Role of Na⁺/Ca²⁺ Exchanger in Ca²⁺ Homeostasis in the Rat Suprachiasmatic Nucleus Neurons

Yi-Chi Wang, Ya-Shuan Chen, Ruo-Ciao Cheng, and Rong-Chi Huang*

Departments of Physiology and Pharmacology, Chang Gung University College of Medicine, Kwei-San, Tao-Yuan, Taiwan

Running title: Role of NCX in Ca²⁺ Homeostasis in the Rat SCN

*Correspondence should be addressed to:
Dr. Rong-Chi Huang
Department of Physiology and Pharmacology
Chang Gung University School of Medicine
259 Wen-Hwa 1st Road
Kwei-San, Tao-Yuan
Taiwan

Phone: (886)-3-211-8631
Fax: (886)-3-211-8631
E-mail: rongchi@mail.cgu.edu.tw

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Abstract

Intracellular Ca\(^{2+}\) is critical to the central clock of the suprachiasmatic nucleus (SCN). However, the role of Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) in [Ca\(^{2+}\)]\(_i\) homeostasis in the SCN is unknown. Here we show that NCX is an important mechanism for somatic Ca\(^{2+}\) clearance in the SCN neurons. In control conditions Na\(^+\)-free solution lowered [Ca\(^{2+}\)]\(_i\) by inhibiting TTX-sensitive as well as nimodipine-sensitive Ca\(^{2+}\) influx. Using the Na\(^+\) ionophore monensin to raise [Na\(^+\)], Na\(^+\)-free solution provoked rapid Ca\(^{2+}\) uptake via reverse NCX. The peak amplitude of 0 Na\(^+\)-induced [Ca\(^{2+}\)]\(_i\) increase was larger during the day than at night, with no difference between dorsal and ventral SCN neurons. Ca\(^{2+}\) extrusion via forward NCX was studied by determining the effect of Na\(^+\) removal on Ca\(^{2+}\) clearance after high K\(^+\)-induced Ca\(^{2+}\) loads. The clearance of Ca\(^{2+}\) proceeded with two exponential decay phases, with the fast decay having total signal amplitude of ~85% and a time constant of ~7 s. Na\(^+\)-free solution slowed the fast decay rate threefold, whereas mitochondrial protonophore prolonged mostly the slow decay. In contrast, the blockade of plasmalemmal and sarcoplasmic reticulum Ca\(^{2+}\) pumps had little effect on the kinetics of Ca\(^{2+}\) clearance. RT-PCR indicated the expression of NCX1 and NCX2 mRNAs. Immunohistochemical staining showed the presence of NCX1 immunoreactivity in the whole SCN but restricted distribution of NCX2 immunoreactivity in the ventrolateral SCN. Together our results demonstrate an important role of NCX, most likely NCX1, as well as mitochondrial Ca\(^{2+}\) uptake in clearing somatic Ca\(^{2+}\) after depolarization-induced Ca\(^{2+}\) influx in the SCN neurons.
Introduction

Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) is an electrogenic antiporter that exchanges 3 Na\(^+\) for 1 Ca\(^{2+}\) in either forward (Ca\(^{2+}\) exit) mode, or reverse (Ca\(^{2+}\) entry) mode (Blaustein and Lederer 1999). As such, an increase in [Ca\(^{2+}\)]\(_i\) activates the forward mode to extrude Ca\(^{2+}\), and an increase in [Na\(^+\)], activates the reverse mode to uptake Ca\(^{2+}\). Three genes are known to encode three isoforms NCX1, NCX2, and NCX3 (Li et al. 1994; Nicoll et al. 1990; Nicoll et al. 1996), all of which have been found to express in the rat brain (Lee et al. 1994; Papa et al. 2003; Yu and Colvin 1997). While all three isoforms appear to have similar properties (Iwamoto and Shigekawa 1998; Linck et al. 1998), the isoform-specific distribution in different brain regions (Papa et al. 2003) and in different cell types (Thurneysen et al. 2002) suggests distinct functions for different isoforms.

The hypothalamic suprachiasmatic nucleus (SCN) is the central clock controlling circadian rhythms in mammals (Dibner et al. 2010). The SCN clock is synchronized by light information conveyed from the eye to the SCN via the glutamatergic retinohypothalamic tract (Golombek and Rosenstein 2010; Meijer and Schwartz 2003). The SCN neurons exhibit a circadian rhythm 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; Enoki et al. 2012; Ikeda et al. 2003; Irwin and Allen 2009). In the mouse SCN, the rhythmic change in [Ca\(^{2+}\)]\(_i\) involves internal release (Ikeda et al. 2003) and is not altered or reduced by the blockade of action potentials with TTX (Enoki et al. 2012; Ikeda et al. 2003). In the rat SCN, TTX eliminates the day-night variation in [Ca\(^{2+}\)]\(_i\) (Colwell 2000) or similarly lowers baseline Ca\(^{2+}\) ratio between day and night (Irwin and Allen 2009), suggesting an important contribution of action potential (voltage)-dependent...
transmembrane Ca\textsuperscript{2+} influx to basal [Ca\textsuperscript{2+}]. Indeed, transmembrane Ca\textsuperscript{2+} influx appears to be required for maintaining rhythmicity of clock gene *per1* in the rat SCN and of both *per1* gene and PER2 protein in the mouse SCN (Lundkvist et al. 2005). Furthermore, glutamate-mediated phase shifts during the night critically depend on Ca\textsuperscript{2+} influx via voltage-dependent Ca\textsuperscript{2+} channels (Irwin and Allen 2007; Kim et al. 2005) as well as ryanodine receptor-mediated Ca\textsuperscript{2+} signaling (Ding et al. 1998; Gillette and Mitchell 2002). These findings indicate a critical role of intracellular Ca\textsuperscript{2+} in maintaining circadian rhythmicity and mediating photic entrainment in the SCN. Nevertheless, it is not known whether and how NCX regulates [Ca\textsuperscript{2+}]\textsubscript{i} in the SCN neurons.

This study aimed to investigate the role of NCX in the regulation of Ca\textsuperscript{2+} homeostasis in the SCN neurons. Ratiometric Ca\textsuperscript{2+} imaging was used to study the effects of Na\textsuperscript{+}-free solution on [Ca\textsuperscript{2+}]\textsubscript{i}. We also used RT-PCR and immunohistochemistry to determine the expression and distribution of NCX isoforms. Part of the results have been presented in abstract form (Wang et al. 2012a).
METHODS

Hypothalamic brain slices and reduced SCN preparations

All experiments were carried out in accordance with NIH for Care and Use of Laboratory Animals and the experimental protocol was approved by the Institutional Animal Care and Use Committee of Chang Gung University. Sprague-Dawley rats (18–25 days old) were kept in a temperature-controlled room under a 12:12 light:dark cycle (light on 0700–1900 h). Lights-on was designated Zeitgeber time (ZT) 0. For daytime and nighttime recordings, the animal was killed at ZT 2 and ZT 10, respectively. An animal was carefully restrained by hand to reduce stress and killed by decapitation using a small rodent guillotine without anaesthesia, and the brain was put in an ice-cold artificial cerebrospinal fluid (ACSF) prebubbled with 95% O₂-5% CO₂. The ACSF contained (in mM): 125 NaCl, 3.5 KCl, 2 CaCl₂, 1.5 MgCl₂, 26 NaHCO₃, 1.2 NaH₂PO₄, 10 glucose. A coronal slice (200–300 µm) containing the SCN and the optic chiasm was cut with a Vibroslice (Campden Instruments, Lafayette, IN, USA) or a DSK microslicer (Ted Pella, Redding, CA, USA), and was then incubated at room temperature (22–25°C) in the incubation solution, which contained (in mM): 140 NaCl, 3.5 KCl, 2 CaCl₂, 1.5 MgCl₂, 10 glucose, 10 HEPES, pH 7.4, bubbled with 100% O₂.

For electrical recordings and fluorescent Ca²⁺ and Na⁺ imaging, a reduced SCN preparation was obtained by excising a small piece of tissue (circa one-ninth the size of SCN) from the medial SCN using a fine needle (Cat no. 26002-10, Fine Science Tools, Foster City, CA, USA), followed by further trimming down to 4–10 smaller pieces with a short strip of razor blade. The reduced preparation (containing tens of cells, see Fig. 1) was then transferred to a coverslip precoated with poly-D-lysine (Sigma-Aldrich, St Louis, MO, USA).
in a recording chamber for recording. The SCN neurons of the reduced preparation could be identified visually with an inverted microscope (Olympus IX70 and IX71, Japan). The preparation thus obtained allows rapid application of drugs (Chen et al. 2009) and has been used for fluorescent Na\(^+\) imaging (Wang et al. 2012\(b\)) and to demonstrate diurnal rhythms in both spontaneous firing and Na/K pump activity (Wang and Huang 2004).

