Intracellular Chloride Regulation in AVP+ and VIP+ Neurons of the Suprachiasmatic Nucleus

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Several reports have described excitatory GABA transmission in the suprachiasmatic nucleus (SCN), the master pacemaker of circadian physiology. However, there is disagreement regarding the prevalence, timing, and neuronal location of excitatory GABA transmission in the SCN. Whether GABA is inhibitory or excitatory depends, in part, on the intracellular concentration of chloride (\([\text{Cl}^-]_i\)). Here, using ratiometric Cl− imaging, we have investigated intracellular chloride regulation in AVP and VIP-expressing SCN neurons and found evidence suggesting that \([\text{Cl}^-]_i\) is higher during the day than during the night in both AVP+ and VIP+ neurons. We then investigated the contribution of the cation chloride cotransporters to setting \([\text{Cl}^-]_i\) in these SCN neurons and found that the chloride uptake transporter NKCC1 contributes to \([\text{Cl}^-]_i\) regulation in SCN neurons, but that the KCCs are the primary regulators of \([\text{Cl}^-]_i\) in SCN neurons. Interestingly, we observed that \([\text{Cl}^-]_i\) is differentially regulated between AVP+ and VIP+ neurons—a low concentration of the loop diuretic bumetanide had differential effects on AVP+ and VIP+ neurons, while blocking the KCCs with VU0240551 had a larger effect on VIP+ neurons compared to AVP+ neurons.

The suprachiasmatic nucleus (SCN) of the anterior hypothalamus is the master pacemaker of the circadian system. Besides a cohort of neuropeptides, SCN neurons synthesize and package the neurotransmitter GABA. GABA transmission regulates synaptic input from the RHT1, mediates phase shifts2, 3, regulates firing frequency4, and contributes to circadian synchrony5–9.

GABA is the primary inhibitory neurotransmitter in the central nervous system, but has been observed to be excitatory during embryonic and neonatal development, in certain pathologies, as well as in several areas of the adult brain10–12. Interestingly, excitatory GABA transmission has been observed in the mature SCN6, 7, 13–20. However, reports have disagreed on the prevalence, timing, and neuronal location of excitatory GABA transmission. GABA has been reported to be exclusively inhibitory, inhibitory during the day and excitatory during the night, and excitatory during the night and inhibitory during the day7, 13–15, 17, 20–22. Additionally, the proportion of neurons within the SCN neural network that are excited by GABA may be involved in encoding day length18, 23.

The GABAA receptor is permeable to both \(\text{Cl}^-\) and \(\text{HCO}_3^-\) ions with a relative permeability ratio of approximately 0.824–26. Because the GABAA receptor is primarily permeable to \(\text{Cl}^-\) ions, whether GABA is depolarizing or hyperpolarizing depends on the intracellular concentration of chloride (\([\text{Cl}^-]_i\)) and the membrane potential. \([\text{Cl}^-]_i\) is regulated by a family of cation chloride cotransporters (CCCs) which use the concentration gradients of \(\text{Na}^+\) and \(\text{K}^+\) ions to transport \(\text{Cl}^-\) ions into (the sodium-potassium-chloride cotransporter 1, NKCC1) or out of (the potassium-chloride cotransporters, KCC) neurons. Normally, \([\text{Cl}^-]_i\) is kept low in neurons by the action of the neuron-specific27, 28 isotonically-active29–31 KCC2. However, a role for NKCC1 in \([\text{Cl}^-]_i\) regulation has been demonstrated in the SCN—blocking NKCC1 with bumetanide decreased the amplitude of GABA-induced \(\text{Ca}^{2+}\) transients15, 16, 18 and hyperpolarized the GABAergic reversal potential15, 17.

Interestingly, immunohistochemistry has revealed differential expression of chloride transporters throughout the SCN32. KCC2 expression was most dense in the ventrolateral SCN, and correlated with vasoactive intestinal peptide (VIP) expression. Alternatively, KCC3 and KCC4 expression was concentrated in the dorsomedial SCN. NKCC1 was expressed throughout the SCN, but was concentrated in the dorsomedial SCN, and correlated with...
vasopressin (AVP) expression. The differential expression of the CCCs throughout the SCN suggests that [Cl\textsuperscript{−}]
and the GABAergic equilibrium potential (E\textsubscript{GABA}) may vary regionally throughout the SCN. Indeed, Albus et al. observed that GABA is exclusively inhibitory in the ventral SCN, but is excitatory in the dorsal SCN during the late day and early night. Similarly, GABA-induced Ca\textsuperscript{2+} transients were found to be most prevalent in the dorsomedial SCN, and using the Cl\textsuperscript{−}-sensitive dye MQAE, [Cl\textsuperscript{−}] was found to be elevated in the dorsomedial SCN. Therefore, there is mounting evidence to support the idea that subpopulations of SCN neurons differ in their regulation of the intracellular chloride concentration.

