In the adult brain, chloride (Cl\textsuperscript{−}) influx through GABA\textsubscript{A} receptors is an important mechanism of synaptic inhibition. However, under a variety of circumstances, including acquired epilepsy, neuropathic pain, after trains of action potentials or trauma, and during normal early brain development, GABA\textsubscript{A} receptor activation excites neurons by gating Cl\textsuperscript{−} efflux because the intracellular Cl\textsuperscript{−} concentration (Cli) is elevated. These findings require an inducible, active mechanism of chloride accumulation. We used gramicidin-perforated patch recordings to characterize Cl\textsuperscript{−} transport via NKCC1, the principal neuronal Cl\textsuperscript{−} accumulator, in neonatal CA1 pyramidal neurons. NKCC1 activity was required to maintain elevated Cli, such that GABA\textsubscript{A} receptor activation was depolarizing. Kinetic analysis of NKCC1 revealed reversible transmembrane Cl\textsuperscript{−} transport characterized by a large maximum velocity (v\textsubscript{max}) and high affinity (K\textsubscript{m}), so that NKCC1 transport was limited only by the net electrochemical driving force for Na\textsuperscript{+}, K\textsuperscript{+}, and Cl\textsuperscript{−}. At the steady-state Cli, NKCC1 was at thermodynamic equilibrium, and there was no evidence of net Cl\textsuperscript{−} transport. Trains of action potentials that have been previously shown to induce persistent changes in neuronal E\textsubscript{CI} (reversal potential for Cl\textsuperscript{−}) did not alter v\textsubscript{max} or K\textsubscript{m} of NKCC1. Rather, action potentials shifted the thermodynamic set point, the steady-state Cli, at which there was no net NKCC1-mediated Cl\textsuperscript{−} transport. The persistent increase in Cli required intact α2/α3 Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity, indicating that trains of action potentials reset the thermodynamic equilibrium for NKCC1 transport by lowering Na\textsuperscript{+}. Activity-induced changes in Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity comprise a novel mechanism for persistent alterations in synaptic signaling mediated by GABA.

Key words: seizure; sodium pump; development; long-term potentiation; dendrite; action potential; gramicidin-perforated patch

Introduction
Mature neurons are typically hyperpolarized and inhibited by the net chloride (Cl\textsuperscript{−}) influx mediated by GABA\textsubscript{A} receptor activation. In contrast, in the developing brain, GABA depolarizes and excites neurons by gating Cl\textsuperscript{−} efflux. Whether Cl\textsuperscript{−} flows into or out of a cell through GABA\textsubscript{A} receptor-operated channels depends on the transmembrane Cl\textsuperscript{−} gradient. Cation chloride cotransporters expressed at different stages of development are thought to regulate intracellular Cl\textsuperscript{−} concentration (Cli), and therefore, the response to GABA (Lu et al., 1999; Marty et al., 2002; Stein et al., 2004; Wang et al., 2005). NKCC1, expressed early in development, transports Na\textsuperscript{+}, K\textsuperscript{+}, and Cl\textsuperscript{−} into the cell in various electroneutral stoichiometries (Geck et al., 1980; Russell, 2000). In the mature brain, KCC2 moves 1 K\textsuperscript{+} and 1 Cl\textsuperscript{−} across the membrane. Normally transport is outward, but transport by KCC2 is reversible and the direction of transport is dictated by the free energy gradients for K\textsuperscript{+} and Cl\textsuperscript{−} (Staley and Proctor, 1999).

In response to a variety of conditions, the polarity of the GABA response can change from hyperpolarizing and inhibitory to depolarizing and excitatory. Examples of such conditions include trauma to postsynaptic (van den Pol et al., 1996) and presynaptic (Coull et al., 2003) neurons, animal models of neuropathic pain (Coull et al., 2003), seizures (Khalilov et al., 2003), and human epilepsy (Cohen et al., 2002). It is clear from developmental studies that reduced expression of NKCC1 (Vardi et al., 2000; Marty et al., 2002; Wang et al., 2002; Ikeda et al., 2003) and increased expression of KCC2 (Clayton et al., 1998; Rivera et al., 1999; Wang et al., 2002) can reverse the polarity of GABA responses.

However, persistent changes in the response to GABA can also occur immediately after trains of action potentials (APs) (Fiumelli et al., 2005). The mechanism by which this rapid and persistent change in the direction of Cl\textsuperscript{−} flux occurs is not well understood. The purpose of the present work was first to understand the regulation of Cl\textsuperscript{−} in the developing brain, and then to determine the mechanism by which Cl\textsuperscript{−} regulation changes after trains of action potentials.

NKCC1-mediated Cl\textsuperscript{−} transport has been demonstrated and characterized in multiple systems, but not in neonatal neurons, where it contributes to normal development and also to seizure activity (Russell, 2000; Dzhala et al., 2005). Here, we show that NKCC1-mediated Cl\textsuperscript{−} transport is the principle mechanism by
which neonatal hippocampal pyramidal neurons accumulate Cl¹ to make GABA polarizing. We describe NKCC1 Cl¹ cotransport with a model analogous to Ohm’s law, in which the combined electrochemical gradients of the transported ions provide the driving force for Cl¹ transport and transporter kinetics represent conductance. This thermodynamic model of NKCC1-mediated Cl¹ transport is supported by evidence that transport is reversible and the direction is sensitive to changes in intracellular Na⁺ and Cl¹. Finally, we show that the α2/α3 isoform of Na⁺-K⁺-ATPase is required for the rapid and persistent increase in Cl¹ that occurs after a train of action potentials. Activity-induced changes in Na⁺ and K⁺ gradients represent a novel form of plasticity in GABAergic signaling.

Materials and Methods

Slice preparation. All animals were housed and cared for according to institutional guidelines. We prepared acute hippocampal slices from male Sprague Dawley rats, age postnatal day 4 (P4) through P6, when NKCC1 expression is high and KCC2 expression is low (Dzhala et al., 2005). The brain was removed and submerged in ice-cold solution containing (in mM) 87 NaCl, 75 sucrose, 2.5 KCl, 25 NaHCO₃, 0.5 CaCl₂, 2 H₂O, 0.7 MgCl₂·6H₂O, 2.25 NaH₂PO₄·H₂O, and 25 glucose. The solution was continuously bubbled with 95% O₂/5% CO₂. The cerebellum was removed with a razor blade, and the brain was cut sagittally into two hemispheres. For each hemisphere, the diencephalon, midbrain, and remaining hindbrain were bluntly dissected away and discarded. Hemispheres were affixed to an agar block (4% agar) attached to the stage of a vibrating knife microtome (Leica Microsystems, Wetzlar, Germany) and submersed in the above ice-cold solution. Horizontal hippocampal slices (400 μm) were cut and placed in a slice chamber at room temperature in a nominally bicarbonate-free incubation solution containing (in mM) 77 NaCl, 73.5 sucrose, 2.5 KCl, 26 HEPES, 1.25 CaCl₂·2 H₂O, 4.5 MgCl₂·6 H₂O, 1.75 NaH₂PO₄·H₂O, and 17.5 glucose. pH was titrated to 7.4 with NaOH. The solution was continuously bubbled with 100% O₂. Slices recovered at room temperature for at least 1 h before experiments.

