na/K pump is activated by intracellular Na$^+$, and the rapid activation of the Na/K pump by intracellular Na$^+$ allows the pump to vary its activity according to intracellular Na$^+$ levels (see, for examples, Haber et al. 1987; Nakao and Gadsby 1989; Senatovor et al. 2000). As such, the Na/K pump may cooperate with the Na$^+$ leak to regulate intracellular Na$^+$ concentrations, as well as to regulate intracellular Ca$^{2+}$ homeostasis via Na/Ca exchangers (Blaustein and Lederer 1999).

The regulation of Na/K pump activity by intracellular Na$^+$ and ATP, along with its ability to regulate intracellular Na$^+$ and membrane excitability, suggests that the Na/K pump may play an important role in the integration of energy metabolism, intracellular ion homeostasis, and neuronal excitability in the SCN. In particular, in view of recent evidence indicating that metabolic signals can regulate the circadian functioning of the SCN (see Challet 2010; Green et al. 2008), we sought to know if the SCN neurons are sensitive to metabolic perturbation via metabolic regulation of Na/K pump activity. The purpose of this study was to investigate the role of intracellular Na$^+$ and energy metabolism in regulating Na/K pump activity and neuronal excitability. We first determined the effects of pump blockade with zero external K$^+$ on membrane excitability and intracellular Na$^+$ in the SCN neurons. We then examined the effects of Na$^+$ loading with the Na$^+$ ionophore monensin and of Na$^+$ depletion with Na$^+$ omission from the pipette solution, followed by investigation of the roles of glycolysis and oxidative phosphorylation in the regulation of Na/K pump activity. The cell-attached and perforated-patch recording techniques were used to determine the firing rate, membrane potential, and membrane current, whereas the ratiometric Na$^+$ imaging technique was used to monitor intracellular Na$^+$ changes in the SCN neurons. Part of these results have been presented in abstract form (Hsu et al. 2010; Wang and Huang 2006a).
**METHODS**

**Animals.** All experiments were carried out and approved according to the guidelines of the Institutional Animal Care and Use Committee of Chang Gung University College of Medicine. Sprague-Dawley rats (17–25 days old) were kept in a temperature-controlled room under a 12:12-h light-dark cycle (light on 0700–1900). An animal was carefully restrained by hand to reduce stress and killed by decapitation using a small rodent guillotine without anesthesia, and the brain was put in an ice-cold artificial cerebrospinal fluid (ACSF) pre bubbled with 95% O_2-5% CO_2. The ACSF contained (in mM) 125 NaCl, 3.5 KCl, 2 CaCl_2, 1.5 MgCl_2, 26 NaHCO_3, 1.2 NaH_2PO_4, and 10 glucose.

**Reduced SCN preparations.** A coronal slice (200–300 μm) containing the SCN and the optic chiasm was cut with a Vibroslicer (Campden Instruments, Lafayette, IN) and was then incubated at room temperature (22–25°C) in the incubation solution, which contained (in mM) 140 NaCl, 3.5 KCl, 2 CaCl_2, 1.5 MgCl_2, 10 glucose, and 10 HEPES, pH 7.4, bubbled with 100% O_2. A reduced preparation was obtained by excising a small piece of tissue (circa one-ninth the size of SCN) from the SCN using a fine needle (catalog no. 26002-10; Fine Science Tools, Foster City, CA), followed by further trimming down to 4–10 smaller pieces with a short strip of razor blade. The reduced preparation (containing tens of cells) was then transferred to a coverslip precoated with poly-D-lysine (Sigma-Aldrich, St. Louis, MO) in a recording chamber for recording. The SCN neurons of the reduced preparation could be identified visually with an inverted microscope (IX70; Olympus, Tokyo, Japan). The preparation thus obtained allows rapid application of drugs (Chen et al. 2009) and has been used to demonstrate diurnal rhythms in both spontaneous firing and Na/K pump activity (Wang and Huang 2004).

**Electrical recordings.** All experiments were performed during the daytime, with the animal killed near zeitgeber time (ZT 4 (4 h into lights-on at ZT 0). The reduced SCN preparation was perfused with bath solution containing (in mM) 140 NaCl, 3.5 KCl, 2 CaCl_2, 1.5 MgCl_2, 10 glucose, and 10 HEPES, pH adjusted to 7.4 with NaOH. The perfusion rate was at 0.6 ml/min and solution change was completed in ~1 s judging from the measurement of junction potential. The patch solution contained (in mM) 20 NaCl, 1 CaCl_2, 2 MgCl_2, 110 K-glucanate, 11 EGTA, 10 HEPES, 3 Na-ATP, and 0.3 Na-GTP, pH adjusted to 7.3 with KOH. The measured liquid junction potential was ~12 mV (Neher 1992) and was corrected for in the presentation of data obtained with perforated-patch recordings. Pipette resistance was 4–6 MΩ. For perforated-patch recordings, the patch pipette also included nystatin (Sigma-Aldrich) at a final concentration of 250 μg/ml prepared from a stock solution (25 mg/ml DMSO). All recordings were made with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) at room temperature (22–25°C). The spontaneous firing rate was recorded in the cell-attached and perforated-patch current-clamped configurations. The spike counts, in 6-s epochs, always began only after stable recordings were made. At least 1 or 2 min of spontaneous firing rate were counted before the application of drugs. Membrane potentials and membrane currents were recorded in the presence of 0.3 μM tetrodotoxin (TTX) using the perforated-patch recording technique. The signal was low-pass filtered at 1–5 kHz and digitized online at 2–10 kHz via a 12-bit analog-to-digital digitizing board (DT2821F-DI; Data Translation, Marlboro, MA) with a custom-made program written in the C language. Data were analyzed and plotted with custom-made programs written in Visual Basic 6.0 and the commercial software GraphPad Prism (GraphPad Software, San Diego, CA). Data are means ± SE.