Electrical recording

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, 10 HEPES, pH adjusted to 7.4 with NaOH. All recordings were made with Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA) at room temperature (22–25\(^\circ\)C). The spontaneous firing rate was recorded in the cell-attached configuration. The patch electrode was filled with the bath solution or with the patch solution containing (in mM): 20 NaCl, 1 CaCl\(_2\), 2 MgCl\(_2\), 110 K-gluconate, 11 EGTA, 10 HEPES, 3 Na-ATP, 0.3 Na-GTP, pH adjusted to 7.3 with KOH. The spike counts, in 6-s epochs, always began only after stable recordings were made. At least one or two minutes of spontaneous firing rate were counted before the application of drugs. The signal was low-pass filtered at 1–5 KHz and digitized on-line at 2–10 KHz via a 12-bit A/D digitising board (DT2821F-DI, Data Translation, Marboro, MA, USA) with a custom-made program written in the C Language.

Ca\(^{2+}\) and Na\(^+\) imaging

Fluorescent Ca\(^{2+}\) and Na\(^+\) imaging were performed by pre-loading the SCN neurons with the Ca\(^{2+}\)-sensitive fluorescent indicator Fura2-acetoxymethyl ester (Fura2-AM) (Gryniewicz et al. 1985) or the Na\(^+\)-sensitive fluorescent indicator sodium-binding benzofuran isophthalate (SBFI-AM) (Harootunian et al. 1989; Minta and Tsien 1989). The reduced SCN preparation
was incubated in 10 µM Fura2-AM or 15 µM SBFI-AM mixed with a nonionic surfactant Pluronic F-127 (0.02–0.04 % wt/vol; Molecular Probe, Invitrogen, Carlsbad, CA, USA) in 50 µl of bath solution in the dark for 60 min at 37°C. Incubation was terminated by washing with 6 ml of bath solution and at least 60 min was 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 using a charge-coupled device camera (Olympus XM10, Japan) attached to an inverted microscope (Olympus IX71, Japan) and recorded with Xcellence imaging software integrated with the CellIR MT20 illumination system (Olympus Biosystems, Planegg, Germany). The system used a 150 W xenon arc burner as the light source to illuminate the loaded cells. The excitation wavelengths were 340 (± 12) nm and 380 (± 14) nm, and emitted fluorescence was collected at 510 nm. Pairs of 340/380 nm images were sampled at 0.2 Hz for Na⁺ and 0.5 Hz for Ca²⁺ signals. Ca²⁺ and Na⁺ levels in regions of interest (ROI) were spatially averaged and presented by fluorescence ratios (F340/F380) after background subtraction. The average F340/F380 trace of Fig. 3A was smoothed by a moving average of nine successive points to reduce noise. 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, USA). Data were given as means ± SEM and analyzed with Student’s t-test and paired t-test or with ANOVA, followed by Tukey’s test for comparison of selected pairs.
Drugs

Stock solutions of monensin (10 mM in 100% ethanol), KB-R7943 (10 mM in DMSO), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP; 1 mM in DMSO), thapsigargin (1 mM in DMSO), dantrolene (10 mM in DMSO), and aminoethoxydiphenyl borate (100 mM in DMSO) were stored at –20°C, and were diluted 10,000 or 1000 times to reach desired final concentrations. Ni²⁺ and La³⁺ were directly added to the bath to achieve the final concentration. These chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA), except for KB-R7943 and dantrolene which were from Tocris Cookson (Ellisville, MO, USA) and Alomone Labs (Jerusalem, Israel), respectively. Na⁺-free solutions were prepared with total replacement of extracellular Na⁺ with Li⁺ or N-methyl-D-glucamine (NMDG⁺), Ca²⁺-free solutions were prepared with omission of extracellular Ca²⁺ and the addition of 1 mM EGTA, and high (50 mM) K⁺ solutions were prepared with equal molar substitution of K⁺ for Na⁺.

RT-PCR analysis of NCX1, NCX2, and NCX3 expression

Total RNA of SCN was extracted using the Absolutely RNA Nanoprep kit (Stratagene, La Jolla, CA, USA) according to the manufacturer’s guide; total RNA of rat brain was purchased from BioChain Institute Inc (Newark, CA, USA). RNA samples were treated with DNaseI for 13–15 min at 25°C to eliminate genomic DNA contamination. The resulting RNA was reverse-transcribed (RT) to cDNA using ReverTra Ace (TOYOBO, Osaka, Japan) with oligo(dT) primers in a total volume of 20 μl. One-tenth of RT products were used as templates (2 μl) to perform PCR reaction. RT reaction with omission of reverse transcriptase was used as templates for negative control PCR. Primers used for RT-PCR were as follows: NCX1 forward 5’- ACCACCAAGACTACAGTGCG-3’ and reverse 5’-
TTGGAAGCTGTGTCTCC-3', NCX2 forward 5'-GCGTGTGGCGATGCTCA-3' and reverse 5'- GACCTCGAGGCGACAGTTC-3', and NCX3 forward 5'-CTGGAAGGGGATGACC-3' and reverse 5'-GTTTAGGGTGTTCACCAATA-3'.

The thermal cycling condition of RT-PCR was 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and then 72°C for 7 min. PCR amplified products were electrophoresed in 1.5% agarose gels, stained with ethidium bromide, and photographed.

Immunohistochemistry

Sprague-Dawley rats (23–25 days old) were deeply anesthetized with Zoletil (40 mg/kg, i.p.; Virbac Laboratories, Carros, France) and fixed by transcardial perfusion with PBS and then with 4% paraformaldehyde (500 ml/animal). Brains were removed and post-fixed for at least 4 hr in 4% paraformaldehyde, followed by dehydration with 30% sucrose in PBS for another 24 h. Twenty-micrometer-thick coronal sections through the hypothalamus region containing the SCN were cut on a cryostat (–20°C), collected in antifreeze solution, and stored in –20°C freezer until further processing.

For immunohistochemical staining, sections (20 µm) were treated with 0.3% H2O2 for 15 min to quench endogenous peroxidase, and then incubated overnight at 4°C in PBS containing 2% serum, 0.3% Triton X-100, and primary antibodies against NCX1 (mouse anti-NCX1, against epitope between amino acid 371 and 525 on intracellular side of plasma membrane; 1:100; AB2869; Abcam, MA, USA) (Markova et al. 2013) or NCX2 (goat anti-NCX2, against a peptide mapping within an extracellular domain of human origin; 1:1000; SC-33528; Santa Cruz, CA, USA) (Engelhardt et al. 2013). After incubation with primary antibodies, sections were treated with respective biotinylated secondary antibodies for 1 h at room temperature (22–25°C). Sections were then rinsed in PBS and incubated in avidin-
biotin complex (ABC Elite Kit, Vector Labs, Burlingame, CA, USA) for 1 h according to the manufacturer’s instructions. After two 10-min washes in 0.1 M sodium acetate, sections were stained with diaminobenzidine. Sections were photographed and analyzed with an inverted microscope (Olympus IX71, Japan). Immunoreactivity for NCX1 or NCX2 was quantified by calculating the relative optical density with ImageJ 1.43u (NIH, USA).
RESULTS

Na⁺-free solution induced variable $[\text{Ca}^{2+}]_i$, responses in the SCN neurons

Ratiometric fluorescence recordings were performed to determine changes in $[\text{Ca}^{2+}]_i$, when external Na⁺ was removed to block Ca²⁺ extrusion via forward NCX and/or to promote Ca²⁺ uptake via reverse NCX. The experiment began with the application of K⁺-free solution to determine the condition of reduced preparations and to confirm the cells being recorded are indeed neurons. The removal of extracellular K⁺ has been shown to block the Na/K pump to depolarize the membrane potential and increase spontaneous firing of SCN neurons, followed by rebound hyperpolarization and inhibition of spontaneous firing on return to normal K⁺ (Wang and Huang 2006; Wang et al. 2012b). Figure 1 shows the effects of K⁺-free (0 K⁺) and Na⁺-free (0 Na⁺; Li⁺ substituted) solution on levels of $[\text{Ca}^{2+}]_i$ in the SCN neurons in a reduced preparation. The center picture shows part of a reduced preparation isolated from ventrolateral region of the SCN, with selected cells circled to represent the ROI for averaging fluorescence signals. The surrounding plots of F340/F380 ratio ($a$–$h$) indicate the change in $[\text{Ca}^{2+}]_i$. In all circled cells, 0 K⁺ increased $[\text{Ca}^{2+}]_i$, and then lowered $[\text{Ca}^{2+}]_i$ below basal levels on return to normal K⁺, reminiscent of its effects on membrane potential and spontaneous firing in the SCN neurons (Wang and Huang, 2006; Wang et al., 2012b). The result is consistent with previous finding that basal $[\text{Ca}^{2+}]_i$ in the rat SCN neurons is dependent on action potential-mediated Ca²⁺ influx (Colwell, 2000; Irwin and Allen, 2009). Interestingly, 0 Na⁺ decreased $[\text{Ca}^{2+}]_i$, and return to normal Na⁺ produced rebound increase in $[\text{Ca}^{2+}]_i$. Note the gradual (f) and more marked (h) delayed increase in $[\text{Ca}^{2+}]_i$ during the application of Na⁺-free solution (marked by arrowheads).