In this work, we used epifluorescent imaging of a genetically-encoded chloride indicator to examine regional and circadian regulation of [Cl\textsuperscript{−}] in the SCN. Cl-Sensor is a ratiometric chloride indicator composed of a CFP moiety linked to a YFP moiety whose emission is sensitive to chloride in a physiological range (YFP\textsubscript{Cl}). Ratiometric Cl\textsuperscript{−} imaging allows estimation of [Cl\textsuperscript{−}] from multiple cells simultaneously without disrupting the native cellular milieu. Using a transgenic strategy, we targeted Cl-Sensor to AVP or VIP-expressing SCN neurons, and were able to detect both transient and persistent changes in [Cl\textsuperscript{−}].

Our results indicate that GABA\textsubscript{A} receptor activation elicits Cl\textsuperscript{−} influx in AVP\textsuperscript{+} and VIP\textsuperscript{+} neurons in the SCN. Accordingly, we show that the KCCs play a major role in [Cl\textsuperscript{−}] regulation in SCN neurons, while the chloride importer NKCC1 has a relatively minor role in setting resting [Cl\textsuperscript{−}]. Further, we show that [Cl\textsuperscript{−}] is differentially regulated in AVP and VIP-expressing neurons.

**Results**

Vasoactive intestinal peptide (VIP) and vasopressin (AVP) mark the “core” and “shell” partition that has served as a useful anatomical model for dissecting SCN function. Generally, sensory inputs project to the ventrolateral SCN core, while core neurons synapse unto neurons in the dorsomedial shell. Several reports have suggested that excitatory GABA transmission may correlate with this anatomical feature. Therefore, we performed Cl\textsuperscript{−} imaging in VIP\textsuperscript{+} and AVP\textsuperscript{+} neurons of the SCN to look for differences in [Cl\textsuperscript{−}], regulation between these two populations of neurons. To measure [Cl\textsuperscript{−}] in SCN neurons, we used a newly-developed Cre-inducible mouse line, with a floxed Cl-Sensor allele inserted into the Rosa26 locus. To obtain Cl-Sensor expression in the SCN, we crossed these mice with either VIP-IRESCre mice or AVP-IRESCre mice to give Cl-Sensor expression in either VIP\textsuperscript{+} or AVP\textsuperscript{+} neurons. The resultant VIP::Cl-Sensor mice displayed Cl-Sensor expression in the ventrolateral SCN, while the AVP::Cl-Sensor mice displayed Cl-Sensor expression in the dorsomedial SCN, as expected for VIP and AVP expression (Fig. 1). Several studies have indicated that excitatory GABA transmission demonstrates circadian rhythmicity, and we first compared baseline values of R\textsubscript{Cl} during the day (ZT 2 to 8) and night (ZT 12 to 18) in AVP\textsuperscript{+} and VIP\textsuperscript{+} SCN neurons. Interestingly, R\textsubscript{Cl} was higher during the day in both AVP\textsuperscript{+} (generalized estimating equations (GEE), \(p < 0.05\)) and VIP\textsuperscript{+} (GEE, \(p < 0.001\)) neurons (Fig. 1E) suggesting that [Cl\textsuperscript{−}] is higher during the day in these neurons. These results indicate that [Cl\textsuperscript{−}] is higher in SCN neurons, while the chloride transporter NKCC1 has a relatively minor role in setting resting [Cl\textsuperscript{−}]. The timecourse of these Cl\textsuperscript{−} transients reported in other cells. The timecourse of these Cl\textsuperscript{−} transients may represent the activity of chloride transporters. Indeed, it has been shown that transient shifts in GABA polarity can last for minutes and that chloride transporters mediate this equilibration process. Therefore, we next examined the degree to which the CCCs set [Cl\textsuperscript{−}] in SCN neurons. Since our GABA\textsubscript{A}-induced Cl\textsuperscript{−} transients indicated inhibitory Cl\textsuperscript{−} influx upon GABA\textsubscript{A} receptor activation, we investigated the contribution of KCC2, the neuron-specific CCC responsible for keeping [Cl\textsuperscript{−}] levels low in neurons throughout the brain. To test for the activity of KCC2 in setting resting [Cl\textsuperscript{−}], we used VU0240551, an antagonist that selectively targets the KCCs. VU increased R\textsubscript{Cl} by 0.18 in AVP\textsuperscript{+} and by 0.27 in VIP\textsuperscript{+} neurons (Fig. 3B). Based on our calibration curve, we estimate these changes in R\textsubscript{Cl} to reflect a 15 mM increase in [Cl\textsuperscript{−}] in AVP\textsuperscript{+} neurons and a 29 mM increase in VIP\textsuperscript{+} neurons. VU had a significantly greater effect in VIP\textsuperscript{+} neurons compared to AVP\textsuperscript{+} neurons (GEE, \(p < 0.05\)), but there were no day/night differences within neuron type (Fig. 3C). Because several Cl\textsuperscript{−} transporters are also transporters for the bicarbonate ion, we wondered if removing bicarbonate ions from the extracellular solution might alter R\textsubscript{Cl} and therefore the efficacy of VU. Therefore, we repeated VU application in a separate set of experiments using a HEPES-buffered solution (Fig. 3D). Blocking the KCCs with VU gave a pattern of results similar to that observed with solution containing bicarbonate. VU elicited an increase in R\textsubscript{Cl} in all conditions, indicative of [Cl\textsuperscript{−}] increase. When comparing the effect of VU across solutions, we observed a difference in the amplitude of VU’s effect in AVP\textsuperscript{+} neurons during the day (two-sample z-test, \(p < 0.05\)). Furthermore, experiments in HEPES solution revealed a day/night difference in the effect of VU that was not present in the bicarbonate solution (Fig. 3D, GEE, \(p < 0.05\)). In a separate set of experiments, we examined the effect of VU on the GABAAergic reversal potential (E\textsubscript{GABA}) using perforated-patch recording. In these experiments, VU elicited a depolarization of E\textsubscript{GABA} by approximately 23 mV (paired t-test, \(p < 0.001\)), and slowed the recovery of resting E\textsubscript{GABA} after Cl\textsuperscript{−} loading (paired t-test, \(p < 0.05\)).
Collectively, we interpret these findings to indicate that the KCC family of chloride cotransporters play a major role in $[\text{Cl}^-]$ regulation in SCN neurons. Previous studies have implicated NKCC1 in $[\text{Cl}^-]$ regulation in SCN neurons\cite{15-18,32}. To test for a contribution of NKCC1 to resting $[\text{Cl}^-]$, we used the loop diuretic bumetanide which selectively targets NKCC1 when used at $10 \mu\text{M}$\cite{45}. Bumetanide increased $R_{\text{Cl}}$ in AVP+ neurons by approximately $0.04$ ($\sim4 \text{mM}$) and decreased $R_{\text{Cl}}$ in VIP+ neurons by $0.04$ ($\sim3 \text{mM}$) (Fig. 4). These changes were small but significantly different from baseline (GEE, $p < 0.005$). As with VU, we observed differences in the effect of bumetanide between AVP+ and VIP+