The GABA A channel conducts anions, mainly Cl¹ and bicarbonate (Bormann et al., 1987). In the present work, we estimate the Clι by measuring the reversal potential of GABA-evoked currents (E GABA). E GABA approximates the reversal potential for Cl¹ (E Cl) when the bicarbonate flux through the GABA A channel is minimized. This is achieved by buffering extracellular solutions with HEPES instead of bicarbonate and CO₂ (Staley et al., 1995; Staley and Proctor, 1999). Therefore, Cl¹ currents were recorded at the soma in response to pressure application of GABA (100 μM in ACSF) to the apical dendrites (~100 μm from the soma (5–15 ms, 5 s; Picospirter II, Parker, Fairfield, NJ) (Staley and Proctor, 1999). The optimal location for the GABA puffer pipette was determined by monitoring the size of GABA-evoked currents in the voltage-clamped neuron while advancing the pipette into the slice.

Dendritic currents can be underestimated if the dendrites are inadequately voltage clamped; this is often improved by using Cs⁺ in the recording electrode. We chose not to use Cs⁺ in these experiments, because we did not want to alter K⁺ gradients or NKCC1 transport. To minimize the error, we integrated the GABA-evoked responses over time to obtain the Cl¹ charge transfer (Staley and Mody, 1992). Space-clamp error should not affect the accuracy of the estimated Clι transport rates because each test current should be affected to the same degree.

Quantification of GABA-evoked currents and calculation of Clι. To determine the steady-state E Cl, GABA-evoked currents were recorded during a series of 1 s voltage steps. Nominally bicarbonate-free ACSF was used in all experiments to minimize bicarbonate flux through the GABA channel so that the E GABA would approximate E Cl. Clι currents were evoked at five to seven different membrane potentials, in 5 mV increments with the middle step approximately where the currents reversed (E Cl). We usually limited the membrane potential (V m) range to within ±15 mV of E Cl to minimize the possibility of altering Clι with large Clι influxes or effluxes. Ten seconds elapsed between each voltage step to prevent receptor desensitization or changes in Clι from confounding our results.

We calculated the charge transfer of GABA A receptor-mediated currents by calculating the integral of the GABA-evoked current waveform after subtracting the baseline current. Charge transfer/voltage plots were obtained by fitting the data to the Goldman–Hodgkin–Katz constant field equation:

\[
I = G \times C_{Li} \times \frac{V_m}{RT} \times \frac{1 - e^{-\frac{E_{Cl} - V_m}{RT}}}{1 - e^{-\frac{E_{Cl}}{RT}}}
\]

where I is current; C Lι is extracellular Cl¹ concentration; G = GABA A channel Cl¹ permeability; F is Faraday’s constant (96,487 C·mol⁻¹);
R is gas constant (8.315 J·mol⁻¹·K⁻¹); and T is temperature (in Kelvins). $E_{\text{Cl}}$ is the value of $V_m$ that corresponds to the zero-current condition for the constant field equation (Eq. 1):

$$E_{\text{Cl}} = -\frac{RT}{F} \times \ln \left( \frac{C_{\text{lo}}}{C_{\text{li}}} \right).$$ (2)

We calculated the $C_{\text{li}}$ based on $E_{\text{Cl}}$ using Equation 2.

**Estimating $Cl^-$ transport.** To estimate $C_{\text{li}}$ transport, we transiently changed $C_{\text{li}}$ and measured the rate at which $C_{\text{li}}$ returned to baseline. The experimental protocol is illustrated in Figure 1B ("$C_{\text{li}}$ depletion") and Figure 2 ("$C_{\text{li}}$ load"). To transiently change $C_{\text{li}}$, we created a large driving force for $Cl^-$ to flow into or out of the cell by stepping the $V_m$ to potentials negative (Fig. 1B) or positive (Fig. 2) to $E_{\text{Cl}}$ for 1 s. Two hundred milliseconds into the voltage step, GABA was puffed onto the apical dendrites. When $V_m$ was hyperpolarized to $E_{\text{GABA}}$, $Cl^-$ efflux through GABA$_A$ channels transiently lowered $C_{\text{li}}$ ("$C_{\text{li}}$ depletion"). Conversely, when GABA was applied as $V_m$ was depolarized to $E_{\text{GABA}}$, $Cl^-$ influx transiently increased $C_{\text{li}}$ ("$C_{\text{li}}$ load"). We coupled each $C_{\text{li}}$ load or depletion current with a single "test" $Cl^-$ current evoked 1–6 s later. The test current was evoked at a $V_m$ near $E_{\text{Cl}}$ so that small changes in $C_{\text{li}}$, caused by the $C_{\text{li}}$ load or depletion would be easily seen as a change in the direction and/or size of the test current. By measuring the charge transfer of the test currents at 1 s intervals after the $C_{\text{li}}$ load or depletion, we estimated the rate at which $C_{\text{li}}$ recovered to baseline. We assumed that any voltage-gated currents activated during the voltage steps did not significantly alter our results for two reasons. First, $C_{\text{li}}$ consistently returned to baseline within seconds of the $C_{\text{li}}$ load or depletion; and second, the test potential had no effect on the time constant of $C_{\text{li}}$ recovery.

$C_{\text{li}}$ was calculated for each test point by estimating the change in $E_{\text{Cl}}$ that would account for the direction and size of each GABA-evoked test current. For the experiments shown in Figure 1B–D, $C_{\text{li}}$ was calculated at each time interval according to the steady-state permeability (estimated from the data in Fig. 1A and Eq. 1) and the direction and charge transfer of the GABA-evoked $Cl^-$ current at each time interval after $C_{\text{li}}$ depletion (Staley and Proctor, 1999). By combining Equations 1 and 2, we estimate $C_{\text{li}}$ for each test current:

$$C_{\text{li}} = \Delta C_{\text{li}} \times (1 - e^{-t/\tau}) + C_{\text{li},t=0},$$ (4)

To determine the NKCC1-specific component of $C_{\text{li}}$ transport, this protocol was repeated in the same cell after selectively blocking NKCC1 with 10 μM bumetanide (Russell, 2000). In control conditions, $C_{\text{li}}$ returns to baseline via NKCC1 and diffusion; in the presence of 10 μM bumetanide, there is only diffusion. To estimate $C_{\text{li}}$ transport by NKCC1 alone, we performed a "bumetanide subtraction." In the presence and absence of bumetanide, $C_{\text{li}}$ recovered to baseline with monoeponential kinetics (Eq. 4, Fig. 3B). Monoeponential rate constants are additive; therefore, the rate constants for NKCC1-mediated $Cl^-$ transport and diffusion were additive:

$$\frac{1}{\tau_{\text{NKCC1}}} = \frac{1}{\tau_{\text{control}}} - \frac{1}{\tau_{\text{diffusion}}},$$ (5)

where $\tau_{\text{control}}$ represents the time constant of $C_{\text{li}}$ recovery in the absence of bumetanide and $\tau_{\text{diffusion}}$ represents the time constant of $C_{\text{li}}$ recovery in the presence of 10 μM bumetanide (Fig. 3). Repetitive GABA application can cause GABA$_A$ receptor desensitization. Because receptor desensitization is insensitive to 10 μM bumetanide, it was subtracted out as part of the bumetanide-insensitive change in $C_{\text{li}}$.