The results of 0 Na⁺-induced $[\text{Ca}^{2+}]_i$ decrease and rebound $[\text{Ca}^{2+}]_i$ increase on return to
normal Na\(^+\) are opposite to what would be expected for 0 Na\(^+\)-promoted reverse NCX to raise [Ca\(^{2+}\)]\(_i\) and Na\(^+\)-promoted forward NCX to lower [Ca\(^{2+}\)]\(_i\), respectively. This suggests that the effects of 0 Na\(^+\) on [Ca\(^{2+}\)]\(_i\) must be mediated by acting on targets more than the NCX. The important contribution of membrane potential- and action potential-mediated Ca\(^{2+}\) influx to basal [Ca\(^{2+}\)]\(_i\) prompted us to use cell-attached recordings to determine the effect of 0 Na\(^+\) on spontaneous firing. Figure 2A shows the results obtained from two representative SCN neurons. For both cells, the firing was completely inhibited upon the removal of Na\(^+\) (top panels). Nevertheless, the firing responses to more prolonged absence of Na\(^+\) (equal molar substitution of Li\(^+\) for Na\(^+\)) differed between the two cells. The cell on the left panel remained silent, and the cell on the right resumed firing action potentials with decreasing amplitude and increasing frequency. Note that Li\(^+\) can permeate Na\(^+\) channels to generate action potentials. For a total of 33 cells, 42% (14/33) exhibited the latter type of firing response to 4 min application of Na\(^+\)-free solution. The reason for the two different responses is not clear at this moment. Bottom panels plot the time courses of change in the spontaneous firing rate. For comparison, Ca\(^{2+}\) signals from two cells shown in Fig. 1 (a, h) are re-plotted in Fig. 2B. The resemblance of 0 Na\(^+\)-induced alterations of spontaneous firing rate (Fig. 2A, bottom panels) and [Ca\(^{2+}\)]\(_i\) (Fig. 2B) suggests that the effect of 0 Na\(^+\) on [Ca\(^{2+}\)]\(_i\) was mediated by altering action potential-mediated Ca\(^{2+}\) influx.

To test this idea, we compared the effect of 0 Na\(^+\) on [Ca\(^{2+}\)]\(_i\) in control and in the presence of 0.3 µM TTX to block the generation of action potentials. Figure 2C superimposes the [Ca\(^{2+}\)]\(_i\) responses thus obtained from a representative experiment (n = 10 cells, grey traces; averaged trace in black). The result indicates that TTX, which lowered basal [Ca\(^{2+}\)]\(_i\), blocked most of 0 Na\(^+\)-induced [Ca\(^{2+}\)]\(_i\) decrease and eliminated delayed increase in [Ca\(^{2+}\)]\(_i\) (marked by arrowheads), leaving a small but sustained lowering effect on [Ca\(^{2+}\)]\(_i\) (marked by arrow). For a total of 5 experiments (n = 134 cells), 0 Na\(^+\)-induced [Ca\(^{2+}\)]\(_i\)
decrease was 0.013 ± 0.001 in control and reduced to 0.003 ± 0.000 in TTX \((P < 0.001\), paired \(t\)-test). The result suggests that 0 Na\(^+\) lowered \([Ca^{2+}]_i\) by blocking both TTX-sensitive (action potential-mediated) and TTX-resistant Ca\(^{2+}\) influx. Furthermore, the rebound \([Ca^{2+}]_i\) increase on return to normal Na\(^+\) was virtually abolished by TTX, suggesting an origin of action potential-mediated Ca\(^{2+}\) influx. As the nimodipine-sensitive L-type Ca\(^{2+}\) channels have been shown to play a role in maintaining basal \([Ca^{2+}]_i\) (Irwin and Allen 2009), we suspected that these channels may mediate the TTX-resistant \([Ca^{2+}]_i\) component. Indeed, the 0 Na\(^+\)-induced \([Ca^{2+}]_i\) decrease was virtually eliminated in the combined presence of 0.3 µM TTX and 20 µM nimodipine \((n = 8\) cells, grey traces and averaged trace in black; Fig. 2D).

Together the results indicate that in control conditions, 0 Na\(^+\) inhibited TTX-sensitive as well as nimodipine-sensitive Ca\(^{2+}\) influx, but failed to promote significant Ca\(^{2+}\) uptake via reverse NCX.

Na\(^+\)-free solution induced Ca\(^{2+}\) uptake via reverse NCX in monensin

Two factors may be responsible for the inability of 0 Na\(^+\) to induce Ca\(^{2+}\) uptake via reverse NCX in control conditions. First, internal Ca\(^{2+}\) is required for allosteric activation of NCX (DiPolo 1979; Hilgemann et al. 1992a) and as such the lowering of \([Ca^{2+}]_i\) by 0 Na\(^+\) may prevent the activation of reverse NCX. Second, the lowering of \([Na^+]_i\) during the application of Na\(^+\)-free solution may also inhibit the reverse mode of NCX. Since increasing internal Na\(^+\) has been shown to reduce or eliminate the need for Ca\(^{2+}\) allosteric activation of NCX in intact cells (Urbanczyk et al. 2006), we investigated the effect of 0 Na\(^+\) on \([Ca^{2+}]_i\) with the addition of the Na\(^+\) ionophore monensin to increase \([Na^+]_i\).

For the experiment, we compared the effects of 0 Na\(^+\) in control and in elevated \([Na^+]_i\), with 1 µM monensin. Figure 3A shows the increase in \([Na^+]_i\) in response to 1 µM monensin \((n = 10\) cells, grey data points and smoothed averaged trace in black). For comparison, K\(^+\)-
free solution was also applied for 2 min to transiently block the Na/K pump to increase [Na\(^+\)]\(_i\) (see also Wang et al. 2012b). Figure 3B shows a typical experiment to demonstrate the ability of 0 Na\(^+\) to uptake Ca\(^{2+}\) in elevated [Na\(^+\)]\(_i\), with monensin (\(n = 9\) cells, grey traces; averaged trace in black). As expected, 0 Na\(^+\) in TTX slightly lowered basal [Ca\(^{2+}\)]\(_i\). In contrast, in the presence of 1 µM monensin to raise [Na\(^+\)]\(_i\), 0 Na\(^+\) rapidly increased [Ca\(^{2+}\)]\(_i\). The 0 Na\(^+\)-induced increase of [Ca\(^{2+}\)]\(_i\) inactivated during the 4-min period of Na\(^+\) removal, likely mediated by Na\(^+\)-dependent inactivation of reverse NCX (Hilgemann et al. 1992b).

Pharmacological treatments commonly used for blocking reverse NCX-mediated Ca\(^{2+}\) uptake includes the removal of external Ca\(^{2+}\) (0 Ca\(^{2+}\)) and the addition of the isothiourea derivative KB-R7943 at low micromolar concentrations which preferentially blocks the reverse mode of Ca\(^{2+}\) uptake catalyzed by NCX1 (Iwamoto et al. 1996; Watano et al. 1996). The divalent cation Ni\(^{2+}\) has also been shown to block Ca\(^{2+}\) uptake via NCX1 and NCX2 with an IC\(_{50}\) of ~50 µM (Iwamoto and Shigekawa 1998). For the experiments, the peak magnitude of 0 Na\(^+\)-induced [Ca\(^{2+}\)]\(_i\) rise in monensin was determined before and after the treatment of 0 Ca\(^{2+}\), 10 µM KB-R7943, or 50 µM Ni\(^{2+}\). Figure 3C summarizes the results. On average, the peak amplitude of 0 Na\(^+\)-induced [Ca\(^{2+}\)]\(_i\) rise was reduced from 100 ± 0.0% (\(n = 305\) cells from 20 experiments) to –6.7 ± 3.5% (\(n = 95\) cells from 5 experiments; \(P < 0.001\), ANOVA), 54.9 ± 2.3% (\(n = 147\) cells from 9 experiments; \(P < 0.001\), ANOVA), and 57.7 ± 2.1% (\(n = 63\) cells from 6 experiments; \(P < 0.001\), ANOVA), respectively, in 0 Ca\(^{2+}\), 10 µM KB-R7943, and 50 µM Ni\(^{2+}\).