To determine the extent of zero K^-induced excitation and rebound inhibition, relative firing rate was calculated by taking the ratios of firing rates in zero K^+ and following washout to that in control (see Fig. 1). Only cells with control firing rate ≥0.5 Hz were selected for analysis to prevent the occurrence of unusual large ratio values. The control firing rate was determined by averaging over a period of 30 s right before each application of K^-free solution. The firing rate in zero K^+ was determined similarly by averaging over a period of 30 s during the application of K^-free solution. The rebound firing rate was determined over two time periods, early phase (first 5 s) and late phase (next 24 s), after return to 3.5 mM K^+. Membrane potentials and currents in perforated-patch recordings were determined similarly, with the magnitude of zero K^-induced depolarizations or inward currents and rebound hyperpolarizations or rebound outward currents measured by subtracting from the control values. Data were analyzed with ANOVA, followed by Tukey’s test for comparison of selected pairs. Statistical comparisons of means were performed with Student’s t-test or paired t-test.

**Na^+ imaging.** Intracellular Na^+ concentration ([Na^+]_i) was measured using the Na^-sensitive fluorescent indicator sodium-binding benzofuran isophthalate (SBFI) (Harootunian et al. 1989; Minta et al. 1989). The reduced SCN preparation was incubated in 10 or 15 μM SBFI-AM mixed with a nonionic surfactant, Pluronic F-127 (0.02–0.04% wt/vol; Molecular Probes, Invitrogen, Carlsbad, CA) in 50 μl of bath solution in the dark for 60 min at 37°C. Incubation was terminated by washing twice with 3 ml of bath solution, and at least 30 min were allowed for de-esterification of the dye. All imaging experiments were performed at room temperature (22–25°C). For the experiments, the reduced SCN preparation was gently pressed on the edge against the coverslip to allow adherence of the tissue to the surface. Fluorescence signals were imaged with the use of a charge-coupled device camera attached to an inverted microscope (Olympus IX71) and recorded with Xcelsense imaging software integrated with the CellIR MT20 illumination system (Olympus Biosystems, Planegg, Germany). The excitation wavelengths were 340 and 380 nm, and emitted fluorescence was collected at 510 nm. Pairs of 340-/380-nm images were sampled at 0.1–0.5 Hz. Na^+ levels in regions of interest (ROI) were spatially averaged and presented as fluorescence ratios (F340/F380) after background subtraction. Some of the record was smoothed by a moving average of five or nine successive points to reduce noise (see Fig. 7, A and B).

**Drugs.** Stock solutions of strophanthidin (10 mM) and monensin (10 mM) were prepared with 100% ethanol and stored at ~20°C; they were allowed to reach room temperature before being added to the bath to achieve the final concentrations during experiments. Sodium cyanide and iodoacetate were directly added to the bath to achieve the desired concentrations. These chemicals were purchased from Sigma-Aldrich. K^-free (zero K^+) solutions were prepared with omission of extracellular Na^+ and Na^-free solutions were prepared total replacement of extracellular Na^+ with N-methyl-D-glucamine (NMDG).

**RESULTS**

Effects of zero K^+ on the SCN neurons with perforated-patch recordings. In a previous study we used K^-free solution (zero K^+) and the cardiac glycoside strophanthidin to block the Na/K pump to investigate their effects on spontaneous firing in the SCN neurons with cell-attached and whole cell recordings (Wang and Huang 2006b). Whereas either treatment blocks the Na/K pump to increase spontaneous firing, zero K^+ additionally enhances K^- currents to increase peak afterhyperpolarization potential during action potential firing. As a result, zero K^+ increases spontaneous firing to a lesser degree than strophanthidin in cell-attached recordings and reduces firing in the presence of strophanthidin to block the Na/K pump (Wang and Huang 2006b). Nevertheless, the slow washout of strophanthidin prevents us from using this drug to do repetitive applications as often required in this study to monitor and compare Na/K pump activity during the course of experiments. For this reason, we used zero K^+ to block the Na/K pump, with occasional use of strophanthidin for comparison. Because the depolarizing effects of zero K^+ on resting membrane potential
are similar to or slightly larger than those of strophanthidin in the presence of TTX to block action potentials (see Fig. 1C), the contribution of K⁺ currents is probably not significant near resting membrane potential. The presentation of this study begins with the determination of zero-K⁺ effects on membrane excitability and intracellular Na⁺ (Figs. 1A and 2).