**Estimating the regulation of $Cl^-$ transport by NKCC1.** The rate of $Cl^-$ transport by NKCC1 can be expressed in analogy to Ohm’s law:

$$\text{current flow} = \text{driving force} \times \text{conductance},$$ (6)
transport. The only free variable in the driving force calculation is steady-state $N_a$.

The transport capacity ($v_{\text{SEM}}$) analogous to the conductance term in Ohm’s law can be modeled using Michaelis–Menten kinetics for enzymatic activity (Läuger, 1987; Alvarez-Leefmans and Russell, 1990; Russell, 2000):

$$v_{\text{SEM}} = \frac{v_{\text{max}} \times [\text{Cl}^-]}{K_m + [\text{Cl}^-]},$$

where $[\text{Cl}^-]$ is the substrate Cl$^-$ concentration ($C_{li}$ for inward transport; $C_{o}$ for outward transport), $v_{\text{max}}$ is the maximum velocity of transport, and $K_m$ is the Cl$^-$ affinity of the transporter (substrate Cl$^-$ concentration at which Cl$^-$ transport is half-maximal). When Cl$^-$ is expressed as a function of seconds after a Cl$^-$ load or depletion, extrapolating the monoeponential fit line (Eq. 4) back to $t = 0$ gives an estimate of $v_{\text{max}}$ (the velocity at $t = 0$). Therefore, $K_m$ was the only free variable in calculating transport capacity.

In analogy to Ohm’s law (Eq. 6), the velocity of NKCC1-mediated Cl$^-$ transport ($v_{\text{NKCC1}}$) is predicted by multiplying the normalized driving force $\Delta G/\Delta G_{\text{max}}$ by the conductance term $v_{\text{SEM}}$ (Läuger, 1987):

$$v_{\text{NKCC1}} = \frac{\Delta G}{\Delta G_{\text{max}}} \times v_{\text{SEM}}.$$  

**Activity-induced changes in $C_{li}$.** For the experiments presented in Figure 6, neurons were switched from voltage clamp to current clamp; action potentials were evoked by injecting depolarizing current pulses (1.5 ms, 2 nA) at 20 Hz for 2.5 min as described previously (Fiunelli et al., 2005). Recordings were switched back to voltage-clamp mode after the train of action potentials.

**Statistical analyses.** Student’s $t$ test was used to test for significant differences between control and experimental conditions. SigmaPlot (SyStat Software, Point Richmond, CA) was used to test whether data were normally distributed. ANOVA with correction for the mean of the observations was used to test the validity of correlations between two variables. In all analyses, $p < 0.05$ was considered significant. All data are presented as mean ± SEM unless noted otherwise.

**Drugs.** The NKCC1 inhibitor bumetanide was stored as a 50 mM stock solution in ethanol at 4°C (protected from light) and then diluted to 10 μM in ACSF daily. The α2/α3 Na$^+$–K$^+$–ATPase inhibitor dihydro-ouabain (DHO) was diluted directly into ACSF before each experiment. Bumetanide and DHO were bath applied. All chemicals were purchased from Sigma (St. Louis, MO) except QX-314 and CGP 55845A (Tocris, Ellisville, MO).

**Junction potentials.** Junction potentials were calculated using pClamp 9.2 (Molecular Devices) and subtracted during data analysis: 0 mM Na$_{\text{pipette}}$ −3.7 mV; 9 mM Na$_{\text{pipette}}$ −3.5 mV; whole cell, −15.2 mV.

**Results**

**NKCC1 is required for depolarizing GABA**

The Na$^+$–K$^+$–Cl$^-$ transporter NKCC1 is highly expressed in neonatal neurons when GABA is depolarizing and excitatory (Dzhala et al., 2005). To test whether NKCC1 is required for neonatal hippocampal neurons to maintain elevated Cl$^-$, and therefore depolarize in response to GABA, we measured the resting membrane potential (RMP) and $E_{\text{Cl}}$ of voltage-clamped hippocampal CA1 pyramidal cells from postnatal day 4–6 rats. Neurons were recorded in the absence and presence of 10 μM bumetanide, a selective inhibitor of NKCC1 (Gillen et al., 1996; Russell, 2000). We used the gramicidin-perforated patch technique to maintain the native Cl$^-$, Gramicidin channels are impermeable to anions such as Cl$^-$, but allow cations (such as Na$^+$ and K$^+$) and small uncharged molecules to pass through (Akaike, 1996). The GABA$_{A}$ channel conducts anions, mainly Cl$^-$ and bicarbonate. To minimize the bicarbonate flux through the GABA$_{A}$ channel so that $E_{\text{GABA}} \approx E_{\text{Cl}}$, we used HEPES-buffered (nominally CO$_2$- and bicarbonate-free) ACSF in all experiments.
by −37 ± 9% to 8.0 ± 1.5 mM \((n = 5; p = 0.02)\). Bumetanide had no effect on the GABA\(_A\) conductance \((\text{control: } 20.3 ± 5.7 \mu\text{S}; +\text{Bum: } 25.0 ± 6.7 \mu\text{S}; n = 5; p = 0.62)\) or holding current \((\text{control: } −24.7 ± 8.5 \text{ pA}; +\text{Bum: } −31.6 ± 13.8 \text{ pA}; n = 6; p = 0.47)\). All cells were recorded with 0 mM Na\(^+\) in the recording pipette \((N_{\text{pipette}})\). These results indicate that NKCC1 is necessary for neonatal CA1 pyramidal cells to maintain elevated Cl\(_i\), and thus enable depolarizing responses to GABA\(_A\) receptor activation.