The ability of 0 Na\(^+\) to promote Ca\(^{2+}\) uptake via reverse NCX in monensin allows us to use the peak amplitude as a measure of NCX activity. To determine if there is a day-night variation in NCX activity, we compared the peak amplitude of 0 Na\(^+\)-induced Ca\(^{2+}\) uptake in the presence of 1 µM monensin (~10 min into the application) recorded between the day (ZT 4–11) and the night (ZT 13–18). Our results indicate a larger value of 0 Na\(^+\)-induced Ca\(^{2+}\)
uptake during the day than at night \((F = 55.19, P < 0.001, \text{two-way ANOVA})\), irrespective of neurons from the dorsal or ventral SCN (Fig. 3D). In contrast, the amplitude of 0 Na\(^+\)-induced Ca\(^{2+}\) uptake was similar between the two regions \((F = 3.45, P = 0.06, \text{two-way ANOVA})\) irrespective of day or night. Together, the results suggest a diurnal rhythm in NCX activity in either dorsal or ventral SCN neurons, but no regional variation in NCX activity.

**Regulation of Ca\(^{2+}\) clearance by forward NCX**

To determine the role of forward NCX in the regulation of [Ca\(^{2+}\)], in the SCN neurons, we investigated the effect of Na\(^+\)-free solution (Li\(^+\) or NMDG\(^+\) substituted) on Ca\(^{2+}\) clearance after depolarization-induced increase in [Ca\(^{2+}\)]. For the experiment high K\(^+\) (50 mM) solution was applied for 10 s to elicit Ca\(^{2+}\) transients. Figure 4A superimposes the [Ca\(^{2+}\)], responses thus obtained from a representative experiment \((n = 10 \text{ cells, grey traces; averaged trace in black})\). The result indicated a rapid increase in [Ca\(^{2+}\)], in response to high K\(^+\) solution. The clearance of [Ca\(^{2+}\)], on return to control solution, which completed within several minutes, appeared to proceed with both a fast and slow time course. Figure 4B replots the averaged Ca\(^{2+}\) response to determine the decay phases. The theoretic black curve fitted to the average Ca\(^{2+}\) response was calculated with two exponential decay phases: \(A1*\exp(-t/\tau1) + A2*\exp(-t/\tau2)\), where \(A1 + A2 = 100\%\). For this particular experiment, the fast phase had total signal amplitude of 85% (A1) and a time constant of 9 s (\(\tau1\)), and the slow phase had amplitude of 15% (A2) and a time constant of 1.6 min (\(\tau2\)). The grey curve assuming only the fast decay phase (A1 = 100% and \(\tau1 = 9\) s) cannot account for the slow clearance of Ca\(^{2+}\).

Figure 5A shows the average Ca\(^{2+}\) responses \((n = 20 \text{ cells})\) to demonstrate the slowing effect of Na\(^+\)-free solution on Ca\(^{2+}\) clearance after high K\(^+\)-induced Ca\(^{2+}\) transients. For the experiment, high K\(^+\) solution was applied for 10 s to elicit Ca\(^{2+}\) transients followed
by return to control solution (140 mM Na\(^+\), trace a) and to Na\(^+\)-free solution (140 mM Li\(^+\), trace c); 140 mM NMDG\(^+\), trace e). As Na\(^+\)-free solution (Li\(^+\) or NMDG\(^+\)) also altered basal [Ca\(^{2+}\)]\(_i\), the Ca\(^{2+}\) responses to 140 mM Li\(^+\) (trace b) or 140 mM NMDG\(^+\) (trace d) alone were obtained for digital subtraction. To compare Ca\(^{2+}\) clearance in Na\(^+\) and Na\(^+\)-free solution, the Ca\(^{2+}\) responses to Li\(^+\) (trace b) or NMDG\(^+\) (trace d) alone were then subtracted from Ca\(^{2+}\) transients that return to Li\(^+\) (trace c) or NMDG\(^+\) (trace e), respectively. Figure 5B superimposes the high K\(^+\)-induced Ca\(^{2+}\) transients that return to 140 mM Na\(^+\) (a), 140 mM Li\(^+\) (c – b), and to 140 mM NMDG\(^+\) (e – d) (left panel). The result indicates a similar slowing of the fast decay by both Na\(^+\)-free solutions. Right panel superimposes the normalized Ca\(^{2+}\) responses to reveal the time when the decay in Li\(^+\) (marked by grey arrow) and in NMDG\(^+\) (marked by black arrow) began to deviate from that in Na\(^+\). It appears that Na\(^+\)-free solution-induced slowing of the fast decay phase occurred earlier in NMDG\(^+\) than in Li\(^+\). The reason for the earlier action of NMDG\(^+\) is not clear at this moment.

To quantify the slowing effect of Na\(^+\)-free solution on Ca\(^{2+}\) clearance, the decay time courses of Ca\(^{2+}\) transients were further expanded in Fig. 5C. The theoretic curves fitted to the data points were calculated with two exponential decay phases as described above. The fast decay time constants used to calculate the curves were 7.2 s, 24 s, and 25.2 s in 140 mM Na\(^+\), 140 mM Li\(^+\), and 140 mM NMDG\(^+\), respectively, with the slow decay time constants set to be 1.5 min. The result indicates a threefold increase in the fast decay time constant by Na\(^+\)-free solutions. It should be noted that Na\(^+\)-free solution may appear to alter the slow decay phase in some experiments. Nevertheless, as the slow Ca\(^{2+}\) clearance is prone to subtraction artifact due to its small amplitude, no further attempt will be made to pursue this matter.

Figure 5D summarizes the effects of Na\(^+\)-free solution on the fast decay time constant determined from each of the 20 cells in this particular experiment. On average, the fast decay time constants increased from 7.2 ± 0.1 s (n = 20), to 23.8 ± 0.5 s (n = 20; P < 0.001,
ANOVA), and to 25.0 ± 0.5 s (n = 20; P < 0.001, ANOVA) in Na\(^+\), Li\(^+\), and NMDG\(^+\), respectively.

To summarize the data from different experiments, only average Ca\(^{2+}\) signal from each experiment was fitted with theoretic curves to obtain the value of fast decay time constant. For a total of 26 experiments (n = 271 cells), the fast decay phase in control solution (140 mM Na\(^+\)) had total signal amplitude of 86 ± 1% and a time constant of 7.3 ± 0.3 s. Two-way ANOVA reveals no difference in the fast decay time constant between cells from dorsal and ventral SCN (F = 0.57, P = 0.46) or between experiments performed at day and at night (F = 0.12, P = 0.73), with the values of fast decay time constant being 7.2 ± 0.6 s (n = 6 experiments, 94 cells; dorsal SCN at day), 7.4 ± 1.0 s (n = 5 experiments, 55 cells; dorsal SCN at night), 7.5 ± 0.4 s (n = 10 experiments, 71 cells; ventral SCN at day), and 6.8 ± 0.4 s (n = 5 experiments, 51 cells; ventral SCN at night). Among them 23 experiments were performed to determine the effects of Na\(^+\)-free solution on the fast decay time constant, the values being 7.6 ± 0.3 s (n = 23 experiments for a total of 261 cells), 24.7 ± 1.9 s (n = 15 experiments, 202 cells; P < 0.001, ANOVA), and 25.6 ± 2.8 s (n = 12 experiments, 110 cells; P < 0.001, ANOVA), in Na\(^+\), Li\(^+\), and NMDG\(^+\), respectively.

In contrast to the marked effects of Na\(^+\)-free solution on slowing the fast Ca\(^{2+}\) decay phase, the blockade of plasmalemmal Ca\(^{2+}\) pumps (PMCA) with 250 or 300 µM La\(^{3+}\) (Shimizu et al. 1997) did not slow the rate of Ca\(^{2+}\) clearance (Fig. 6). Figure 6A shows the average Ca\(^{2+}\) signals (n = 20 cells) from an experiment designed to determine the effects of La\(^{3+}\) on Ca\(^{2+}\) clearance (top panel). Note that La\(^{3+}\) lowered basal [Ca\(^{2+}\)]\(_i\), which returned slowly to baseline for ~10 min. Bottom left panel superimposes the high K\(^+\)-induced Ca\(^{2+}\) transients that return to control (a) and to 250 µM La\(^{3+}\) solution (b), as well as the Ca\(^{2+}\) response to La\(^{3+}\) alone (c) for subtraction purpose. Bottom right panel superimposes the normalized Ca\(^{2+}\) responses that return to control (a) and to 250 µM La\(^{3+}\) solution after
subtraction \((b - c)\). On average, the fast decay time constant was \(7.4 \pm 0.3\) s \((n = 5\) experiments, 123 cells) in control and \(7.7 \pm 1.1\) s in \(\text{La}^{3+}\) \((n = 5\) experiments, 123 cells; \(P > 0.05\), paired \(t\)-test). Furthermore, while the fast \(\text{Ca}^{2+}\) decay phase was little altered by \(\text{La}^{3+}\) inhibition of PMCA, it was again slowed by the removal of external \(\text{Na}^+\) (\(\text{Li}^+\) substituted) to block forward NCX in another experiment \((n = 20\) cells) (Fig. 6B). Taken together, the results indicate that in the SCN neurons NCX, as opposed to PMCA, plays an important role in the clearance of \(\text{Ca}^{2+}\) after depolarization-induced \(\text{Ca}^{2+}\) influx.

