Activity-Dependent Layer-Specific Changes in the Extracellular Chloride Concentration and ChlorideDriving Force in the Rat Hippocampus

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Kroeger D, Tamburri A, Amzica F, Sik A. Activity-dependent layerspecific changes in the extracellular chloride concentration and chloride driving force in the rat hippocampus. J Neurophysiol 103: 1905–1914, 2010. First published February 3, 2010; doi:10.1152/jn.00497.2009. The transmembrane distribution of chloride anions (Cl−) determines the direction of the Cl− flux through GABA A receptors; this establishes whether GABA A receptor-mediated responses are hyperpolarizing or depolarizing in neurons. Thus an activity-dependent reduction in the efficacy of inhibitory responses can be the result of an activity-induced reduction of the Cl− driving force. Using Cl−-sensitive electrodes, we measured the extracellular Cl− concentration ([Cl−] o) in each layer of the hippocampus under control conditions and after stimulation. In the control condition, [Cl−] o was lower within the CA1 region (112.9 ± 1.3 mM; mean ± SD) than in the CA3 dentate gyrus areas (117.7 ± 1.2 mM). Stimulation of CA3 pyramidal cells led to an increase in the [Cl−] o. The maximum values were observed in the stratum lacunosum-moleculare (253.4 ± 51.1 mM) and in the hilar (261 ± 43.7 mM) whereas in the granular cell layer, it reached only 159.5 ± 41 mM. The stimulation-induced [Cl−] o increase was followed by a period of decreasing [Cl−] o that fell below the control values. The maximum undershoot (21.6 ± 0.7 mM) was observed in the stratum radiatum. Systemic application of the gap junction blocker carbenoxolone significantly decreased the stimulation-induced Cl− extrusion in the dentate gyrus but only slightly modified it in the CA1 area. Carbenoxolone also drastically reduced the Cl− clearance. The time constant of [Cl−] o clearance was similar between layers (83.4 ± 15.9 ms) but increased after carbenoxolone application (207.1 ± 44.4 ms). Stimulation-induced changes in the [Cl−] o significantly decreased the Cl− driving force and resulted in large fluctuations between layers (Δ = 9.4 mV). The lowest value was observed in the stratum radiatum of the CA1 and the hilar area (7.7 mV), whereas the highest value was calculated for the granule cell layer (16.3 mV). We suggest that a decrease of the extracellular space is mainly responsible for the rapid [Cl−] o increase while the gap junction coupled astrocytic network plays a key role in the activity-dependent redistribution and clearance of Cl− across layers of the hippocampus.

INTRODUCTION

Chloride is the most abundant anion in plant and animal tissue and plays major roles in the repolarization of depolarized cells as well as cellular pH regulation and volume regulation (Hille 1992; Inoue et al. 2005). The transmembrane distribution of chloride anions (Cl−) determines the direction of the Cl− flux through gated GABA A receptors. The gradient between the extra- and intracellular Cl− concentrations establishes whether GABA A receptor-mediated responses are hyperpolarizing or depolarizing in neurons (Kaila 1994). Activity-dependent disinhibition is a reduction in the efficacy of synaptically activated GABA A receptor-mediated inhibitory responses (McCarren and Alger 1985). Activity-dependent decreases in the conductance underlying the inhibitory postsynaptic current (gIPSC) can result from a decrease in presynaptic GABA release (Deisz and Prince 1989; Thompson and Gahwiler 1989a), desensitization of postsynaptic GABA A receptors (Krnjevic 1981; Numann and Wong 1984), or increase of intracellular Cl− ([Cl−] i) (Huguenard and Alger 1986; Thompson and Gahwiler 1989b). Several previous results indicated that the [Cl−] i within neurons is not distributed homogeneously (Hara et al. 1992; Kuner and Augustine 2000). Thus if the extracellular Cl− ([Cl−] o) is to be considered homogeneous (Dietzel et al. 1982), the driving force for Cl− flux, which is the difference between the membrane potential (V M) and Cl− equilibrium potential (E Cl), varies from cell compartment to compartment. On the other hand, if the [Cl−] i is not as homogeneous as suggested by previous reports (Dietzel et al. 1982), then the driving force for Cl− can vary considerably. Thus [Cl−] i is an important factor in the activity-dependent disinhibition, which can vary from one neuronal compartment to another. Because activation of GABA-gated channels permits not only Cl− influx but also a significant efflux of bicarbonate (Kaila and Voipio 1987; Staley et al. 1995), we calculated the overall E GABA value that influences the neuronal membrane potential.

Chloride homeostasis is regulated by various pumps and carrier mechanisms. Cation-coupled secondarily active cotransporters, e.g., the potassium-chloride co-transporter (KCC2), and the sodium potassium chloride co-transporter (NKCC) actively transport Cl− across membranes, while Cl− channels (CIC-2 in neurons and astroglial cells) only passively distribute Cl−. Although the main function of astroglial cells in ion homeostasis is to spatially buffer and redistribute potassium ions through glial syncytia (Newman et al. 1984; Orkand et al. 1966), they are also proposed to balance this cationic influx by coupling it with Cl− influx to neutralize electrical charges (Bormann and Kettenmann 1988; Hodgkin and Horowicz 1959; Kimmelberg and Kettenmann 1990; Kimmelberg et al. 1990; Sik et al. 2000). It is therefore hypothesized that a redistribution mechanism for Cl− might operate through the gap junction connected astrocytic network (Sik et al. 2000). Previously we have suggested that the interconnected astrocytic network may play a key role in the activity-dependent redistribution of Cl− across layers of the hippocampus. Thus the astroglial syncytium may take potassium and Cl− up.
where high glutamatergic synaptic activity occurs and deliver chloride to other layers, where effective GABA_A receptor-mediated inhibition is required (Sik et al. 2000).