**Cl\(^−\)** transport quantified

NKCC1 is required for neonatal neurons to maintain elevated Cl\(_i\), and depolarize in response to GABA. However, NKCC1-mediated Cl\(^−\) transport has not been demonstrated or characterized in the developing hippocampus, where it contributes to both normal development and seizures (Dzhala et al., 2005). We measured inward Cl\(^−\) transport by transiently lowering Cl\(_i\) and monitoring Cl\(_i\) at 1 s intervals as it returned to baseline. The experimental protocol is illustrated in Figure 1B. First, we measure steady-state \((\text{i.e., baseline}) E_{\text{Cl}}\), as in Figure 1A. Then, to measure Cl\(^−\) transport, we applied a 1 s hyperpolarizing voltage step \((\text{usually } 60 \text{ mV negative to } E_{\text{Cl}})\). Two hundred milliseconds into the voltage step, GABA was puffed onto the apical dendrites. The rationale was that the hyperpolarized \(V_{m}\) created a large driving force for Cl\(^−\) efflux; in the small compartment of the dendrite, Cl\(^−\) efflux will decrease Cl\(_i\). For the cell in Figure 1B, the Cl\(_i\) depletion current evokes a Cl\(^−\) efflux \((\text{shown by the large inward current})\). \(V_{m}\) was then stepped to a test potential near \(E_{\text{Cl}}\), and a second GABA puff was applied. We performed six trials of paired GABA puffs, each trial having a different time interval \((\text{range } 1–6 \text{ s})\) between the Cl\(_i\) depleting current \((\text{first GABA puff})\) and the test current \((\text{second GABA puff})\). Immediately after Cl\(_i\) depletion when Cl\(_i\) is low, the first test current reflects Cl\(^−\) influx. Over a few seconds, as Cl\(_i\) increases back to its steady-state level, the test currents reverse and return to baseline. Fifteen seconds elapsed between each trial. We set \(t = 0 \text{ s}\) to be the time at which the hyperpolarizing voltage step ends and \(V_{m}\) is stepped to a potential near \(E_{\text{Cl}}\).

The charge transfer of each test current was calculated by measuring the area under the current waveform after baseline subtraction. Figure 1C shows charge transfer as a function of time after Cl\(_i\) depletion for the cell illustrated in Figure 1B. The first test current is positive, indicating a negative shift in \(E_{\text{Cl}}\) immediately after Cl\(_i\) depletion. Within a few seconds, charge transfer returns to its negative baseline value. To estimate the Cl\(_i\) at each time interval, we calculated the change in \(E_{\text{Cl}}\) that would account for the size and direction of the charge transfer when compared with baseline (please see Materials and Methods for further de-
tails). Figure 1D shows that Cl and returns to its baseline value within a few seconds after Cl depletion for the cell in Figure 1, B and C.

NKCC1-mediated ion transport is reversible, meaning Cl can be transported in or out of the cell (Russell, 2000). We estimated the rate of outward Cl transport by calculating Cl for a function of time after an increase in Cl (“Cl load”). Figure 2A illustrates the experimental protocol. To measure outward Cl transport, we performed a similar experiment to that shown in Figure 1B–D. The protocol was the same as in Figure 1B, except that V was stepped to a depolarizing potential (60 mV positive to E) to provide a large driving force for Cl influx to transiently raise Cl (“Cl load”). Then, at 1 s intervals, we measured the direction and charge transfer of GABA-evoked currents as described above. For the cell in Figure 2, when V was depolarized to E, GABA evoked a large outward current (inward Cl flux). One second later, GABA evokes a large inward test current, which indicates that the Cl load induced a positive shift in E. The size of the test currents decreases back to steady state over the next few seconds. Figure 2B shows the charge transfer of the test currents recovering to baseline following the Cl load. Figure 2C shows the Cl at each time interval after the Cl load, calculated based on the estimated change in E compared with baseline. After a transient increase, Cl decreases back to its steady-state value within several seconds.

NKCC1 Cl transport quantified

The techniques described above allow us to measure the rate at which Cl returns to baseline after a Cl depletion (Fig. 1B–D) or load (Fig. 2). In these control conditions, Cl, most likely returns to steady state by the combination of NKCC1-mediated transport plus non-NKCC1 mechanisms (such as diffusion from neighboring sections of dendrite). This is illustrated in Figure 3A. To estimate the NKCC1-specific component of Cl transport, we repeated the Cl load and depletion experiments in each cell after bath applying 10 μM bumetanide, a selective inhibitor of NKCC1. In the presence of 10 μM bumetanide, Cl recovers to baseline by non-NKCC1 mechanisms (Fig. 3A). The method used to calculate NKCC1-mediated Cl transport is illustrated in Figure 3 and described further in Materials and Methods. Figure 3B shows that in control and bumetanide, Cl returns to baseline via a monoexponential kinetics (Eq. 4). Monoexponential rate constants (k = 1/v) are additive, so the rate of NKCC1-specific transport (1/τ) was calculated by subtracting non-NKCC1 transport (1/τ) from the total (1/τ). This calculation is shown in Figure 3C. Figure 3D shows averaged NKCC1-mediated Cl transport. After an average decrease in dendritic Cl of −1.0 ± 0.3 mM, NKCC1 transports Cl into the cell with the time constant (τNKCC1) of 0.6 ± 0.1 s and vmax of 1.6 ± 0.3 mM/s (n = 5; each recorded with 0 mM Npipette). When Cl was transiently increased by evoking the first GABA-gated current at a membrane potential positive to E, monoexponential NKCC1-mediated transport kinetics were also demonstrated for outward Cl transport when 9 mM Na was included in the perforated patch electrode solution. After an increase in dendritic Cl of +1.3 ± 0.4 mM, NKCC1 transported Cl out of the cell with τNKCC1 = 0.9 ± 0.3 s and vmax = 1.6 ± 0.5 mM/s (n = 3). For both inward and outward Cl transport, NKCC1 activity caused Cl to relax to the same steady-state value of 12.9 ± 1.3 mM (n = 8). NKCC1 transport requires all three transported ions to be present on the side of the membrane from which ions are being transported (Russell, 2000). Consistent with this, no outward NKCC1-mediated Cl transport took place in neurons recorded with perforated patch-pipette solution containing 0 mM Na (τNKCC1 = 0.1 ± 2.6 s; n = 5). Data presented in Figures 1, 2, and 3A–C are from single cells.

Thermodynamic regulation of NKCC1 and Cl

The mechanism by which NKCC1-mediated Cl transport sets a particular Cl, is unknown (Alvarez-Leefmans and Russell, 1990; Russell, 2000). Michaelis–Menten enzyme kinetics models describe the affinity of an enzyme for its substrate (Km) based on the substrate concentration (C) and the observed initial/maximum velocity of transport. These models are often used to model Cl transport (Michaelis and Menten, 1913; Tats, et al., 1987; Gasbjerg and Brahm, 1991; Lauf et al., 1992; Staley and Proctor, 1999). Therefore, we attempted to fit NKCC1-mediated Cl transport using Michaelis–Menten kinetic models. Figure 4A shows the average data of NKCC1-mediated inward Cl transport from Figure 3D fit with various transport models. We found that the initial/maximum velocity of Cl transport was well described by the Michaelis–Menten model (Eq. 9). However, this model did not fit with the relaxation back to steady-state Cl that we observed (Fig. 4A, inset), even when the model was modified to include noncompetitive or uncompetitive antagonism by intracellular chloride (data not shown).