To investigate the role of mitochondria, the protonophore carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) was used to reduce driving force for mitochondrial \(\text{Ca}^{2+}\) uptake. Figure 7A shows such a representative experiment (averaged response from 16 cells). The result indicates that FCCP \((0.1\) µM) increased basal \([\text{Ca}^{2+}]_i\) (top panel), and increased the peak amplitude as well as slowed the decay of high \(\text{K}^+\)-induced \(\text{Ca}^{2+}\) transients (bottom panels). This concentration was chosen because FCCP at higher concentrations of 0.3 and 1 µM have marked often biphasic, excitatory followed by inhibitory, effects on spontaneous firing. On average, 0.1 µM FCCP increased basal \([\text{Ca}^{2+}]_i\) signals by \(0.056 \pm 0.004\) \((n = 133\) cells from 6 experiments) and the peak amplitude by \(10 \pm 3\%\) \((n = 133\) cells from 6 experiments; \(P < 0.05\), paired \(t\)-test). Note that 140 mM NMDG\(^+\) induced \(\text{Ca}^{2+}\) uptake in FCCP (trace \(b\)) but not in control (traces \(a\) and \(f\)), an effect most likely mediated by reverse NCX due to FCCP-induced elevated \([\text{Na}^+]_i\) (unpublished observation, Y-C Wang and R-C Huang; see Tretter et al. 1998). Superimposition of \(\text{Ca}^{2+}\) transients indicates that FCCP prolonged both fast (marked by arrowhead) and slow decay phase in 140 mM \(\text{Na}^+\) (bottom left panel), but mostly the slow decay phase in \(\text{Na}^+\)-free (NMDG\(^+\) substituted) solution (bottom right panel). Together the FCCP results suggest a role of mitochondrial \(\text{Ca}^{2+}\) uptake in regulating basal \([\text{Ca}^{2+}]_i\) and, in particular, the slow decay after depolarization-induced \(\text{Ca}^{2+}\) influx.
To investigate the role of endoplasmic reticulum, the experiment was done with thapsigargin to block the sarco/endoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA) in a way similar to that shown in Fig. 7A. Superimposition of Ca\textsuperscript{2+} transients (average response from 9 cells) indicates that thapsigargin (1 µM) reversibly reduced the peak amplitude but had no effect on Ca\textsuperscript{2+} clearance kinetics (Fig. 7B), with the latter effect suggesting that SERCA may not play an important role in shaping the Ca\textsuperscript{2+} transient. Thapsigargin also reversibly lowered basal [Ca\textsuperscript{2+}]\textsubscript{i} to various degrees. On average, 1 µM thapsigargin decreased basal [Ca\textsuperscript{2+}]\textsubscript{i} signals by 0.006 ± 0.003 (n = 83 cells from 6 experiments) and reduced peak amplitude by 19 ± 2% (n = 83 cells from 6 experiments; P < 0.001, paired t-test). As the blockade of SERCA by thapsigargin is irreversible, the reversible inhibition by thapsigargin in basal [Ca\textsuperscript{2+}]\textsubscript{i} and peak Ca\textsuperscript{2+} transients is most likely due to its block of voltage-dependent Ca\textsuperscript{2+} channels (Shmigol et al. 1995).

To determine the role of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release, the experiment was done with a cocktail of blockers for ryanodine receptor (10 µM dantrolene), IP\textsubscript{3} receptor (100 µM aminoethoxydiphenyl borate (2-APB)), and SERCA (1 µM thapsigargin). Superimposition of Ca\textsuperscript{2+} transients (average response from 12 cells) indicates that the blocker cocktail markedly reduced the peak amplitude (Fig. 7C). The blocker cocktail also lowered basal [Ca\textsuperscript{2+}]\textsubscript{i}. On average, the blocker cocktail decreased basal [Ca\textsuperscript{2+}]\textsubscript{i} signals by 0.07 ± 0.01 (n = 40 cells from 4 experiments) and reduced peak amplitude by 64 ± 2% (n = 40 cells from 4 experiments; P < 0.001, paired t-test). The marked inhibition of peak Ca\textsuperscript{2+} transients was a result of combined inhibition by thapsigargin, dantrolene, and 2-APB, as individually 10 µM dantrolene and 100 µM 2-APB (inset, left panel) reduced peak transients by 45 ± 2% (n = 91 cells from 4 experiments; P < 0.001, paired t-test) and 42 ± 2% (n = 99 cells from 4 experiments; P < 0.001, paired t-test), respectively. On the other hand, the cocktail effect on basal [Ca\textsuperscript{2+}]\textsubscript{i} was mostly due to dantrolene, which decreased basal [Ca\textsuperscript{2+}]\textsubscript{i} signals by 0.06 ±
0.01 \( (n = 91 \text{ cells from 4 experiments}) \), whereas 2-APB had biphasic (both increasing and
decreasing) effect and slightly increased basal \([\text{Ca}^{2+}]_i\) signals by \(0.004 \pm 0.004 \) \( (n = 99 \text{ cells}
\text{from 4 experiments}) \). Neither drug appreciably altered the \(\text{Ca}^{2+}\) clearance kinetics,
suggesting not an important role of \(\text{Ca}^{2+}\)-inuced \(\text{Ca}^{2+}\) release in shaping the \(\text{Ca}^{2+}\)
transient.

Note the good superimposition of the slow decay phase of \(\text{Ca}^{2+}\) transients in spite of marked
difference in their peak amplitude \((\text{left panel})\). Furthermore, \(\text{Na}^+\)-free solution had little
effect on the remaining \(\text{Ca}^{2+}\) transient in the presence of the blocker cocktail \((\text{right panel})\), as
seen by the excellent superimposition (at least for the first 30 s) with the \(\text{Ca}^{2+}\) transient that
return to 140 mM \(\text{Na}^+\) (thin dark-grey curve, re-plotted from \(\text{left panel}\)). The lower-than-
baseline \(\text{Ca}^{2+}\) level during the slow decay phase (marked by arrowhead) was an artifact due
to subtraction.

Expression and distribution of NCX isoforms in the SCN

We used RT-PCR to determine the expression of NCX isoforms in the SCN \((\text{Fig. 8A})\).
Positive control reactions were performed using cDNA of rat brain NCX1, NCX2, and
NCX3 to determine the primer efficiency and anneal temperature. These primers were then
used to examine the gene transcription of NCX1-3 isoforms in the SCN. The positive signals
with NCX1 and NCX2 primers, as compared with the RT- \((\text{with omission of reverse}
\text{transcriptase})\) control, indicate the presence of mRNA for NCX1 and NCX2 in the SCN.

Immunohistochemistry with NCX isoform-specific antibodies was also used to study
the distribution pattern of NCX1 and NCX2 isoforms \((\text{Fig. 8B})\). The result shows the
presence of NCX1 immunoreactivity throughout the rostrocaudal axis of the SCN \((\text{top}
\text{panels, } a-c)\). In the medial SCN the NCX1 immunoreactivity is present in the whole SCN,
albeit with gradually decreasing staining intensity in the direction from ventral to dorsal area
\((\text{Fig. 8Bb})\). The more intense NCX1 immunoreactivity in the ventrolateral SCN is associated
with more intense neuropil staining in this area, as seen on high magnification (not shown).

In contrast to the wide distribution of NCX1 immunoreactivity, the NCX2 immunoreactivity is restricted to the ventrolateral region of the SCN (bottom panels, e–g) and is best seen in the middle section of the nucleus (Fig. 8Bf). This region is dominated by vasoactive intestinal peptide (VIP)- and gastrin releasing peptide (GRP)-containing neurons, which receive photic inputs (Moore et al. 2002). Notably, NCX2 immunoreactivity was weak or absent in the area adjacent to the optic chiasm, where the VIP-containing neurons are situated (Moore et al. 2002). Negative controls were without primary antibody against NCX1 (Fig. 8Bd) or NCX2 (Fig. 8Bh).