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**Figure 1.** $R_{\text{Cl}}$ measurement in genetically-identified SCN neurons. Confocal micrographs demonstrating regional expression of Cl-Sensor in the SCN of AVP::Cl-Sensor (A) and VIP::Cl-Sensor (B) mice. Native Cl-Sensor fluorescence is depicted in green, and DAPI stain is shown in blue. Pseudocolored epifluorescent micrographs of an AVP::Cl-Sensor SCN slice exhibiting YFP (C) and CFP (D) emission. (E) Baseline $R_{\text{Cl}}$ was higher during the day (ZT 2 to 8) compared to the night (ZT 12 to 18) for both AVP+ (GEE, $p < 0.05$) and VIP+ (GEE, $p < 0.001$) neurons.
neurons (GEE, \( p < 0.001 \)), but no day/night differences within neuron type. Surprisingly, bumetanide elicited a small increase in \([\text{Cl}^-]\) in AVP\(^+\) neurons, contrary to its expected role in blocking \([\text{Cl}^-]\) uptake. This result may be due to off-target effects of bumetanide, which is known to inhibit the KCCs at higher concentrations\(^{10, 44} \). In a separate series of experiments, we tested the efficacy of bumetanide in a HEPES-buffered solution, which should diminish \([\text{Cl}^-]/\text{HCO}_3^-\) exchange. In HEPES, bumetanide reduced \( R_{\text{Cl}} \) in VIP\(^+\) neurons but had little effect on AVP\(^+\) neurons (Fig. 4D). However, the effect of bumetanide on AVP\(^+\) neurons was significantly greater in the bicarbonate-buffered solution compared to the HEPES-buffered solution (two-sample \( z \)-test, \( p < 0.05 \)). As in the bicarbonate-buffered experiments, bumetanide elicited a greater effect in VIP\(^+\) neurons compared to AVP\(^+\) neurons (GEE, \( p < 0.05 \)). Using perforated-patch recording, we also investigated the effect of bumetanide on \( E_{\text{GABA}} \). In these experiments, bumetanide did not significantly alter \( E_{\text{GABA}} \) or the timecourse for the recovery of \( E_{\text{GABA}} \) following a \([\text{Cl}^-]\) depletion protocol (Supplementary Fig. 3). Overall, we observed relatively small effects of bumetanide compared to VU, suggesting that the KCCs are the major regulators of resting \([\text{Cl}^-]\) in SCN neurons.