Another cation-chloride cotransporter, the neuronal K+-Cl cotransporter KCC2 (Payne, 1997), is at or near thermodynamic equilibrium at the steady-state K and Cl concentrations (Staley and Proctor, 1999). This means that no net transport takes place until a change in the electrochemical gradient of one of the transported ions creates a driving force for ion transport. This is illustrated by the fact that KCC2 Cl transport follows a monoexponential relaxation back to the steady-state Cl, after a Cl load or depletion (Staley and Proctor, 1999). We have observed this...
same behavior following Cl\textsuperscript{−} load or depletion with NKCC1-mediated Cl\textsuperscript{−} transport (Fig. 3D). This implies that NKCC1 transport may be thermodynamically regulated as well, meaning that ion transport only takes place when there is an electrochemical driving force to do so (Alvarez-Leefmans and Russell, 1990; Russell, 2000).

We tested the hypothesis that Cl\textsuperscript{−} transport by NKCC1 is thermodynamically regulated by modeling transport as an analog to Ohm’s law [current = conductance \times driving force (Eqs. 6, 10)] (Läuger, 1987). Current represents the rate of NKCC1-mediated Cl\textsuperscript{−} transport; conductance represents the affinity of the transporter for substrate [calculated by the Michaelis–Menten equation (Eq. 9)], and driving force is provided by the combined electrochemical gradient of the transported ions as a function of the transport ratio (Eq. 7). The only free variables are the steady-state intracellular Na\textsuperscript{+} concentration (N\textsubscript{a}) and K\textsubscript{o}, NKCC1’s affinity for Cl\textsuperscript{−}, which was consistently high in all experiments (<1 mm). Figure 4A shows the NKCC1-mediated Cl\textsuperscript{−} transport data modeled with various transport ratios. These are the same data points as those shown in Figure 3D, now fit with the thermodynamic model (Eq. 10). The data are best described by a thermodynamic model incorporating a transport stoichiometry of 1 Na\textsuperscript{+}:4 K\textsuperscript{+}:5 Cl\textsuperscript{−}. This model predicts thermodynamic equilibrium at a physiologically reasonable N\textsubscript{a} of 3–5 mM (Rose et al., 2000) and chloride-steady Cl\textsubscript{i} equal to the experimentally observed steady state. The inset to Figure 4A shows that other transport ratios (1:1.2 and 1:2:3) predict Cl\textsubscript{i} to be much higher than the steady-state value we observed. Figure 4B shows that the driving force (ΔG) for Cl\textsuperscript{−} transport and the Cl\textsuperscript{−} transport velocity are maximal immediately following a Cl\textsubscript{i} depletion. As NKCC1 transports Cl\textsuperscript{−} into the cell, the driving force for transport, and therefore the velocity of transport, decrease to zero as Cl\textsubscript{i} returns to steady state. In summary, the results presented thus far show that NKCC1 transports Cl\textsuperscript{−} in the direction that allows the free energy of the combined Na\textsuperscript{+}:K\textsuperscript{+}:Cl\textsuperscript{−} gradient to return to zero after a transient change in Cl\textsubscript{i}.

Our results suggest that NKCC1 transport is regulated by the combined driving force created by the electrochemical gradients of the transported ions. We reasoned that if this were true, changing the transmembrane Na\textsuperscript{+} or K\textsuperscript{+} gradients should change the driving force for coupled Na\textsuperscript{+} K\textsuperscript{+}:Cl\textsuperscript{−} transport, and therefore, should change steady-state Cl\textsubscript{i} (Eq. 8). Changes in Na\textsubscript{o} and K\textsubscript{o} would have the most influence on E\textsubscript{Na} and E\textsubscript{K} and thus the most influence on NKCC1-mediated transport and Cl\textsubscript{i}; of the two, Na\textsubscript{o} is more labile in single-cell experiments performed in perfused slices. The inward Cl\textsuperscript{−} transport data presented thus far have been from recordings using 0 mM Na\textsuperscript{+} in the recording pipette. To test the hypothesis that altering steady-state Na\textsubscript{o} changes steady-state Cl\textsubscript{i}, we repeated our gramicidin-perforated patch recordings with 0 mM Na\textsubscript{pipette} increased Na\textsubscript{o} would decrease steady-state Cl\textsubscript{i} by altering the combined electrochemical driving force for NKCC1 transport.

In contrast to our hypothesis, the steady-state Cl\textsubscript{i} was not significantly changed (9 mM Na\textsubscript{pipette} Cl\textsubscript{i} = 13.9 ± 1.1 mM; n = 6; 0 mM Na\textsubscript{pipette}; Cl\textsubscript{i} = 13.0 ± 1.5 mM; n = 8; p = 0.65). We had assumed that Na\textsubscript{o} would equal Na\textsubscript{pipette} but that is probably not the case. It has been shown that dialysis from the recording pipette solution is progressively less effective at changing ionic equilibria in dendrites because of transmembrane transport along the length of the dendrite (Rose and Ransom, 1997; Yu and Salter, 1998; Jarolimek et al., 1999). Consistent with this, RMP was significantly more negative with 9 mM Na\textsubscript{pipette} (−74.0 ± 1.2 mV; n = 6) than with 0 mM Na\textsubscript{pipette} (−66.1 ± 1.5 mV; n = 8; p = 0.002), indicating increased activity of an electrogenic transporter such as Na\textsuperscript{+}K\textsuperscript{−}ATPase in the cells recorded with 9 mM Na\textsubscript{pipette} (Jarolimek et al., 1999). Values for Cl\textsubscript{i} and RMP here differ slightly from those presented earlier in Results because this data set includes all cells in which we measured Cl\textsubscript{i} and RMP, not just those cells in which we measured Cl\textsuperscript{−} transport kinetics.

Although the steady-state Cl\textsubscript{i} was unaffected by 9 mM Na\textsubscript{pipette}, NKCC1-mediated inward Cl\textsuperscript{−} transport was significantly slower (t\textsubscript{NKCC1} = 2.2 ± 0.03 s; n = 3) than transport with 0 mM Na\textsubscript{pipette} (t\textsubscript{NKCC1} = 0.6 ± 0.1 s; n = 5; p = 0.000006). These data are shown in Figure 5A.