To investigate variation in NCX1 and NCX2 immunoreactivity, relative optical density was determined from the ventrolateral and dorsomedial region of the SCN obtained from animals sacrificed during the day (ZT 8) and at night (ZT 14). The statistics for NCX1 and NCX2 immunostaining intensity are shown, respectively, in Fig. 8D and 8E. Comparison of relative optical density between ZT 8 and ZT 14 indicates a lack of day-night variation in immunostaining intensity for either NCX1 (Fig. 8D) or NCX2 (Fig. 8E). Nevertheless, the immunostaining intensity for either isoform was higher in the ventrolateral than the dorsomedial SCN, irrespective of day or night.
DISCUSSION

*NCX1 is the major NCX isoform involved in regulating somatic [Ca\(^{2+}\)], in the SCN*

This study demonstrates the expression of mRNAs for NCX1 and NCX2 in the SCN. Furthermore, NCX1 is distributed throughout the whole SCN along the rostrocaudal axis, but NCX2 is restricted to the ventrolateral SCN, a region corresponding to the VIP- and GRP-positive area that receives major sensory inputs (Moore et al. 2002). The different distribution pattern of NCX1 and NCX2 suggests distinct roles played by the two isoforms. Importantly, we find that NCX regulate the fast recovery of Ca\(^{2+}\) transients in response to high K\(^{+}\)-induced Ca\(^{2+}\) influx. Together our results indicate an important role of NCX, most likely NCX1, in the regulation of somatic Ca\(^{2+}\) homeostasis in the SCN neurons.

Comparison of immunoreactivity for NCX1 and NCX2 between subregions of the nucleus indicates stronger intensity for either isoforms in the ventrolateral than dorsomedial SCN. A stronger NCX2 immunoreactivity is obvious due to its preferential distribution to the ventrolateral SCN, whereas the more intense NCX1 immunoreactivity is likely due to more intense neuropil staining. Nonetheless, no day-night difference was observed in the immunoreactivity for either isoform, at least, between ZT 8 and ZT 14.

*Reverse NCX can be demonstrated in elevated [Na\(^{+}\)], by monensin*

Na\(^{+}\)-free solution was used to study Ca\(^{2+}\) uptake via reverse NCX in the SCN neurons. Without a prior increase in [Na\(^{+}\)], however, 0 Na\(^{+}\) decreases [Ca\(^{2+}\)], and then produces rebound increase in [Ca\(^{2+}\)], on return to normal Na\(^{+}\). Zero Na\(^{+}\)-induced [Ca\(^{2+}\)] decrease is mediated by blocking TTX-sensitive as well as nimodipine-sensitive Ca\(^{2+}\) influx. The result accords with previous findings of both action potential-mediated Ca\(^{2+}\) influx and
nimodipine-sensitive basal Ca$^{2+}$ influx in the rat SCN neurons (Colwell 2000; Irwin and Allen 2007, 2009). As the CaV1.3 L-type Ca$^{2+}$ channel is activated at low voltages (Xu and Lipscombe 2001) and present in the SCN neurons (Huang et al. 2012), the 0 Na$^+$-induced inhibition of TTX-resistant, nimodipine-sensitive Ca$^{2+}$ influx is likely mediated by deactivation of these channels as a result of membrane hyperpolarization. This explanation is reasonable, because the replacing ion Li$^+$ has K$^+$-like action on the Na/K pump (Glitsch 2001) and at a concentration of 140 mM may enhance the Na/K pump to hyperpolarize the membrane potential of the SCN neurons.

The inability of 0 Na$^+$ in control conditions to activate reverse NCX to uptake Ca$^{2+}$ has also been observed in other intact cells such as Chinese hamster ovary (CHO) cells (Reeves and Condrescu 2008; Urbanczyk et al. 2006) and myocytes (Ginsburg et al. 2013). This has been attributed to the low concentrations of intracellular Ca$^{2+}$ and Na$^+$ in CHO cells (Reeves and Condrescu 2008; Urbanczyk et al. 2006), and is likely to be the case for SCN neurons as well owing to the lowering effect of 0 Na$^+$ on [Na$^+$]$_i$ and [Ca$^{2+}$]$_i$.

In the presence of monensin to raise [Na$^+$]$_i$, 0 Na$^+$ produces rapid Ca$^{2+}$ uptake, which is eliminated in 0 Ca$^{2+}$ and partially blocked by 10 µM KB-R7943 or 50 µM Ni$^{2+}$, suggesting the activation of reverse NCX. The ability of monensin to allow for 0 Na$^+$-induced activation of reverse NCX is likely mediated by the elevated [Na$^+$]$_i$, which has been shown to activate NCX by reducing or eliminating the need for Ca$^{2+}$ allosteric activation in intact cells (Reeves and Condrescu 2008; Urbanczyk et al. 2006).

Comparison of 0 Na$^+$-induced Ca$^{2+}$ uptake in monensin between day and night indicates a diurnal rhythm in Ca$^{2+}$ uptake for both ventral and dorsal SCN neurons, suggesting higher daytime NCX activity in the somatic plasma membrane in both regions. In contrast, the peak amplitude of 0 Na$^+$-induced Ca$^{2+}$ uptake in monensin is similar between dorsal and ventral SCN neurons, suggesting similar NCX activity in cells of the two regions.
Forward NCX plays an important role in the regulation of Ca\(^{2+}\) clearance

To determine the role of forward NCX in the regulation of Ca\(^{2+}\) clearance, high K\(^{+}\) solution was used to evoke depolarization-induce Ca\(^{2+}\) entry. Our results show that following depolarization-induced Ca\(^{2+}\) influx the clearance of Ca\(^{2+}\) is best described by two exponential decay phases. The fast decay phase constitutes ~85% of total signal amplitude and has a time constant of ~7 s, and the remainder slower decay phase has a time constant of ~2 min. No difference in the fast decay time constant is found between cells from dorsal and ventral SCN or between experiments performed at day and at night, suggesting a similar rate of Ca\(^{2+}\) extrusion. In other words, the higher daytime 0 Na\(^{+}\)-induced Ca\(^{2+}\) uptake in monensin may be a result of daytime increase in the amount, but not the turnover rate, of NCX transporters.

We show that the fast decay rate is decreased threefold by the blockade of forward NCX with the removal of extracellular Na\(^{+}\). The result indicates an important role of NCX in mediating rapid clearance of Ca\(^{2+}\). In contrast, the blockade of PMCA with La\(^{3+}\) has little effect on Ca\(^{2+}\) clearance. Together the result is consistent with the distinct properties of NCX and PMCA in handling Ca\(^{2+}\), with NCX being a low-affinity but high-capacity transporter able to handle large amount of Ca\(^{2+}\) and PMCA a high-affinity but low-capacity transporter better suited for extruding Ca\(^{2+}\) at low levels (Blaustein and Lederer 1999).

On the other hand, the slow decay phase is mostly not affected by the blockade of PMCA or forward NCX. Although it is altered in some experiments, its small total signal amplitude (~15%) is prone to subtraction artifact and thus preventing us from making meaningful inference. Instead, reducing mitochondrial Ca\(^{2+}\) uptake with FCCP prolongs mostly the slow decay of Ca\(^{2+}\) transients, in particular, in the absence of external Na\(^{+}\) to block forward NCX. The apparent slowing of the fast decay by FCCP in 140 mM Na\(^{+}\) could...
be a result of reduced forward NCX activity due to FCCP-induced elevated $[\text{Na}^+]_i$.

Alternatively, FCCP may increase the relative amplitude of the slow decay phase without altering fast decay kinetics, suggesting that FCCP may preferentially increase the $\text{Ca}^{2+}$ component that contributes to the slow decay. The exact mechanism for this regulation is not certain at this moment. Further experiments are needed to better elucidate the mechanism.

In contrast to a role of mitochondrial $\text{Ca}^{2+}$ uptake, endoplasmic reticulum $\text{Ca}^{2+}$ uptake does not appear to play an important role in clearing $\text{Ca}^{2+}$ following high $\text{K}^+$-induced $\text{Ca}^{2+}$ transients. The reversible effect of thapsigargin on both basal $[\text{Ca}^{2+}]_i$ and the peak $\text{Ca}^{2+}$ transient can be accounted for by its inhibition of voltage-dependent $\text{Ca}^{2+}$ channels (Shmigol et al. 1995). Similarly, $\text{Ca}^{2+}$-induced $\text{Ca}^{2+}$ release also appears not to play an important role in shaping the $\text{Ca}^{2+}$ transient. The marked inhibition (by ~65%) of peak $\text{Ca}^{2+}$ transient by the cocktail of blockers for ryanodine receptor, IP$_3$ receptor, and SERCA is most likely a combined effect of dantrolene and 2-APB, each reducing peak $\text{Ca}^{2+}$ transients by ~45%, which have been shown to block L-type $\text{Ca}^{2+}$ channels (Bannister et al. 2008). Interestingly, the relatively unaltered slow decay phase of remaining $\text{Ca}^{2+}$ transients in the blocker cocktail, which presumably blocks the L-type $\text{Ca}^{2+}$ channels, suggests an origin of $\text{Ca}^{2+}$ influx mostly from non-L-type $\text{Ca}^{2+}$ channels. This is to say that $\text{Ca}^{2+}$ influx via L-type $\text{Ca}^{2+}$ channels may be responsible for the fast decay phase and is extruded by NCX, an idea consistent with the insignificant effect of $\text{Na}^+$-free solution on the remaining $\text{Ca}^{2+}$ transient in the blocker cocktail (Fig. 7C). Together with the role of mitochondria in the regulation of basal $\text{Ca}^{2+}$ and slow decay of depolarization-induced $\text{Ca}^{2+}$ transients, our results suggest that different sources of voltage-dependent $\text{Ca}^{2+}$ influx may be differentially regulated by NCX and mitochondria.