Figure 1 shows the effect of zero K⁺ on the SCN neurons recorded under perforated-patch conditions. As shown in Fig. 1A, a 30-s elimination of extracellular K⁺ excited the cell to fire at a higher rate, and return to normal (3.5 mM) K⁺ inhibited the firing to a level below control (top left). The inhibition of firing was associated with rebound hyperpolarization (arrowhead) on return to normal K⁺. Figure 1A, bottom left, plots the corresponding time course of change in the firing rate. To quantify the extent of zero K⁺-induced excitation and rebound inhibition of firing, relative firing rate was calculated by taking the ratios of firing rates in zero K⁺ and on return to 3.5 mM K⁺ to that in control, with the rebound firing rates determined over two time periods: the early phase (first 5 s) and the late phase (next 24 s). For a total of 23 cells, the mean spontaneous firing rate (2.8 ± 0.4 Hz, n = 23) was enhanced to 201 ± 22% (n = 23; P < 0.001, ANOVA) in zero K⁺ and inhibited to 37 ± 8% (early phase; n = 23; P < 0.01, ANOVA) and then to 62 ± 9% (late phase; n = 23; P > 0.05, ANOVA) after return to 3.5 mM K⁺ (Fig. 1A, right).

To better determine the effects of zero K⁺ on membrane potentials and currents, the experiments were done in the presence of 0.3 μM TTX to block the Na⁺-dependent action potentials. Figure 1B shows the effects of zero K⁺ on the membrane potential and current in a representative SCN neuron by switching between current- and voltage-clamp modes (2 rows of traces). For this particular cell, removal of extracellular K⁺ depolarized the membrane potential by 8 mV (from −59 to −51 mV), and return to 3.5 mM K⁺ transiently hyperpolarized the membrane potential by 7 mV (from −59 to −66 mV) before return to the control level (top left trace). At a holding potential of −52 mV (after correction of −12 mV junction potential), removal of extracellular K⁺ induced an inward current of 4.6 pA (from 1.1 to −3.5 pA) and return to 3.5 mM K⁺ produced rebound outward current of 1.9 pA (from 1.1 to 3.0 pA) (top right trace). The traces in Fig. 1B, middle, expand the time course of voltage and current traces during the removal and reappearance of extracellular K⁺. On average, the resting potential was depolarized by 10.4 ± 1.7 mV (n = 7; **P < 0.001, ANOVA) in zero K⁺, followed by hyperpolarizations of −9.9 ± 0.9 mV (early phase; n = 7; **P < 0.001, ANOVA) and −5.8 ± 0.7 mV (late phase; n = 7; P < 0.001, ANOVA) on return to 3.5 mM K⁺ (bottom left), and the holding current was shifted inwardly by 3.3 ± 0.4 pA (n = 7; **P < 0.001, ANOVA) in zero K⁺, followed by outward shifts of 1.8 ± 0.2 pA (early phase; n = 7; P < 0.001, ANOVA) and 1.1 ± 0.2 pA (late phase; n = 7; P < 0.001, ANOVA) on return to 3.5 mM K⁺ (bottom right).

Consistent with the blockade of Na/K pumps with zero K⁺, the cardiac glycoside strophanthidin at a concentration of 30 μM (IC₅₀ = 4 μM during daytime; Wang and Huang 2004) almost completely eliminated the zero K⁺ effects (Fig. 1C). Figure 1C, left, shows such results obtained from a representative SCN neuron. For this particular cell, the application of 30 μM strophanthidin depolarized the membrane potential to the same level as zero K⁺ did and completely abolished the
zero K⁺ effects. Note that in 50% (4/8) cells, zero K⁺ can still produce a small depolarization with negligible rebound hyperpolarization in the presence of 30 μM strophanthidin. On average, zero K⁺-induced depolarization was 7.5 ± 1.4 mV (n = 8) in control and was reduced to 1.3 ± 0.9 mV (n = 8; P < 0.05, paired t-test) in strophanthidin, whereas rebound hyperpolarization (early phase) was −7.3 ± 1.5 mV (n = 8) in control and reduced to −0.2 ± 1.0 mV (n = 8; P < 0.01, paired t-test) in strophanthidin (Fig. 1C, right).