The major regulator of Na\textsubscript{i} is Na\textsuperscript{+}K\textsuperscript{−}ATPase. Therefore, Na\textsuperscript{+} imported by NKCC1 during coupled Na\textsuperscript{+}K\textsuperscript{−}:Cl\textsuperscript{−} transport will be cleared from the dendrite via Na\textsuperscript{+}K\textsuperscript{−}ATPase activity. We hypothesized that the additional 9 mM Na\textsuperscript{+} in the recording pipette saturated the cell’s Na\textsuperscript{+}K\textsuperscript{−}ATPase activity, which prevented the Na\textsuperscript{+} imported by NKCC1 from being rapidly cleared from the dendrite. We reasoned that the reduced ability to clear imported Na\textsuperscript{+} from the dendrite led to a transient Na\textsuperscript{+} accumulation that reduced the driving force for further NKCC1-mediated transport. According to our thermodynamic model, further Na\textsuperscript{+}K\textsuperscript{−}Cl\textsuperscript{−} transport would take place only as quickly as Na\textsuperscript{+}K\textsuperscript{−}ATPase and diffusion remove the excess Na\textsuperscript{+}.

To test this hypothesis, we modeled the 9 mM Na\textsubscript{pipette} Cl\textsuperscript{−} transport data shown in Figure 5A according to a modified version of our thermodynamic model. In previous experiments, Na\textsubscript{i} was a free variable with a single steady-state value. To test whether a transient increase in Na\textsubscript{i} could account for the difference in Cl\textsuperscript{−} transport rate between 0 mM Na\textsubscript{pipette} and 9 mM Na\textsubscript{pipette} we modified the model to allow Na\textsubscript{i} to change over time. We found that the slower transport rate with 9 mM Na\textsubscript{pipette} was well described when the 0 mM Na\textsubscript{pipette} Cl\textsuperscript{−} transport data were combined with a transient increase in Na\textsubscript{i} that returns to baseline with a τ of 1.85 s. The predicted Na\textsubscript{i} transient is shown in the inset to Figure 5A. The transient increase in Na\textsubscript{i} that predicts the observed
transport rates is physiologically reasonable, which supports the idea that Na\(^+\) and Na\(^+\)-K\(^+\)-ATPase are important regulators of NKCC1 transport, and therefore, Cl\(_i\).

These results suggest that Na\(^+\) imported by NKCC1 itself can decrease the driving force for further NKCC1 transport if Na\(^+\) is not cleared from the dendrite quickly enough. According to our thermodynamic model, large Cl\(_i\) depletions create a large driving force for combined inward Na\(^+\)-K\(^+\)-Cl\(^-\) transport. In the small compartment of the dendrite, a large Cl\(_i\) depletion could therefore increase Na\(_i\) to the extent that further inward NKCC1 transport is inhibited.

To test the hypothesis that large Cl\(_i\) depletions correlate with slow transport, we examined the relationship between \(r_{\text{NKCC1}}\) and the size of the Cl\(_i\) depletion in recordings made with 0 or 9 mM Na\(^+\) in the recording pipette (Na\(_{\text{pipette}}\)). These data are shown in Figure 5B. In recordings with 0 mM Na\(_{\text{pipette}}\), reduced NKCC1 transport rate (increased \(r_{\text{NKCC1}}\)) correlated with increased size of the Cl\(_i\) depletion (\(R^2 = 0.99\); \(n = 5\); \(p = 0.0002\)). This suggests that transient increases in Na\(_i\) by NKCC1-mediated Na\(^+\)-K\(^+\)-Cl\(^-\) transport limit the velocity of further NKCC1 transport. In contrast, no relationship between Cl\(_i\) depletion size and NKCC1 transport rate was seen in recordings with 9 mM Na\(_{\text{pipette}}\) (\(R^2 = 0.067\); \(n = 3\); \(p = 0.83\)). This is consistent with Na\(^+\) export (presumably by Na\(^+\)-K\(^+\)-ATPase) that was already saturated by the combination of pipette Na\(^+\) and the NKCC1-mediated Na\(^+\) influx triggered by even the smallest of Cl\(_i\) depletions. Together, these results support the thermodynamic model of NKCC1 Cl\(^-\) transport and provide further evidence that Na\(_i\) is a key regulator of NKCC1, and therefore, Cl\(_i\).

**Action potentials increase steady-state Cl\(_i\) and improve Na\(^+\) handling**

Thus far, we have examined the thermodynamic regulation of NKCC1 transport, the major Cl\(_i\) accumulation mechanism in neonatal neurons. Our thermodynamic model of Cl\(^-\) transport provides the tools to test specific hypotheses about activity-dependent changes in Cl\(_i\). Consider the following: trains of action potentials have been shown to cause persistent increases in neuronal Cl\(_i\) (Woodin et al., 2003; Fiumelli et al., 2005). Trains of action potentials are also known to cause long-lasting increases in Na\(^+\)-K\(^+\)-ATPase activity, which lowers Na\(_i\) (Ritchie and Straub, 1957; McDougall and Osborn, 1976; Thompson and Prince, 1986; Morita et al., 1993; Parker et al., 1996). The results we have presented thus far have shown that NKCC1 transport is thermodynamically regulated and sensitive to changes in Na\(_i\). If Na\(_i\) can limit inward Cl\(^-\) transport as our data suggest, then Na\(^+\)-K\(^+\)-ATPase activity, which sets Na\(_i\), must be integral to controlling the concentration of Cl\(^-\) in the cell. Because trains of action potentials increase Na\(^+\)-K\(^+\)-ATPase activity, the changes in Cl\(_i\) observed following action potential trains could be caused by altered Na\(^+\) and K\(^+\) transmembrane gradients from increased Na\(^+\)-K\(^+\)-ATPase activity. A decreased steady-state Na\(_i\) would shift NKCC1’s thermodynamic equilibrium such that net Cl\(^-\) transport would come to rest at a higher steady-state Cl\(_i\) (Eq. 8; see Fig. 7).

We hypothesized that if NKCC1-mediated transport is thermodynamically limited, as our results suggest, changes in the Na\(^+\) and K\(^+\) gradients mediated by increased Na\(^+\)-K\(^+\)-ATPase activity should result in persistent changes in Cl\(_i\). To test this hypothesis, steady-state \(E_{\text{Cl}}\) and Cl\(^-\) transport kinetics were measured before and after a 20 Hz, 2.5 min train of action potentials in the recorded cell (Fiumelli et al., 2005). Data from a representative cell are shown in Figure 6, A and B. The action potential train caused a persistent increase in Cl\(_i\) of 3.7 ± 0.5 mM following the action potentials (Cl\(_i\) before APs, 13.6 ± 3.1 mM; Cl\(_i\) after APs, 17.3 ± 3.4 mM; \(n = 3\); \(p = 0.02\)). Based on steady-state Cl\(_i\), the calculated steady-state Na\(_i\) decreased from 2.7 mM before APs to 0.8 mM after APs for NKCC1 at thermodynamic equilibrium (Eq. 8). We monitored Cl\(_i\) in these cells for 27, 33, and 42 min following the train of action potentials. Therefore, in reference to these results, “persistent” means up to 42 min after action potentials. There was no change in holding current during that time (before APs: -22 ± 15 pA; after APs: -23 ± 13 pA; \(n = 3\); \(p = 0.69\)). In control conditions, as in previous experiments with 9 mM Na\(^+\) in the patch pipette (Fig. 5B), there was no correlation between the Cl\(^-\) transport rate and the size of the Cl\(_i\) depletion (\(R^2 = 0.02\); \(p = 0.87\)). However, in the same cells after the train of action potentials, slower Cl\(^-\) transport correlated with larger Cl\(_i\) transients, as it had in previous experiments with 0 mM Na\(^+\) in the recording pipette (\(R^2 = 0.97\); \(p = 0.02\)). In other words, action potentials caused Cl\(^-\) transport in neurons recorded with 9 mM Na\(_{\text{pipette}}\) to behave as if they were being recorded with 0 mM Na\(_{\text{pipette}}\) (compare with Fig. 5B). These results imply that in control conditions, the Na\(^+\) load from the recording pipette had saturated the mechanisms by which Na\(^+\) is cleared from the dendrite. Therefore, the Na\(^+\) imported by NKCC1 after even the smallest Cl\(_i\) depletion was sufficient to slow down further inward Cl\(^-\) transport. In contrast, after the action potentials, larger Cl\(_i\) depletions correlated with slower Cl\(^-\) transport (as we saw in recordings with 0 mM Na\(_{\text{pipette}}\)). This implies a persistently lower
Na$^+$ and/or an improved ability to rapidly clear Na$^+$ from the dendrite. Either mechanism could be explained by an increase in Na$^+$/K$^+$-ATPase activity (Eq. 7).