While NCX plays an important role in rapid clearance of $\text{Ca}^{2+}$ following depolarization-induced $\text{Ca}^{2+}$ loads, its involvement in the regulation of basal $[\text{Ca}^{2+}]_i$,
although very likely (see later), is difficult to establish in this study. On the one hand, replacement of Na\(^+\) with Li\(^+\) has complex actions on spontaneous firing to alter basal \([\text{Ca}^{2+}]_i\), and thus render impossible to distinguish its effect on forward NCX from that on membrane potential and spontaneous firing. On the other hand, replacement of Na\(^+\) with NMDG\(^+\) lowers basal \([\text{Ca}^{2+}]_i\), by blocking the generation of action potentials and also hyperpolarizing the membrane potential to \(\sim -80\) mV (Wang et al. 2012b), which would completely close both the high- and low-threshold voltage-dependent \(\text{Ca}^{2+}\) channels (Huang 1993) to prevent \(\text{Ca}^{2+}\) entry via these channels. Future study with cells clamped at resting potentials may help better resolve this issue.

**Functional implications**

The ability of NCX to rapidly clear depolarization-induced \(\text{Ca}^{2+}\) loads suggests a critical role of forward NCX in the regulation of \(\text{Ca}^{2+}\) homeostasis, in particular, in relation to glutamate-induced \(\text{Ca}^{2+}\) signaling in the SCN neurons. By studying \(\text{Ca}^{2+}\) response to retinohypothalamic tract synaptic transmission, Irwin and Allen (2007) conclude that the increase in somatic \([\text{Ca}^{2+}]_i\), due to activation of glutamate receptors requires the generation of action potentials and activation of voltage-dependent \(\text{Ca}^{2+}\) channels. As somatic \([\text{Ca}^{2+}]_i\) summates in response to a train of action potentials (Irwin and Allen 2007), the rate of \(\text{Ca}^{2+}\) clearance may dictate the kinetics of \(\text{Ca}^{2+}\) accumulation and thus the \([\text{Ca}^{2+}]_i\) levels. The rate of \(\text{Ca}^{2+}\) clearance following voltage step-induced \(\text{Ca}^{2+}\) transients has been determined to have a single decay time constant of \(\sim 8\) s in the soma (Irwin and Allen 2007), a value close to the fast decay time constant determined in this study, suggesting the involvement of NCX in clearing somatic \(\text{Ca}^{2+}\) during interspike periods. Together with the observation that action potentials contribute to basal \([\text{Ca}^{2+}]_i\) in rat SCN neurons (Colwell 2000; Irwin and Allen 2007, 2009; this study), it follows that NCX most likely also participates in the regulation of
basal [Ca$^{2+}$].

On the other hand, Irwin and Allen (2007) also show that in the dendrites depolarization-induced Ca$^{2+}$ loads are larger and decay with a time constant of ~0.8 s, 10 times faster than in the soma. A similar 10 times difference in Ca$^{2+}$ decay time constants (6 versus 0.6 s) has also been observed in hippocampal pyramidal neurons and attributed to the different surface-to-volume ratios between the soma and dendrites (Jaffe et al. 1994). This is to say that the same Ca$^{2+}$ extrusion mechanism, i.e. the NCX, maybe at work in the soma and dendrites in the SCN neurons. The intense NCX1 immunoreactivity in the neuropil is consistent with this suggestion. Incidentally, in CA1 pyramidal cells the density of NCX1 is significantly higher in the dendritic shafts than in the soma (Lőrincz et al. 2007).

In the rat supraoptic nucleus neurons, somatodendritic release of vasopressin (AVP) and oxytocin is closely associated with intracellular Ca$^{2+}$ concentrations (Dayanithi et al. 2012). Importantly, NCX plays a role in the regulation of somatodendritic Ca$^{2+}$ and AVP release (Komori et al. 2010). Along the same line of thinking, the ability of NCX to regulate somatic, and likely also dendritic, Ca$^{2+}$ in the SCN neurons also suggests a possible involvement in the release of neuropeptides such as AVP, VIP, and GRP. Indeed, high K$^+$-induced, Ca$^{2+}$-dependent somatodendritic release of dense-core vesicles has been previously demonstrated in the rat SCN (Castel et al. 1996). Furthermore, experiments with reverse-microdialysis perfusion also show high K$^+$-induced, Ca$^{2+}$-dependent release of VIP, GRP, and AVP from the hamster SCN (Francl et al. 2010a, 2010b). How and to what extents the NCX may participate in the regulation of neuropeptide release will await further investigations.
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Figure Legends

Figure 1. Effects of K\(^+\) and Na\(^+\)-free solution on \([\text{Ca}^{2+}]_i\) in a reduced SCN preparation.

Center, fluorescence micrograph of a reduced SCN preparation loaded with the Ca\(^{2+}\)-sensitive fluorescent indicator Fura2-acetoxymethyl ester (Fura2-AM). Regions of interest (ROI) are indicated with circles. The image was taken in the resting condition. Scale bar, 20 \(\mu\)m. a–h, the time course of change in the F340/F380 fluorescence ratio recorded from 8 selected ROIs as indicated in center picture. Arrowheads (f and h) indicate the delayed increase in \([\text{Ca}^{2+}]_i\) in Na\(^+\)-free (0 Na\(^+\); Li\(^+\) substituted) solution. Similar results were obtained from four other experiments (\(n = 54\) cells). Daytime recordings (ZT 8).

Figure 2. Zero Na\(^+\) in control conditions lowers \([\text{Ca}^{2+}]_i\) in the SCN neurons

A, two representative cells showing the effects of 0 Na\(^+\) (equal molar substitution of Li\(^+\) for Na\(^+\)) on spontaneous firing (top panels). Bottom panels plot the time course of change in spontaneous firing rate (SFR). B, two representative cells showing the effects of 0 Na\(^+\) on \([\text{Ca}^{2+}]_i\) (from Fig. 1, a & h). Note the similar effects of 0 Na\(^+\) on SFR (A) and on \([\text{Ca}^{2+}]_i\). C, 0 Na\(^+\) effect on \([\text{Ca}^{2+}]_i\) in control and in the presence of TTX. Note that TTX markedly reduced the magnitude of 0 Na\(^+\)-induced \([\text{Ca}^{2+}]_i\) decrease. D, 0 Na\(^+\) effect on \([\text{Ca}^{2+}]_i\) in control and in the combined presence of 0.3 \(\mu\)M TTX and 20 \(\mu\)M nimodipine, the values of 0 Na\(^+\)-induced \([\text{Ca}^{2+}]_i\) decrease being, respectively, 0.012 ± 0.001 (\(n = 50\) cells from 4 experiments) and 0.002 ± 0.000 (\(n = 50\) cells from 4 experiments; \(P < 0.001\), paired \(t\)-test). Daytime recordings (ZT 5–9).
Figure 3. Zero Na\textsuperscript{+} in monensin induces rapid Ca\textsuperscript{2+} uptake via reverse NCX

A, effects of 0 K\textsuperscript{+} and 1 µM monensin on [Na\textsuperscript{+}]\textsubscript{i}. Similar results were obtained from three other experiments (n = 40 cells). B, effects of 0 Na\textsuperscript{+} on [Ca\textsuperscript{2+}]\textsubscript{i} in control and in the presence of 1 µM monensin. Note the rapid uptake of Ca\textsuperscript{2+} by 0 Na\textsuperscript{+} in the presence, but not absence, of monensin. C, statistics to show the relative peak amplitude of 0 Na\textsuperscript{+}-induced Ca\textsuperscript{2+} uptake in control (1 µM monensin), 0 Ca\textsuperscript{2+}, 10 µM KB-R7943, and in 50 µM Ni\textsuperscript{2+}. D, statistics showing the average peak amplitude of 0 Na\textsuperscript{+}-induced Ca\textsuperscript{2+} uptake, the values being 0.085 ± 0.003 (n = 168 cells from six experiments), 0.064 ± 0.001 (n = 325 cells from eight experiments), 0.082 ± 0.006 (n = 178 cells from nine experiments), and 0.055 ± 0.003 (n = 251 cells from seven experiments), respectively, for dorsal SCN (dSCN) neurons at day (ZT 4–11), dSCN at night (ZT 13–18), ventral SCN (vSCN) neurons at day, and vSCN at night. *** P < 0.001.