**Pump blockade with zero K⁺ produces intracellular Na⁺ loading.** In addition to its effect on membrane excitability, pump blockade with zero K⁺ is expected to produce accumulation of intracellular Na⁺. To test this idea, we used fluorescence ratiometric recordings to monitor change in [Na⁺]. During pump blockade in zero K⁺ (Fig. 2), Figure 2A shows a portion of a reduced SCN preparation with selected cells circled to represent the ROI for averaging fluorescence signals. Figure 2B, top, plots the F340/F380 ratio to indicate the change of intracellular Na⁺ in five cells in response to the application of K⁺-free solution. As expected, pump blockade in zero K⁺ gradually increased intracellular Na⁺ levels as reflected by the increase in F340/F380. Figure 2B, bottom, plots the average signal from a total of 22 cells. The result indicates an initial linear increase in [Na⁺], in the first few minutes, followed by gradual leveling off until reaching a steady state between 10 and 15 min after the removal of extracellular K⁺. The zero K⁺-induced Na⁺ loading was not due to the increase in the rate of action potential firing, because similar results were also obtained with zero K⁺ in the presence of TTX (see Fig. 7).

**Intracellular Na⁺ regulation of Na/K pumps.** The Na/K pump is sensitive to intracellular Na⁺ and ATP (Therien and Blostein 2000). To demonstrate intracellular Na⁺ regulation of the Na/K pump in the SCN neurons, we investigated the effects of Na⁺ loading with the Na⁺ ionophore monensin (Fig. 3) and of Na⁺ depletion with Na⁺ omission from the pipette solution (Fig. 4). Monensin is a carboxylic polyether ionophore that catalyzes the electroneutral exchange of extracellular Na⁺ for intracellular H⁺ (Pressman and Fahim 1982). Figure 3 shows the monensin effects on the SCN neurons. As indicated in Fig. 3A, monensin at a concentration of 10 μM reversibly hyperpolarized the resting potential of a representative cell, and the hyperpolarizing effect was abolished by the blockade of the Na/K pump with zero K⁺ or the addition of 30 μM strophanthidin. Note the more depolarized resting potentials in zero K⁺ and strophanthidin. For a total of 8 cells, the monensin-induced

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**Fig. 2. Zero K⁺ induces intracellular Na⁺ accumulation.** A: Normarski (left) and fluorescence (right) micrographs of a reduced SCN preparation loaded with the fluorescent sodium indicator sodium-binding benzofuran isophthalate (SBFI). Regions of interest (ROI) are indicated with circles. B: the time course of change in the F340/F380 fluorescence ratio recorded from 5 selected ROIs (top) as indicated in A. Bottom: the average F340/F380 signal from a total of 22 cells. The standard error of the mean for each point (sampled at 0.5 Hz) was not shown for clarity. Scale bars, 20 μm.

**Fig. 3. Monensin effects on the SCN neurons.** A: recordings from a representative cell showing the voltage responses to 10 μM monensin are shown in control and in zero K⁺ or 30 μM strophanthidin to block the Na/K pump (traces). Note the more depolarized resting potentials as indicated in zero K⁺ and strophanthidin. Bottom: summary of experiments showing the dependence of hyperpolarizing effects of monensin on Na/K pump activity (n = 8). B: the average F340/F380 ratio (from 49 cells) indicating the rapid increase in intracellular Na⁺ concentration ([Na⁺]i) in response to the application of 10 μM monensin. C: recordings from a representative cell showing the voltage responses to 10 μM monensin in control and in the absence of extracellular Na⁺ (NMDG-substituted) (traces). Note the more hyperpolarized resting potential as indicated in zero Na⁺. Bottom: summary of experiments showing the dependence of monensin effects on extracellular Na⁺ (n = 6). *P < 0.01; ***P < 0.001.
The absence of extracellular K\(^+\) (Na\(^+\) in the control) led to more depolarized resting potentials with Na\(^+\), indicating Na\(^+\) influx via the Na\(^+\)/K\(^+\) pump. Because 10 \(\mu\)M monensin also increased [Na\(^+\)]\(\text{i}\) in the SCN neurons (Fig. 3B), the hyperpolarizing effect of monensin was most likely mediated by enhanced pump activity as a result of monensin load. Indeed, total replacement of extracellular Na\(^+\) with NMDG blocked the monensin-induced hyperpolarization as indicated in another cell shown in Fig. 3C. Note the more hyperpolarized membrane potential in zero Na\(^+\) (Na\(^+\) pipette solution). Thus, the participation of the Na\(^+\) leak in regulating the resting potential is evident. On average the monensin-induced potential change was −3.4 ± 0.6 mV (n = 6) in control and 0.6 ± 0.4 mV (n = 6; P < 0.001, paired t-test) in the absence of extracellular Na\(^+\). Together, the results indicate that Na\(^+\) influx via the ionophore activates the Na/K pump and thereby hyperpolarizes the resting membrane potential.