**a2/a3 Na$^+$/K$^+$-ATPase is required for activity-induced increase in Cl$^-$**

We proposed that an activity-dependent increase in Na$^+$/K$^+$-ATPase activity was responsible for altering Na$^+$, and thus the Cl$^-$, at which NKCC1 comes to electrochemical equilibrium. We reasoned that if this were true, then blocking Na$^+$/K$^+$-ATPase should prevent the persistent increase in Cl$^-$ observed following trains of action potentials. We hypothesized that the “auxiliary” a2 and/or a3 Na$^+$/K$^+$-ATPase isoforms were responsible for the activity-induced change in Na$^+$ handling by the cell and the change in Cl$^-$ transport. To test this hypothesis, we bath applied the selective a2/a3 Na$^+$/K$^+$-ATPase inhibitor DHO (10 $\mu$M), and then measured steady-state $E_{Cl}$ and Cl$^-$ transport kinetics before and after a 20 Hz, 2.5 min train of action potentials in the recorded cell. Representative data are illustrated in Figure 6C.

In control conditions, DHO had no effect on Cl$^-$ compared with control cells (control: 13.6 ± 3.1 mV; n = 3; DHO: 15.4 ± 3.1 mV; n = 4; p = 0.71). However, in the presence of DHO, action potential trains decreased steady-state Cl$^-$ by 0.9 ± 0.02 mV (n = 4; p = 0.01), corresponding to an increase in Na$^+$ of ~0.6 mV (Eq. 8). These results contrast with those from previous experiments performed without DHO in which action potential increases Cl$^-$ handling by the cell and the change in Cl$^-$ transport.

In the presence of DHO, there was no relationship between the time constant of Cl$^-$ transport ($\tau$) and the size of the Cl$^-$ depletion, neither before nor after the train of action potentials [for the cell in Fig. 6C, $\tau$ versus Cl$^-$ depletion (PC): +DHO before APs: $R^2 = 0.36 \pm 0.97; p = 0.59$; +DHO after APs: $R^2 = 0 \pm 0.68; p = 0.99$]. In other words, selectively blocking the a2/a3 Na$^+$/K$^+$-ATPase with DHO prevented the train of action potentials from making transport with 9 mM Na$_{pipette}$ more closely resemble transport with 0 mM Na$_{pipette}$. These results were in direct contrast to previous experiments showing that with intact a2/a3 Na$^+$/K$^+$-ATPase activity, the train action potentials induced a direct relationship between the rate of Cl$^-$ transport and the size of the Cl$^-$ depletion (Fig. 6B). These results indicate that a2/a3 Na$^+$/K$^+$-ATPase isoforms are required for trains of action potentials to bring about persistent increases in the steady-state Cl$^-$.

After the addition of DHO, there were no significant changes in holding current (control: −8.5 ± 6.1 pA; +DHO: −7.2 ± 7.7 pA; n = 4; p = 0.59), input resistance (control: 159.7 ± 104.3 MΩ; +DHO: 204.8 ± 134.9 MΩ; n = 4; p = 0.24), or resting membrane potential (control: −64.0 ± 2.4 mV; +DHO: −63.9 ± 2.7 mV; n = 4; p = 0.90), implying that the a2/a3 isoforms do not significantly contribute to the cell’s baseline functioning. Furthermore, DHO did not change action potential peak amplitude (control: 99.8 ± 0.01 mV; +DHO: 99.8 ± 0.01 mV; n = 4; p = 0.44) or half-width (control: 4.9 ± 1.1 ms; +DHO: 4.7 ± 0.2 ms; n = 4; p = 0.83), indicating that the lack of activity-dependent changes in the presence of DHO was not an artifact of defective action potential firing.

Finally, we tested the hypothesis that NKCC1 was necessary for the activity-induced increase in Cl$^-$ in neonatal hippocampal pyramidal neurons, NKCC1 transport is controlled by the combined Na$^+$-K$^+$-ATPase isoforms are responsible for the activity-induced increase in Na$^+$ handling by the cell and the change in Cl$^-$ transport. To test this hypothesis, we bath applied the selective a2/a3 Na$^+$/K$^+$-ATPase inhibitor DHO (10 $\mu$M), and then measured steady-state $E_{Cl}$ and Cl$^-$ transport kinetics before and after a 20 Hz, 2.5 min train of action potentials in the recorded cell. Representative data are illustrated in Figure 6C.

**Discussion**

In the present work, we show that neonatal rat CA1 pyramidal neurons require NKCC1 activity to maintain elevated Cl$^-$ to the extent that GABA is depolarizing. When NKCC1 is selectively blocked with 10 $\mu$M bumetanide, action potential trains had no effect on Cl$^-$ (n = 2) (Fiumelli et al., 2005). Together, these results indicate that an activity-dependent increase in Na$^+$/K$^+$-ATPase activity alters the transmembrane gradients of Na$^+$ and K$^+$ such that a new equilibrium point is reached for NKCC1-mediated Cl$^-$ transport.