Figure 4. High K\textsuperscript{+}-induced Ca\textsuperscript{2+} transient has both fast and slow decay components

A, effect of 50 mM K\textsuperscript{+} on [Ca\textsuperscript{2+}]\textsubscript{i} from a representative experiment, with individual responses in grey and averaged response in black. B, Ca\textsuperscript{2+} clearance is better explained with two exponential decay phases as indicated by the theoretic black curve through the average data points (from A). The grey curve was calculated with only the fast exponential decay phase. Daytime recordings (ZT 7).

Figure 5. Zero Na\textsuperscript{+} slows the fast decay phase of high K\textsuperscript{+}-induced Ca\textsuperscript{2+} transients

A, averaged Ca\textsuperscript{2+} responses (n = 20 cells) to 50 mM K\textsuperscript{+} for 10 s followed by return to control solution (140 mM Na\textsuperscript{+}) (a), to 140 mM Li\textsuperscript{+} for 4 min (b), to 50 mM K\textsuperscript{+} followed by return to Li\textsuperscript{+} (c), to 140 mM NMDG\textsuperscript{+} for 4 min (d), and to 50 mM K\textsuperscript{+} followed by return to NMDG\textsuperscript{+} (e). B, comparison of the Ca\textsuperscript{2+} responses that return to Na\textsuperscript{+} (a), Li\textsuperscript{+} (c – b), and to NMDG\textsuperscript{+}
(e – d) indicates a slowing effect of Na\(^+\)-free solutions on the fast decay phase (left panel). Right panel, normalization of Ca\(^{2+}\) responses in Na\(^+\) and Na\(^+\)-free solution indicates the time when Li\(^+\) (marked by grey arrow) and NMDG\(^+\) (marked by black arrow) began to slow the Ca\(^{2+}\) decay. C, the time courses of Ca\(^{2+}\) decay expanded for curve fitting to obtain decay time constants. The smooth curves through the data points were calculated with a fast time constant of 7.2 s, 24 s, and 25.2 s for Na\(^+\), Li\(^+\), and NMDG\(^+\), respectively. D, statistics showing a threefold increase in the fast decay time constants by Na\(^+\)-free solutions (n = 20 cells). *** P < 0.001. Daytime recordings (ZT 7).

Figure 6. La\(^{3+}\) does not slow the decay phase of high K\(^+\)-induced Ca\(^{2+}\) transients

A, averaged Ca\(^{2+}\) responses (n = 20 cells) to 50 mM K\(^+\) for 10 s followed by return to control solution (a), to 50 mM K\(^+\) for 10 s followed by return to 250 µM La\(^{3+}\) for 4 min (b), and to 250 µM La\(^{3+}\) for 4 min (c) for subtraction purpose (top panel). Note that La\(^{3+}\) alone lowered basal [Ca\(^{2+}\)], with slow recovery (c). Bottom left panel, superimposition of Ca\(^{2+}\) responses to high K\(^+\) in control (a) and in 250 µM La\(^{3+}\) (b), and to La\(^{3+}\) alone (c) (left panel). Bottom right panel, normalization of Ca\(^{2+}\) responses in control (black trace, a) and in La\(^{3+}\) after subtraction (grey trace, b – c), indicating a minimal effect of La\(^{3+}\) on Ca\(^{2+}\) decay. B, normalization of average Ca\(^{2+}\) responses (n = 20 cells) that return to control (140 mM Na\(^+\)), 300 µM La\(^{3+}\) (grey trace), and to 300 µM La\(^{3+}\)/140 Li\(^+\). Note that the averaged Ca\(^{2+}\) transients marked by La\(^{3+}\)/140 Na\(^+\) and La\(^{3+}\)/140 Li\(^+\) have been digitally subtracted. Similar results were also obtained from two other experiments (n = 40 cells). Daytime recordings (ZT 6).
Figure 7. Effects of FCCP, thapsigargin, dantrolene, and 2-APB on high K$^+$-induced Ca$^{2+}$ transients

A, effect of FCCP (0.1 µM) on averaged Ca$^{2+}$ responses ($n = 16$ cells) from a representative experiment (top panel). Open horizontal bars represent the duration of Na$^+$-free (NMDG$^+$ substituted) solution ($b$ and $f$ being for subtraction purpose). Bottom left panel, superimposition of Ca$^{2+}$ responses to high K$^+$ in control ($e$) and in FCCP ($d$), showing the prolongation of both fast (marked by arrowhead) and slow decay phase. Bottom right panel, superimposition of Ca$^{2+}$ responses that return to NMDG$^+$ in control ($g$–$f$) and in FCCP ($c$–$b$), showing the prolongation of mostly the slow decay phase. B, effect of thapsigargin (1 µM) on averaged Ca$^{2+}$ responses ($n = 9$ cells) that return to Na$^+$ (left panel, grey trace) and to NMDG$^+$ (right panel, grey trace). Note the Ca$^{2+}$ responses that return to NMDG$^+$ (right panel) have been digitally subtracted. C, effect of a blocker cocktail (1 µM thapsigargin, 10 µM dantrolene, and 100 µM 2-APB) on averaged Ca$^{2+}$ responses ($n = 12$ cells) that return to Na$^+$ (left panel, grey trace) and to NMDG$^+$ (right panel, grey trace). Note the Ca$^{2+}$ responses that return to NMDG$^+$ (right panel) have been digitally subtracted. Inset (left panel): averaged Ca$^{2+}$ responses to 100 µM 2-APB (grey trace) ($n = 20$ cells) and to 10 µM dantrolene (grey trace) ($n = 11$ cells). Daytime recordings (ZT 5–9).

Figure 8. Expression and distribution of NCX isoforms in the SCN. A, RT-PCR analysis of mRNAs for NCX isoforms. Positive controls were performed using cDNA from rat brain. The expected PCR product sizes for NCX1, NCX2, and NCX3 were 494, 529, and 517 bp, respectively. Negative controls were performed using RT products with omission of reverse transcriptase (RT-) to examine the contamination of genomic DNA. The positive signals with NCX1 and NCX2 primers, as compared with the RT- control, indicate the presence of mRNA for NCX1 and NCX2 in the SCN. B, Distribution of immunoreactivity to the NCX1
and NCX2. The NCX1 immunoreactivity is distributed throughout the rostrocaudal axis of
the SCN (a–c). In contrast, the NCX2 immunoreactivity is restricted to the ventrolateral
region of the SCN and is best seen in the middle section of the nucleus (f). Rostral (a, e),
middle (b, f), and caudal (c, g) sections of the SCN (encircled by the dotted lines). Negative
controls were without primary antibodies (d, h). Scale bars: 200 µm. OC: optic chiasm. 3V:
third ventricle. C, statistics showing the average relative optic density for NCX1, the values
being 1.56 ± 0.07 (n = 6), 1.57 ± 0.06 (n = 6), 2.15 ± 0.10 (n = 6), and 2.17 ± 0.08 (n = 6),
respectively, for dorsomedial SCN (dmSCN) at day (ZT 8), dmSCN at night (ZT 14),
ventrolateral SCN (vlSCN) at day, and vlSCN at night. D, statistics showing the average
relative optic density for NCX2, the values being 1.23 ± 0.04 (n = 4), 1.18 ± 0.05 (n = 4),
2.50 ± 0.07 (n = 4), and 2.35 ± 0.09 (n = 4), respectively, for dmSCN at day, dmSCN at
night, vlSCN at day, and vlSCN at night. *** P < 0.001.
Figure 1. Effects of K⁺ and Na⁺-free solution on [Ca²⁺]ᵢ in a reduced SCN preparation.
Figure 2. Zero Na$^+$ in control conditions lowers [Ca$^{2+}$]$_i$ in the SCN neurons
Figure 3. Zero Na\(^+\) in monensin induces rapid Ca\(^{2+}\) uptake via reverse NCX
Figure 4. High K⁺-induced Ca²⁺ transient has both fast and slow decay components
Figure 5. Zero Na\(^+\) slows the fast decay phase of high K\(^+\)-induced Ca\(^{2+}\) transients

A

B

C

D

<table>
<thead>
<tr>
<th>Time constant (s)</th>
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<tbody>
<tr>
<td>Na(^+)</td>
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<td>Li(^+)</td>
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<tr>
<td>NMDG(^+)</td>
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Figure 6. La$^{3+}$ does not slow the decay phase of high K$^+$-induced Ca$^{2+}$ transients
Figure 7. Effects of FCCP, thapsigargin, dantrolene, and 2-APB on high K⁺-induced Ca²⁺ transients
Figure 8. Expression and distribution of NCX isoforms in the SCN.

A

DNA (bp)

NCX1

Brain RT -

SCN RT -

Brain RT -

NCX2

Brain RT -

SCN RT -

Brain RT -

NCX3

Brain RT -

SCN RT -

B

Rostral

3V

Middle

OC

Caudal

C

NCX1 Immunoreactivity

Relative optical density

Day Night

dmSCN vISCN

D

NCX2 Immunoreactivity

Relative optical density

Day Night

dmSCN vISCN