Consistent with the observation of intracellular Na\(^+\) load-induced hyperpolarizations, intracellular Na\(^+\) depletion appeared to depolarize the resting membrane potentials (Fig. 4). The experiment was performed with Na\(^+\) omission from the pipette solution. The resting membrane potential thus recorded averaged −41.8 ± 1.5 mV (n = 17), significantly more depolarized than −53.0 ± 2.0 mV (n = 26; P < 0.001, Student’s t-test) as recorded with 20 mM Na\(^+\) in the pipette solution (Fig. 4A). The result suggests reduced pump activity and thus more depolarized resting potentials with Na\(^+\)-free pipette solutions. Under this condition, however, zero K\(^+\) or strophanthidin can still depolarize the resting potential as indicated in a representative cell shown in Fig. 4B. The result indicates finite pump activity despite the lack of Na\(^+\) inside the pipette, suggesting a finite level of Na\(^+\) around the pump sites, most likely due to constant Na\(^+\) influx via the Na\(^+\) leak (see Discussion).

**Metabolic regulation of Na/K pumps.** To determine how energy metabolism regulates Na/K pump and neuronal excitability, we investigate the effect of mitochondrial inhibition with NaCN on the SCN neurons (Fig. 5). Figure 5A1 shows the excitatory (left) and inhibitory (right) effects of cyanide on spontaneous firing. A2: comparison of SFR in the cyanide-excited SCN neurons in control, cyanide, and zero K\(^+\). The results indicate a stronger effect of cyanide than Na\(^+\) in increasing firing rate. B1: recordings from 2 representative cells showing the depolarizing (left) and hyperpolarizing (right) effects of cyanide on resting potentials in the presence of 0.3 \(\mu\)M TTX. B2: comparison of membrane depolarizations by zero K\(^+\) and by cyanide in control (left) and in the presence of tolbutamide to block ATP-sensitive K\(^-\) (K\(_{ATP}\)) channels (right). Note the stronger effect of zero K\(^+\) than cyanide in depolarizing the resting potential. C: recordings from a representative cell showing the cyanide-induced depolarization in control and in zero K\(^+\) to block the Na/K pump (left). Right: summary of cyanide-induced voltage changes in control and in zero K\(^+\). *P < 0.05, **P < 0.005, ***P < 0.001.

Fig. 5. Effects of mitochondrial inhibition on the SCN neurons. A1: recordings from 2 representative cells showing the excitatory (left) and inhibitory (right) effects of cyanide on spontaneous firing. A2: comparison of SFR in the cyanide-excited SCN neurons in control, cyanide, and zero K\(^+\). Note the stronger effect of zero K\(^+\) than cyanide in increasing firing rate. B1: recordings from 2 representative cells showing the depolarizing (left) and hyperpolarizing (right) effects of cyanide on resting potentials in the presence of 0.3 \(\mu\)M TTX. B2: comparison of membrane depolarizations by zero K\(^+\) and by cyanide in control (left) and in the presence of tolbutamide to block ATP-sensitive K\(^-\) (K\(_{ATP}\)) channels (right). Note the stronger effect of zero K\(^+\) than cyanide in depolarizing the resting potential. C: recordings from a representative cell showing the cyanide-induced depolarization in control and in zero K\(^+\) to block the Na/K pump (left). Right: summary of cyanide-induced voltage changes in control and in zero K\(^+\). *P < 0.05, **P < 0.005, ***P < 0.001.
indicates a stronger effect of zero K\(^+\) than cyanide (n = 19; P < 0.05, ANOVA) in exciting the SCN neurons.

Similarly, in the presence of TTX cyanide also depolarized a majority of cells (54%; 25/46) and hyperpolarized a subset of cells (37%; 17/46), with the remaining (9%; 4/46) cells exhibiting both depolarizing and hyperpolarizing responses to cyanide. Figure 5B1 shows the depolarizing (left) and hyperpolarizing (right) effects of cyanide on resting membrane potentials in two representative cells. On average, cyanide depolarization was 6.3 ± 1.1 mV (n = 25), and cyanide hyperpolarization amounted to −9.8 ± 1.6 mV (n = 17). For comparison, 16 of the 25 cyanide-depolarized cells were also tested with zero K\(^+\).

The result indicates a larger depolarization of membrane potential by zero K\(^+\) than by cyanide, with values of 12.1 ± 2.1 mV and 7.1 ± 1.9 mV (n = 16; P < 0.001, paired t-test), respectively (Fig. 5B2, left). Because cyanide activated SUR1/Kir6.2 combination of ATP-sensitive K\(^+\) (K\(_{\text{ATP}}\)) channels in a subset of SCN neurons (Hsu et al. 2010), we also compared the effects of cyanide and zero K\(^+\) in the presence of 200 \(\mu\)M tolbutamide (or 1 \(\mu\)M glibenclamide) to block the K\(_{\text{ATP}}\) channels. The results also indicate a larger depolarization by zero K\(^+\) than by cyanide, with values of 15.7 ± 2.1 mV (n = 6) and 8.3 ± 1.9 mV (n = 6; P < 0.001, paired t-test), respectively (Fig. 5B2, right).