We investigated activity-dependent changes in $E_{Cl}$ and found that trains of action potentials persistently increased the steady-state Cl$^-$ and made Cl$^-$ transport with 9 mM Na$_{pipette}$ more closely resemble transport with 0 mM Na$_{pipette}$ (Fig. 7). Three Na$^+$/K$^+$-ATPase isoforms have been described in neurons. The $\alpha_1$ isoform is uniformly expressed on the plasma membrane, whereas the $\alpha_2$ and $\alpha_3$ isoforms are expressed in a pattern on the plasma membrane that parallels the underlying endoplasmic reticulum (Juhaszova and Blaustein, 1997). The $\alpha_1$ isoform is thought to control the bulk intracellular Na$^+$ concentration; the $\alpha_2$ and $\alpha_3$ subunits are...
considered “auxiliary” isoforms that are recruited during increased cellular activity (Blanco and Mercer, 1998). The α1 isoform has a low affinity for ouabain and is therefore unaffected by 10 μM DHO (McCarron and Alger, 1987; Sweadner, 1989; Berrebi-Bertrand et al., 1990), whereas the α2/α3 isoforms are relatively ouabain sensitive and selectively inhibited by 10 μM DHO (Gao et al., 1995; Juhaszova and Blaustein, 1997). Dihydro-ouabain prevented the activity-induced changes in Cl−, and the saturating Na+ condition for NKCC1 transport, indicating that α2/α3 isoforms of Na+−K+−ATPase were necessary for the activity-dependent increase in Cl−. Plasticity of Na+−K+−ATPase activity is well established (Thérien and Blostein, 2000), and is a logical means of altering neuronal signaling (Ross and Sotetsu, 2001), but this is the first demonstration of involvement of Na+−K+−ATPase or NKCC1 in long-term synaptic plasticity.

This is also the first quantification of NKCC1-mediated Cl− transport in the neonatal hippocampus. Our results confirm and extend previous reports on the kinetics of NKCC1 transport in a variety of experimental systems (Russell, 2000). A benefit of the electrophysiology technique is that we observed NKCC1 Cl− transport with the time resolution of seconds, rather than minutes. Most estimates of NKCC1 transport rates come from measurement of bumetanide-sensitive 86Rb flux (Rb+ substitutes for K+). Reported transport rates are on the order of pmol·cm−2·s−1. One pmol·cm−2·s−1 corresponds to ~14 m/s in a dendrite of the geometry calculated in supplemental material (available at www.jneurosci.org). These rates of transport are compatible with the rates we observed, given the differences in the time resolution of the techniques, the levels of NKCC1 expression, and the ion gradients at which transport was measured. Our results are also consistent with the change in Cl− transport as a function of developmental age and level of synaptic activity (Ben Ari, 2002; Achilles et al., 2007).

The RMP measurements we report here are slightly depolarized compared with the gramicidin-perforated patch measurements by Tyzio et al. (2003). This could be attributable to the differences between CA3 and CA1 neurons or a difference between Wistar and Sprague Dawley rats, because it has been shown that Long–Evans and Sprague Dawley rats are 2 d off from each other developmentally (Talos et al., 2006). In the absence of NKCC1 (if there are no other mechanisms to change Cl−), Cl− will be passively distributed (ECl = RMP). Here, we show that ECl is 10 mV negative to RMP in the absence of NKCC1 activity. It has been shown in neonatal CA3 pyramidal neurons that the RMP measured with gramicidin-perforated patch is depolarized compared with RMP measured with noninvasive cell-attached techniques (Tyzio et al., 2003). Tyzio et al. (2003) reported that in cell-attached recordings of CA3 pyramidal cells RMP was stable at ~77 mV throughout development, which is the value we report here for ECl in the absence of NKCC1 activity. It is possible that gramicidin recordings distort RMP locally (at the soma), with less distortion in dendrites, such that the dendritic RMP was more negative than our measured somatic RMP (Tyzio et al., 2003).

NKCC2 mediates mostly outward Cl− transport in mature neurons; low levels of expression in this age group could account for the hyperpolarized ECl in the absence of NKCC1 activity (Dzhala et al., 2005). The kinetics we describe for NKCC1 are similar to those reported for KC2 (Staley and Proctor, 1999). Both transporters have maximum transport rates in the range of mmol·L−1·s−1, which has important implications for a “push–pull” model of Cl− homeostasis, where two transporters could work in concert to maintain steady-state Cl−. This hypothesis of coupled inward and outward Cl− transport is attractive from the point of view of precise Cl− control. However, oppositely directed transporters operating at these velocities would waste enormous amounts of ATP so that Na+−K+−ATPase could maintain the necessary transmembrane ion gradients. We have applied 100 μM bumetanide, a concentration that inhibits KCC2, at the end of some experiments (data not shown), and have not observed any change in Cl− beyond what was seen with 10 μM bumetanide, suggesting that KCC2 does not contribute significantly to Cl− in the neonatal period.

Action potentials caused a persistent increase in Cl− that required intact α2/α3 Na+−K+−ATPase activity. It has been shown that the increase in Na+−K+−ATPase activity that occurs following neuronal activity is transient (Thompson and Prince, 1986). Consistent with this, there was no change in holding current up to 42 min following the train of action potentials, suggesting that Na+−K+−ATPase activity was not persistently increased. This implies that once a new lower steady-state Na+ has been reached, the Na+ pump does not need to continue running at an increased rate to maintain the lower steady-state Na+. However, we cannot exclude a longer-lasting change in α2/α3 Na+−K+−ATPase activity because once Na+ is low, the pump current may be so small that it does not make a significant contribution to the resting membrane potential and holding current.

Inducible modifications of ECl mediated by activity-dependent alterations in Na+−K+−ATPase activity represents a fundamental new motif of long-term synaptic plasticity. There are undoubtedly many mechanisms underlying long-term plasticity of GABA signaling as a result of persistent changes in ECl, and these mechanisms are likely to vary depending on the age and conditions under which plasticity is studied (van den Pol et al., 1996; Cohen et al., 2002; Coull et al., 2003, 2005; Khalilov et al., 2003; Woodin et al., 2003; Fiumelli et al., 2005). For example, changes in NKCC1 and KCC2 phosphorylation (Kahle et al., 2005) and membrane trafficking (Del Castillo et al., 2005) as well as changes in KCC2 activity have been demonstrated to be important under appropriate conditions (De Koninck, 2007). Here we establish an entirely new mechanism: activity-dependent long-term changes in ECl in the developing nervous system can be caused by activity-dependent changes in Na+−K+−ATPase activity (McDougal and Osborn, 1976; Thompson and Prince, 1986; Ross and Sotetsu, 2001), which alter the Cl− at which a secondary active Cl− transporter is at equilibrium (Fig. 7). Our findings emphasize the variety of mechanisms that may contribute to plasticity of ECl and the caution that should be used in extrapolating such mechanisms from one experimental condition to another. With these cautions in mind, our findings may be relevant to the correlated circadian fluctuations in ECl and Na+−K+−ATPase activity observed in hypothalamic suprachiasmatic nucleus neurons (Wagner et al., 1997; Wang and Huang, 2004). Finally, the finding that NKCC1 is at thermodynamic equilibrium at the steady-state Cl− in neurons suggests caution in the interpretation of ECl determined by gramicidin-perforated patch recordings, because these recordings may indirectly alter ECl by altering Na+ and K+ gradients.

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