The previous results indicate that both NKCC1 and the KCC family of cotransporters contribute to resting \([\text{Cl}^-]\) in SCN neurons. We next investigated how these cotransporters interact to regulate \([\text{Cl}^-]\). As discussed, blocking the KCCs with VU resulted in a substantial increase in \( R_{\text{Cl}} \) (Fig. 3). However, because of the relatively minor effect of bumetanide, it was not clear what \([\text{Cl}^-]\) uptake pathways were mediating the effect of VU. In order to determine if NKCC1 mediates \([\text{Cl}^-]\) uptake in the absence of the KCCs, we applied VU after bumetanide treatment (Fig. 5A). The effect of VU was largely occluded in the presence of bumetanide (Fig. 5A,B). Therefore, although NKCC1 has a relatively minor role in setting resting \([\text{Cl}^-]\), it does constitute a tonic \([\text{Cl}^-]\) influx pathway in SCN neurons. Conversely, we next examined whether blocking the KCCs could reveal a greater bumetanide effect. However, the amplitude of bumetanide's effect was similar in the presence or absence of VU (Fig. 5C,D), indicating that the KCCs are necessary for \([\text{Cl}^-]\) extrusion.

**Discussion**

We performed somatic \([\text{Cl}^-]\) imaging to investigate \([\text{Cl}^-]\) in two genetically-defined subpopulations of SCN neurons. We found that \( R_{\text{Cl}} \) was higher in both AVP\(^+\) and VIP\(^+\) neurons during the day (ZT 2 to 8) compared to the night (ZT 12 to 18), suggesting that \([\text{Cl}^-]\) is elevated during the day. This observation is in agreement with several reports that have observed increased excitatory GABA transmission during the day and early night\(^{6, 13, 15–17} \). However, we observed \([\text{Cl}^-]\) influx in response to GABA\(_A\) receptor activation, indicative of an inhibitory effect of GABA. Similarly, we observed large changes in \( R_{\text{Cl}} \) after application of the KCC antagonist VU, but small changes after blocking NKCC1 with bumetanide. VU increased \([\text{Cl}^-]\) dramatically, in the range of 15 to 30 mM, suggesting that the KCCs are the major determinants of \([\text{Cl}^-]\) in AVP\(^+\) and VIP\(^+\) SCN neurons. Therefore, our results add to a group of studies that have concluded that GABA is exclusively inhibitory in the mature SCN\(^{5, 46–49} \).
it should be remembered that the SCN is a very heterogeneous nuclei and that AVP and VIP-expressing neurons only constitute ~13% and ~9% of all SCN neurons respectively50, 51, leaving open the possibility that our study did not address the SCN neurons demonstrating excitatory GABA.

The descriptions of excitatory GABA transmission in the SCN have been riddled with discrepancies. Differences in methodology are likely to underlie some of these inconsistencies. Indeed, whole-cell, perforated-patch, cell-attached, and multi-unit recording techniques as well as Ca²⁺ imaging have all been used to address the polarity of GABA transmission in the SCN. The timing of inhibitory post-synaptic currents within the interspike interval is critical in determining whether inhibitory currents will speed up or slow cell firing, further highlighting the nuance of GABA transmission in the SCN4, 9, 52. The issue may also be related to the complexity of intracellular Cl⁻ regulation itself. Besides neurotransmission, [Cl⁻]i is an important cellular feature linked to processes such as pH regulation, cell volume regulation, and even membrane potential 12, 43, 54. Therefore, cell turgidity as well as the osmolarity and pH of solutions are all likely to influence measures of [Cl⁻]i. Further, [Cl⁻]i has been shown to change after neuronal damage, and in relation to the proximity of cells to the surface of a brain slice55, 56. Additionally, previous studies have not adequately ruled out the possibility that disinhibition underlies the observed excitatory effects of GABA transmission in the SCN. The SCN network is known to have diffuse local connectivity7, 19, 57. Therefore, polysynaptic effects must be considered when applying GABA agonists to SCN neurons. Without the inclusion of TTX in the recording media, GABA-mediated inhibition of pre-synaptic inputs could be read out as excitation in the cell of interest. Further, some of the data in support of excitatory GABA transmission has been inferred by the effects of the GABA_A receptor antagonist bicuculline 14, 15, 58. Regrettably, these results are confounded by the off-target effects of bicuculline, which is known to antagonize SK channels at commonly used concentrations59. Indeed, SK channels have been shown to contribute to the resting membrane potential, afterhyperpolarization and spike-frequency adaptation of SCN neurons60, 61.

We have successfully performed Cl⁻ imaging techniques in SCN neurons, and have demonstrated their utility for monitoring Cl⁻ flux and [Cl⁻]i regulation. Cl⁻ imaging offers several advantages compared to gramicidin perforated patch recording by leaving V_m unperturbed and by allowing for the sampling of multiple neurons.