To further determine if cyanide-induced depolarization is indeed mediated by the blockade of Na/K pump, we compared the effect of cyanide on membrane potential in control and during pump blockade in zero K\(^+\). Figure 5C shows such a result. For this particular cell (left), cyanide depolarized the membrane potential by 5 mV by the end of 30-s drug application, and pump blockade in zero K\(^+\) rapidly depolarized the membrane potential by 8 mV and abolished the cyanide-induced depolarization to reveal a small hyperpolarization of −2 mV. On average, cyanide produced a depolarization of 9.3 ± 2.9 mV (n = 8) in control but a small hyperpolarization of −1.5 ± 0.6 mV (n = 8; P < 0.005, paired t-test) during pump blockade in zero K\(^+\) (right). The result suggests that cyanide-induced depolarization is mediated by the blockade of the Na/K pump. Together with the observation of weaker excitatory effects of cyanide than zero K\(^+\) (Fig. 5, A and B), the results suggest that cyanide inhibition of oxidative phosphorylation does not eliminate all Na/K pump activity. In other words, Na/K pump activity in the SCN neurons may be fueled by both oxidative phosphorylation and glycolysis, the two major processes for ATP synthesis.

To test this idea, we first determined the ability of zero K\(^+\) to block the Na/K pump in the presence of cyanide inhibition of oxidative phosphorylation. Figure 6A shows such a result obtained from a cyanide-depolarized cell. The result indicates that zero K\(^+\) depolarized the membrane potential by 10.0 mV in control (left) but still produced a depolarization of 4.1 mV in cyanide (right). Similar results were obtained from nine other cells regardless of their membrane potentials being depolarized (5 cells), hyperpolarized (2 cells), or little affected (by less than 1 mV; 2 cells) by cyanide. On average, the depolarization by zero K\(^+\) was 9.8 ± 0.9 mV (n = 10) in control and 3.1 ± 0.6 mV (n = 10) in cyanide. The results indicate that zero K\(^+\) still blocked the pump to depolarize membrane potentials in cyanide, suggesting an incomplete blockade of Na/K pump activity by mitochondrial inhibition.

The involvement of glycolysis in fueling the Na/K pump was investigated by determining the effects of zero K\(^+\) in the presence of 2 mM iodoacetate to block glycolysis. For the experiment, iodoacetate was applied for at least 10 min, a time previously shown to block the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (Sabri and Ochs 1971). The results indicate that, in the presence of iodoacetate to block glycolysis, zero K\(^+\)-induced depolarization was slowly reduced and became nearly completely blocked at times between 10 and 20 min. Figure 6B shows such a result from a representative cell, indicating the effects of zero K\(^+\) in control (left) and after 10 and 20 min into the application of iodoacetate (right). Similar results were obtained from 13 other cells regardless of their membrane potentials being depolarized (6 cells), hyperpolarized (4 cells), or little affected (by less than 1 mV; 3 cells) by iodoacetate. On average, zero K\(^+\)-induced depolarization was 10.0 ± 1.9 mV (n = 14) in control and 0.0 ± 0.6 mV (n = 14) in iodoacetate. Together, the results of incomplete blockade of Na/K pumps by mitochondrial inhibition (zero K\(^+\)-induced depolarization of 3.1 ± 0.6 mV; n = 10) but complete blockade by glycolytic inhibition (zero K\(^+\)-induced depolarization of 0.0 ± 0.6 mV; n = 14; P < 0.005, Student’s t-test) (Fig. 6C) suggest that the Na/K pump is fueled by both glycolysis and oxidative phosphorylation.

Ratiometric fluorescence imaging of intracellular Na\(^+\) also indicates the involvement of both glycolysis and oxidative phosphorylation in fuelling the Na/K pump (Fig. 7). Figure 7A shows the average [Na\(^+\)] change in response to
5-min application of zero K⁺, cyanide, and iodoacetate. As expected for the blockade of Na/K pump, all three treatments increased [Na⁺], in all SCN neurons. Furthermore, cyanide was less effective than zero K⁺ in raising [Na⁺], suggesting an incomplete blockade of Na/K pump by cyanide. Note the irreversible effect of iodoacetate on increasing [Na⁺]. Consistent with the idea of incomplete blockade of Na/K pump by cyanide inhibition of oxidative phosphorylation, zero K⁺ further increased [Na⁺], in the prolonged presence of cyanide (Fig. 7B). The smooth curve through the data points was drawn to aid the visualization of cyanide-induced saturating accumulation of Na⁺. In contrast, iodoacetate slowly increased [Na⁺] levels and eventually completely blocked the ability of zero K⁺ to further increase [Na⁺] (Fig. 7C). Note the slight increase in [Na⁺] by zero K⁺ after 10 min (arrowhead), but not after 33 or 43 min, into the application of iodoacetate.

**Discussion**

Effects of zero K⁺ on neuronal excitability and intracellular Na⁺. Our results indicate that removal of extracellular K⁺ to block the Na/K pump excited SCN neurons and return to normal K⁺ to re-activate the pump produced rebound hyperpolarization to inhibit firing (Fig. 1). We have previously observed similar effects of zero K⁺ on the SCN neurons with cell-attached recordings and have hypothesized that the rebound inhibition of firing is mediated by enhanced pump activity due to Na⁺ accumulated during prior pump blockade in zero K⁺ (Wang and Huang 2006b). Two observations are in part consistent with this hypothesis. First, pump blockade with zero K⁺ induced saturating accumulation of intracellular Na⁺ as demonstrated with ratiometric Na⁺ imaging (Figs. 2 and 7). Second, the blockade of Na/K pump with strophanthidin blocked zero K⁺-induced depolarization and rebound hyperpolarization. Nevertheless, because rebound hyperpolarization may be caused by activation of outward currents and/or inactivation of inward currents persisting after zero K⁺-induced depolarization, a definite answer to this question must await further experiments.

**Intracellular Na⁺ regulation of Na/K pumps.** Our results indicate that monensin-induced Na⁺ loading enhances pump activity to hyperpolarize the membrane potential, because the hyperpolarizing effect is abolished by the removal of extracellular Na⁺ or by the blockade of Na/K pump with strophanthidin or in zero K⁺. In contrast, Na⁺ depletion with Na⁺ omission from the patch pipette depolarizes the resting membrane potential, suggestive of reduced pump activity with intracellular Na⁺ depletion. Interestingly, the ability of zero K⁺ and strophanthidin to depolarize the resting membrane potential even in the absence of pipette Na⁺ ([Na⁺]pip = 0 mM) indicates finite pump activity supported by a finite level of Na⁺ around the pump sites. Because this level of Na⁺ is most likely maintained by the constant influx of Na⁺ via the Na⁺ leak, one may consider that the Na⁺ leak is functionally coupled to the Na/K pump via local sharing of submembrane Na⁺. In other words, the constant influx of Na⁺ may provide persistent inward Na⁺ current to maintain a more depolarized resting potential in the SCN neurons. Indeed, total replacement of Na⁺ by NMDG markedly hyperpolarized the membrane potential (Fig. 3C), which also has been observed in dissociated SCN neurons (Jackson et al. 2004). Experiments are underway to investigate the nature of the Na⁺ leak and its relation with the Na/K pump.

Because these experiments were done in the presence of TTX to block Na⁺ influx via TTX-sensitive Na⁺ channels, the possible contribution of influxed Na⁺ via action potentials to enhance Na/K pump activity was thus not addressed in this study. It is possible that the higher daytime Na/K pump activity acts to extrude larger influxed Na⁺ via the faster daytime action potential firing. Future experiments should be dedicated to investigating the role of daytime action potential firing in increasing daytime Na/K pump activity.

**Metabolic regulation of Na/K pumps**. Cyanide inhibition of mitochondrial respiration reversibly depolarizes membrane potential and increase spontaneous firing in a majority of SCN neurons. Our results indicate that the excitatory effect of cyanide is mediated by the blockade of the Na/K pump. Consistent with this idea, cyanide, albeit weaker than zero K⁺,
increases intracellular Na\(^+\) in all SCN neurons. The energy produced by oxidative phosphorylation, however, does not support all Na/K pumps. This conclusion comes from results obtained with both electrical recordings and ratiometric Na\(^+\) imaging. First, the depolarization produced by cyanide is smaller than that produced by zero K\(^+\), regardless of the presence of tolbutamide to block K\(_{\text{ATP}}\) channels, and zero K\(^+\) can further produce depolarizations in the presence of cyanide. Second, the increase in intracellular Na\(^+\) levels produced by cyanide is also smaller than that produced by zero K\(^+\), and zero K\(^+\) can further increase intracellular Na\(^+\) on top of elevated [Na\(^+\)], in the presence of cyanide. In contrast to the incomplete blockade of Na/K pumps by cyanide, prolonged application of iodoacetate to block glycolysis eliminates all Na/K pump activity. This is because in the prolonged presence of iodoacetate, zero K\(^+\) can no longer produce any depolarization or further increase intracellular Na\(^+\). Together, the results indicate the involvement of both glycolysis and oxidative phosphorylation in fueling Na/K pumps in the SCN neurons.  

Because the Na/K pump is the major energy consumer in central neurons, the results of both glycolysis and oxidative phosphorylation (this study) fueling the higher daytime pumping activity (Wang and Huang 2004) suggest that the SCN neurons require greater rate of glycolytic flux and respiration to meet greater energy demand during the day. As such, our results may account for the observations of higher daytime 2-deoxyglucose uptake (Newman et al. 1992; Schwartz and Gainer 1977) and cytochrome oxidase activity (López et al. 1997) in the SCN.  