Figure 3. The KCCs regulate [Cl⁻]i in SCN neurons. (A) Example experiment from an AVP::Cl-Sensor mouse recorded during the night demonstrating the effect of 10µM of the KCC antagonist VU. VU caused an increase in R_Cl indicative of a rise in [Cl⁻]i. Each trace represents a R_Cl measurement obtained from a single neuronal soma. (B) Example experiment from a VIP::Cl-Sensor mouse recorded during the day demonstrating the effect of VU. VU caused an increase in R_Cl indicative of a rise in [Cl⁻]i. (C) Summary data of the average change in R_Cl after VU by neuron type and time of day. VU resulted in an increase in R_Cl in all conditions (GEE, p < 0.005), but had a significantly greater effect in VIP+ neurons compared to AVP+ neurons (GEE, p < 0.05). (D) Summary of changes in R_Cl elicited by VU in a HEPES-buffered solution. VU had a larger effect during the day in VIP+ neurons when compared to VIP+ night (GEE, p < 0.05) and AVP+ day neurons (GEE, p < 0.001). For (C) and (D), the number of slices and total regions of interest (in parentheses) is listed for each condition under the x-axis.
simultaneously. Further, the use of a genetically-encoded indicator allowed us to target specific populations of SCN neurons. Nevertheless, Cl-Sensor has room for improvement. Cl-Sensor’s sensitivity to Cl\textsuperscript{−} is not optimal for normal neuronal concentrations of chloride, and the intrinsic H\textsuperscript{+} sensitivity of the indicator within a physiological pH range can be problematic.

Our methodology did not allow us to investigate subcellular differences in R\textsubscript{Cl}. Indeed, intracellular Cl\textsuperscript{−} gradients have been reported in several types of neurons throughout the brain (see\textsuperscript{62} for review). For example, a two-photon Cl\textsuperscript{−} imaging study observed a somatodendritic chloride gradient in a class of retinal bipolar cells, concluding that Cl\textsuperscript{−} is 20 mM higher in dendrites relative to the soma\textsuperscript{63}. A somatodendritic Cl\textsuperscript{−} gradient could explain why previous studies have shown GABA-evoked Ca\textsuperscript{2+} transients in SCN neurons\textsuperscript{15, 16, 18}, supporting an excitatory role of GABA, while we have observed inhibitory Cl\textsuperscript{−} influx. While dendritic depolarization may be able to activate somatic voltage-gated calcium channels, Cl\textsuperscript{−} efflux at dendrites may not be registered by somatic Cl\textsuperscript{−} imaging. Similarly, a somatodendritic Cl\textsuperscript{−} gradient could explain why bumetanide was able to diminish GABA-evoked Ca\textsuperscript{2+} transients, but produced small changes in our measurements of somatic R\textsubscript{Cl}. Higher-resolution imaging techniques will be necessary to address whether somatodendritic Cl\textsuperscript{−} gradients exist in SCN neurons.

Recently, the role of the CCC’s in determining [Cl\textsuperscript{−}]\textsubscript{i} has come under scrutiny by compelling two-photon Cl\textsuperscript{−} imaging results which argue for the primacy of large intracellular anions in setting [Cl\textsuperscript{−}]\textsubscript{i}\textsuperscript{64}. In their study, Glycks et al. observed little effect of blocking either NKCC1 or KCC2 in hippocampal and neocortical pyramidal neurons, while we found a small effect of bumetanide and a clear effect of VU. Neuron type could underlie this incongruity. Alternatively, while we monitored R\textsubscript{Cl} continuously, Glycks et al. sampled R\textsubscript{Cl} before and 30 minutes after application of bumetanide and VU, leaving open the possibility that secondary homeostatic mechanisms reset [Cl\textsuperscript{−}]\textsubscript{i} during that time.