The demonstration of metabolic regulation of Na/K pumps and neuronal excitability suggests that the SCN neurons are sensitive to metabolic disturbance. Additional agents may also be involved, however, in view of the observations that metabolic inhibition of Na/K pumps invariably increases [Na\(^+\)] in all SCN neurons but only excites most, but not all, SCN neurons. It appears that additional activation of K\(_{\text{ATP}}\) channels mostly accounts for the inhibition of a subset of SCN neurons during metabolic stress (Hsu et al. 2010). In other words, in this subset of cells, both Na/K pumps and K\(_{\text{ATP}}\) channels may be involved in metabolic regulation of neuronal excitability.  

**Implications for diurnal rhythm in Na/K pump activity.** Na/K pump activity can be regulated by multiple mechanisms including the concentrations of substrates such as intracellular Na\(^+\) and ATP (Thérien and Blostein 2000). Although this study does not directly address the issue of rhythmic Na/K pump activity, it is worth discussing how our observations of intracellular Na\(^+\) and metabolic regulation may be related to its diurnal rhythmicity. It should be noted that the diurnal rhythm of Na/K pump activity can be determined by measuring the strophanthidin-blocked currents with whole cell recordings (Wang and Huang 2004), in which both [Na\(^+\)], and [ATP], are presumably fixed by the pipette solution. Nevertheless, the finite pump activity with zero Na\(^+\) in pipette solution (Fig. 4) suggests finite concentrations of submembrane Na\(^+\) around the pump sites, most likely mediated by constant flux of Na\(^+\) via the Na\(^+\) leak. In other words, submembrane Na\(^+\) may not be clamped by the pipette solution. If the same holds for our previous study with whole cell recordings, the higher daytime pumping activity could suggest a higher daytime Na\(^+\) leak, an interesting possibility that remains to be tested.

Along the same line of thinking, submembrane ATP concentrations also may not be clamped by the pipette solution due to the actions of localized ATP-producing and ATP-consuming processes. For example, a membrane-compartmented ATP pool has been shown to preferentially fuel the Na/K pump in human red cells (Hoffman et al. 2009; Proverbo and Hoffman 1977). If a similar mechanism is also present in the SCN neurons, the higher daytime pumping activity, fueled by both glycolysis and oxidative phosphorylation, could be a result of higher daytime metabolic rate as demonstrated by 2-deoxyglucose uptake (Newman et al. 1992; Schwartz and Gainer 1977) and cytochrome oxidase activity (López et al. 1997).  

**Functional implications of intracellular Na\(^+\) and metabolic regulation of Na/K pump in SCN neuron.** The Na/K pump is the ultimate active agent responsible for controlling intracellular Na\(^+\) concentrations and as such should be sensitive to regulation by both intracellular Na\(^+\) and energy metabolism. Our results suggest that constant influx of Na\(^+\) via yet to be identified Na\(^+\) leak may raise the submembrane Na\(^+\) concentrations around the pump sites. In other words, the Na\(^+\) leak and the Na/K pump may interact to regulate [Na\(^+\)], which then act on Na/Ca exchangers to regulate intracellular Ca\(^{2+}\) homeostasis (Blaustein and Lederer 1999), as has been demonstrated in myocytes (see, for examples, Lee et al. 2006; Su et al. 1998; Swift et al. 2010). Because Ca\(^{2+}\) signaling is central to SCN physiology, [Na\(^+\)], may regulate Ca\(^{2+}\) handling along the way from Ca\(^{2+}\) channels (Irwin and Allen 2007; Kim et al. 2005) and NMDA-induced Ca\(^{2+}\) influx to ryanodine receptor-mediated Ca\(^{2+}\) signaling as in glutamate-mediated phase shifts (see Gillette and Mitchell 2002). It is thus important to determine the expression of Na/Ca exchangers in the SCN and to know if the Na/K pump indeed regulates Na/Ca exchange activity via [Na\(^+\)], in the SCN neurons.  

Furthermore, the potent regulation of Na/K pump activity by both glycolysis and oxidative phosphorylation suggests that pump activity and thus neuronal excitability may alter as a result of change in energy status of the SCN neurons. In particular, the Na/K pump, as a major consumer of metabolic energy, may be sensitive to the availability of glucose, the alteration of which has been shown to alter circadian functioning including the photic response of the SCN (see Challet 2010; Green et al. 2008). Further work is warranted to examine this possibility.

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**DISCLOSURES**  
No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**  
Y.-C.W., J.-J.Y., and R.-C.H. conception and design of research; Y.-C.W. and J.-J.Y. performed experiments; Y.-C.W., J.-J.Y., and R.-C.H. analyzed data; Y.-C.W., J.-J.Y., and R.-C.H. interpreted results of experiments; Y.-C.W. and R.-C.H. prepared figures; Y.-C.W., J.-J.Y., and R.-C.H. edited and revised manuscript; Y.-C.W., J.-J.Y., and R.-C.H. approved final version of manuscript; R.-C.H. drafted manuscript.
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