Although we did not observe a difference in resting R\textsubscript{Cl} between AVP\textsuperscript{+} and VIP\textsuperscript{+} neurons, we did observe differential regulation of [Cl\textsuperscript{−}]\textsubscript{i} between AVP\textsuperscript{+} and VIP\textsuperscript{+} neurons. We found that AVP\textsuperscript{+} and VIP\textsuperscript{+} neurons differed in their sensitivity to both VU and bumetanide. The increased sensitivity of VIP\textsuperscript{+} neurons to VU suggests that they may have lower resting [Cl\textsuperscript{−}]\textsubscript{i}, relative to AVP\textsuperscript{+} neurons, consistent with studies that have observed a greater prevalence of excitatory GABA transmission in the dorsal SCN\textsuperscript{6,15, 16} as well as with recent data from two groups who, using the Cl\textsuperscript{−} sensitive dye MQAE, concluded that [Cl\textsuperscript{−}]\textsubscript{i} is elevated in the dorsal SCN\textsuperscript{9, 23}. Previous
in situ hybridization and immunocytochemical studies have described regional expression of chloride transporters in the rat SCN. Therefore, these regional differences in expression may explain the differential effects of VU and bumetanide in AVP+ and VIP+ neurons. Specifically, KCC2 expression was limited to the ventrolateral SCN, and colocalized with neurons containing GRP or VIP. Markedly, KCC2 expression was absent from the dorsomedial SCN and did not colocalize with AVP—rather, KCC3 and KCC4 were found in the dorsomedial SCN. This histology is in agreement with our observation that VU had a larger effect in VIP+ neurons compared to AVP+ neurons. Despite the paucity of KCC2 in the dorsomedial SCN, we observed that VU increased [Cl−]i in AVP+ neurons, albeit less than it did in VIP+ neurons. The efficacy of VU in the AVP+ neurons may be explained by the non-specificity of VU for KCC2. VU may have acted on KCC3 or KCC4 in AVP+ neurons.

Generally, resting membrane potential in SCN neurons is approximately −45 mV during the night and exhibits oscillations of roughly 10 to 15 mV throughout the day. For a neuron with a Vm of −45 mV, [Cl−]i is passively distributed at approximately 15 mM. Therefore, our estimates of resting [Cl−]i (greater than 20 mM) indicate that there are constitutively-active uptake mechanisms in SCN neurons. In VIP+ neurons, blocking NKCC1 decreased [Cl−]i, supporting a role for NKCC1-mediated Cl− uptake in setting resting [Cl−]i. However, in AVP+ neurons, bumetanide caused a small increase in [Cl−]i, suggesting that other uptake mechanisms are active in AVP+ neurons. Accordingly, Choi et al. observed that GABA-induced Ca2+ transients remained in NKCC1 knockout mice. This Cl− uptake may be mediated by the anion exchangers (AEs), which exchange intracellular bicarbonate for extracellular chloride, or could be mediated by a Cl− channel. Indeed, our results in HEPES-buffered solutions, which minimize the presence of bicarbonate transport, imply the presence of bicarbonate-dependent Cl− regulation in SCN neurons. Although generally similar, the experiments done in HEPES-buffered solution revealed several interesting differences. The effect of bumetanide in AVP+ neurons differed between solutions, and experiments done in HEPES-buffered solution revealed a day/night difference in VU’s effect on VIP+ neurons (Figs 3 and 4). These findings implicate the activity of the Na+-driven Cl-HCO3

Figure 5. Bumetanide occludes the effect of VU. (A) Example experiment from a VIP::Cl-Sensor mouse recorded during the night in which VU (10 µM) was applied after bumetanide (10 µM). From rest, VU elicits a 0.24 increase in RCl on average (B, same data from Fig. 3). However, the effect of VU was occluded in the presence of bumetanide (GEE, p < 0.001), suggesting that NKCC1 mediates the Cl− accumulation elicited by VU. Conversely, the effect of bumetanide in the presence of VU was similar to the effect of bumetanide alone (Fig. C and D), indicating that the KCCs are necessary to mediate Cl− extrusion in these neurons.
exchanger (NDCBE) or the AE family of cotransporters in SCN neurons. Further research will be necessary to address the role of these transporters in SCN physiology.

Overall, our results demonstrate day/night and regional differences in [Cl$$^\text{−}$$], regulation and highlight the KCC family of chloride co-transporters as regulators of [Cl$$^\text{−}$$], in SCN neurons. Therefore, our results add to a growing number of studies that point to the importance of [Cl$$^\text{−}$$] in SCN function.

Methods

Animal strains and housing. Cl$$^\text{−}$$ imaging experiments were performed with C57BL/6 mice in which a floxed Cl-Sensor allele was inserted into the Rosa26 locus33. We crossed these mice with either AVP-IRESCre or VIP-IRESCre mice34, 35 to yield AVP::Cl-Sensor or VIP::Cl-Sensor mice. Tail snips were sent to an external facility for genotyping (Transnetyx, Inc). Mice were heterozygous for both the Cl-Sensor and Cre transgenes. Tissue was prepared from adult male and female mice between two and six months old. Electrophysiological experiments were performed on wild type male Wistar rats aged 3 to 9 months.

All animals were entrained to 12:12 light-dark (LD) cycles, with the time of lights on represented as ZT 0. All procedures were approved in advance by the Institutional Animal Care and Use Committee of Oregon Health and Science University, and all experiments were performed in accordance with the approved animal protocol.

Confocal microscopy of fixed tissue. Each mouse was deeply anaesthetized with isoflurane and tran-scendally perfused with 10 mL of phosphate buffered saline (PBS) followed by 10 mL of 4% paraformaldehyde solution in PBS (pH 7.4). The brain was removed and post-fixed for 1-2 hours at 4 °C in the same solution. After repeated washes in 0.1 M PB, the brain was blocked and secured to a vibratome insert with cyanoacrylate adhesive and agarose supports. Coronal (40 μm thick) sections were cut with a Leica vibratome in 0.1 M PB and subsequently washed in the same buffer. For optical clearing, we treated the tissue with a glycerol/0.1 M PB gradient (25% to 90%). The tissue was incubated in each solution at 4 °C with light agitation until equilibrated. After clearing, sections were transferred into a 10% glycerol/0.1 M PB solution and counterstained with DAPI. Tissue sections were transferred onto glass slides in 10% glycerol solution and the cover glass was mounted with ProLong Diamond media after removing the excess buffer. Images were taken with a Zeiss Axioskop 2 TM fluorescent microscope using AxioVision 4.8 software (Carl Zeiss Microimaging, Inc.). Confocal micrographs consisted of several 0.4 μm thick optical sections adjusted for optimal brightness and contrast using FIJI software.

Acute slice preparation. During their light phase (ZT 1-3 for day experiments and ZT 10-12 for night experiments), animals were removed from their housing chambers, anaesthetized with isoflurane, and decapitated. The brain was quickly removed and submerged in an ice-cold slicing solution consisting of (in mM): 111 NaCl, 26 NaHCO3, 11 dextrose, 6 Na-gluconate, 4 MgCl2, 3 KCl, 1 NaH2PO4, and 0.5 CaCl2, saturated with 95% O2, 5% CO2. The tissue was incubated in each solution at 4 °C with light agitation until equilibrated. After clearing, sections were transferred into a 10% glycerol/0.1 M PB solution and counterstained with DAPI. Tissue sections were transferred onto glass slides in 10% glycerol solution and the cover glass was mounted with ProLong Diamond media after removing the excess buffer. Images were taken with a Zeiss Axioskop 2 TM fluorescent microscope using AxioVision 4.8 software (Carl Zeiss Microimaging, Inc.). Confocal micrographs consisted of several 0.4 μm thick optical sections adjusted for optimal brightness and contrast using FIJI software.

Cl$$^\text{−}$$ imaging from acute SCN slices. During image acquisition, slices were perfused at 1–2 mL/min with an artificial cerebrospinal fluid (aCSF) with Cl$$^\text{−}$$ adjusted to the physiological concentration (122 mM; ref. 68). aCSF contained (in mM): 114 NaCl, 26 NaHCO3, 11 dextrose, 6 Na-gluconate, 2.7 KCl, 2 CaCl2, 1 MgCl2, and 1 NaOH. Solution was saturated with 95% O2, 5% CO2. The brain was blocked and 175 μm thick coronal slices were prepared with a Leica VT1000S vibratome. Slices were incubated in slicing solution for 1-4 hours at 34 °C before recording.

Calibration of Cl-Sensor and estimation of [Cl$$^\text{−}$$]i. In order to relate RCl to [Cl$$^\text{−}$$]i, it was necessary to construct a calibration curve. Cl-Sensor was calibrated using a 0 mM Cl$$^\text{−}$$ solution consisting of (in mM): 120 Na-glucuronate, 26 NaHCO3, 11 dextrose, 2.7 K-glucuronate, 2 Ca-glucuronate, 1 MgSO4, and 1 NaH2PO4, saturated with 95% O2, 5% CO2. The tissue was incubated in each solution at 4 °C with light agitation until equilibrated. After washing, the tissue was incubated in a 10% glycerol/0.1 M PB solution and counterstained with DAPI. Background values were subtracted for each wavelength independently. Because the YFP moiety of Cl-Sensor is quenched by Cl$$^\text{−}$$, with a higher ratio indicating higher [Cl$$^\text{−}$$]i, it was necessary to correct for this instability, we observed that steady-state RCl remained sensitive to exposure duration. We corrected for this exposure-dependent trend in each condition (AVP+ day, AVP+ night, VIP+ day, VIP+ night) independently in order to avoid any assumptions about the similarities of baseline RCl across conditions. All values were adjusted to a 500 nm exposure of 100 ms.
with 95% O₂, 5% CO₂. This solution was mixed with aCSF to produce solutions of 0, 4, 20, 40, 60, and 80, and 123 mM Cl⁻. 50 μM β-scnic was added to all calibration solutions to permeabilize cells. AVP:Cl-Sensor and VIP:Cl-Sensor day and night–entrained mice were used for analysis. Rₐ values were corrected for exposure as discussed above. Average steady-state Rₐ was plotted against Cl⁻ concentration (Supplementary Fig. 1) and calibration data was fit with the following logistic dose–response sigmoidal curve:

\[ R_{Cl} = \frac{R_{max} + R_{min}}{1 + \left(\frac{[Cl^-]}{K_d}\right)^p} \]

(1)

Where \( R_{Cl} \) is the fluorescence intensity ratio (\( F_{436}/F_{500} \)) for chloride, \( K_d \) is the dissociation constant for Cl⁻ binding, \( R_{min} \) and \( R_{max} \) are the minimum and maximum asymptotic values of \( R_{Cl} \), and \( p \) is the Hill coefficient. To obtain estimates of \([Cl^-]_i\), we re-arranged the equation for \([Cl^-]_i\):

\[ [Cl^-]_i = K_d \times \left( \frac{R_{min} - R_{max}}{R_{Cl} - R_{max}} \right)^{\frac{1}{p}} \]

(2)

After fitting the curve, we obtained the following values: \( K_d = 108.8 \text{ mM}, R_{min} = 0.98, R_{max} = 2.92 \) and \( p = 2.91 \) (Supplementary Fig. 1). Our \( K_d \) is considerably higher than previously-reported values. We also observed substantial variability in \( R_{Cl} \) at specific Cl⁻ concentrations between calibration experiments. Furthermore, \( R_{Cl} \) was fairly non-linear in our range of operation (see Supplementary Fig. 1; our R Cl⁻ values were generally between 1.0 and 1.3). For these reasons, we elected to leave fluorescent measurements in values of \( R_{Cl} \) instead of converting them into estimates of \([Cl^-]_i\), in subsequent analysis.

Perforated-patch electrophysiology. Brain slices were prepared as described. Recordings were performed with an Axopatch-1D amplifier (Axon Instruments), filtered at 2 kHz, digitized at 5 kHz, and acquired with Patchmaster v5.3 (HEKA Elektronik). Pipette solution contained (in mM): 120 KCl, 20 K-glucuronate, 15 HEPES, 2 NaCl, 1 EGTA and either 0.2 of Lucifer Yellow or Texas Red. pH was adjusted to 7.26 with KOH. Gramicidin (Sigma) was dissolved in DMSO to a concentration of 50 mg/mL, aliquoted, and frozen. Before an experiment, this stock solution was diluted to a final pipette concentration of 30–100 μg/mL. A drop of gramicidin-free pipette solution was first applied to the backend of the pipette. After capillary action filled the pipette tip, the pipette was back-filled with the gramicidin-containing solution moments before submersion into the recording chamber. After gigaseal formation, series resistance (Rₛ) was monitored with a – 5 mV voltage step to monitor the progress of perforation. Only recordings with a Rₛ less than 100 MΩ were used for experiments. Cells were voltage clamped at –60 mV and cells with holding currents less than –30 pA were discarded.

\( E_{GABA} \) was determined using voltage ramp protocols. Every 10 seconds, a 400 ms voltage ramp protocol (ΔV ≥ 60 mV) was executed 100 ms after puff-application of 1 mM GABA. A current trace recorded in the absence of GABA was subtracted from currents obtained in the presence of GABA. The subtracted current trace was then plotted against the ramp command potential, and \( E_{GABA} \) was recorded as the x-intercept. \( E_{GABA} \) was not corrected for Rₛ or liquid junction potentials.

Drugs. All drugs used in this study were acquired from Tocris Bioscience. Bumetanide and VU0240551 were dissolved in DMSO, stored as 10 mM stock solutions, and applied through the bath at 10 μM. A 100 mM stock of isoguvacine in water was diluted in aCSF to 1 mM and focally applied (5 psi) through a micropipette connected to a Picospritzer (General Valve Corporation).

Statistics and analysis. Igor Pro (Version 6.22 A; Wavemetrics) was used for plotting, curve-fitting and data analysis. Data are presented as the mean ± standard error. For imaging experiments, generalized estimating equations (GEE) were used to determine statistical significance. GEE models are similar to ANOVA and general linear models in that they estimate a mean response, except standard errors are adjusted for clustered or correlated measurements that originate from multiple observations made from the same brain slice. Therefore, each brain slice is treated as an independent measure, while the ROIs influence the average and standard error of each measurement. GEE test statistics are based on chi-square or z-statistics rather than F- and t-distributions. When comparing drug effects between HEPES-buffered and bicarbonate-buffered solutions, we formed z-statistics equal to the difference between the estimated effects from each separate GEE model (one for each solution) divided by the standard error of the difference. Significance level was Bonferroni-adjusted for these comparisons. For electrophysiology experiments, we used the Student’s t-test. For all tests, a p-value less than 0.05 was considered to be statistically significant.

Data availability. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author Contributions
N.J.K. and C.N.A. designed the experiments. N.J.K. performed the experiments and analyzed the data. N.J.K. and C.N.A. wrote and edited the manuscript.

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