To address the questions whether the distribution of \([\text{Cl}^-]_0\) is indeed heterogeneous and whether this distribution is activity dependent, we recorded \([\text{Cl}^-]_0\) in the extracellular compartment using double-barrel micropipettes filled with a \(\text{Cl}^-\)-sensitive solution. We provide evidence that the \([\text{Cl}^-]_0\) indeed differs layer by layer, that this phenomenon is activity dependent, and that the glial gap junction network participates in this redistribution mechanism.

METHODS

Animal preparation

Eleven Sprague-Dawley rats (250–400 g) of both sexes were anesthetized through injection of urethane (1.3–1.5 g/kg ip) and placed in a stereotaxic apparatus (Narishige). Anesthesia level was kept constant using an 87%-13% ketamine-xylazine cocktail (0.1 ml/100 g) injected intraperitoneally. Body temperature was monitored and kept at 37°C throughout the experiment by an animal-thermoregulation device. Through craniotomy and severing the dura mater, we exposed a 1.2 × 1.2 mm window of cortex above the hippocampus (centered at −3.6 mm anterioposterior, 2.5 mm mediolateral). A pair of tungsten stimulating electrodes with 100 μm tip length and with 0.75 mm tip separation was inserted at the lateral edge of the exposed area and advanced by 2.8 mm from the brain surface to stimulate the CA3 area of the hippocampus.

Double-barrel extracellular recording electrodes were inserted at the medial edge of the window and slowly lowered to the lowest layer of the hippocampus (3.5 mm from the pial surface). A craniotomy was added above the frontal cortex to record the electroencephalogram (EEG) with a tungsten electrode. At the end of the experiments, the animal received a lethal dose of ketamine-xylazine. All experiments were carried out following the National Institutes of Health guiding principles and were also approved by the committee for animal care of Laval University.

Electrode preparation and recording

In this study, we used ion-sensitive double-barrel glass micro pipettes, which were made according to procedures described elsewhere (Massimini and Amzica 2001). The \(\text{Cl}^-\)-sensitive barrels of the micropipettes were pretreated with dimethylchlorosylane and the pipettes were then baked at 120°C for 2 h. The tip was filled with \(\text{Cl}^-\)-sensitive ionophore I-cocktail A (Fluka, Neu-Ulm, Germany), and backfilled with a solution of NaCl (0.1 M). The other nonselective barrel (blank) was filled with NaCl 0.2 M. The \(\text{Cl}^-\)-sensitive barrel was calibrated using the following solutions: 100 mM NaCl solution and 10 mM Cl- solution (250 ml ddH2O with 0.146 g NaCl and 3.805 g NaGlu, Sigma-Aldrich). Pipettes were calibrated before and after each experiment (Kondo et al. 1989).

Because ion potentials could be contaminated through capacitative coupling by local field potentials (LFPs), the latter were measured with the pair electrode and subtracted from the former. The resulting signal was then compared with the points taken from the calibration solutions to assign \(\text{Cl}^-\) values to the signal. The relationship between concentration and voltage was derived in accordance with the Nicolson–Eisenmann equation (Ammann 1986). The \(\text{Cl}^-\) sensitivity was of 15–20 mV per decade. The logarithmic selectivity of the electrode to \(\text{HCO}_3^-\) was −1.5 (Kondo et al. 1989) resulting in ~3% sensitivity. Thus ~97% of the signal recorded by the electrode was originated from \(\text{Cl}^-\).

Drug administration

In three experiments, we systemically administered the wide-spectrum gap junction blocker Carbamaylcholine (CBX; Sigma Aldrich) dissolved in Ringer solution injected into the tail vein. Three injections of CBX (100 mg/kg) were delivered in 2 h intervals. Depth profiles were performed within 60 min after injections, and 1 h of recovery was allowed.

Stimulation

In this study, we recorded the extracellular chloride concentration throughout hippocampal every 100 μm using double-barrel \(\text{Cl}^-\)-sensitive micropipettes. For each 100 μm step, we recorded a 1 min control period prior to stimulation onset. Then each s either single stimulus or a train of 10 stimuli (100 Hz, 1.0 and 1.5 mA) was administered through the stimulating electrodes in the CA3 area, totaling 20 trains of stimulation. We recorded the change of \([\text{Cl}^-]_0\) following each stimulation train and averaged these responses for each depth. After 20 trains of stimulation the pipette was retracted upwards by 100 μm for the next level of the depth profile.

In the 11 rats, a total of 19 depth profiles were recorded. Each depth profile consisted of 20 Cl- measurements per 100 μm totaling to 360 separate Cl- measurements per depth profile.

Analysis

All signals were digitally converted (1 ksamples/s), recorded for off-line analysis and then completed with current source density calculation (CSD) using the software WaveMETRICS (Lake Oswego, OR).

CSD analyses were performed on averaged spontaneous or evoked activity recorded throughout the depth profile. One-minute sweeps were averaged to yield 18 values, which were symmetrically extracted from each point of the depth profile (deepest at 3,900 μm; 100 μm intervals). The current flowing into or out of the cellular membrane is proportional to the second spatial derivative of the potential (Hubbard et al. 1969). The calculation of this second derivative is made according to the following formula (Mitzdorf 1985)

\[
\frac{\partial^2 \varphi}{\partial z^2} = \frac{\varphi(z + n \Delta z) - 2\varphi(z) + \varphi(z - n \Delta z)}{(n \Delta z)^2}
\]

where \(\varphi(z)\) is the potential at location \(z\), \(\Delta z\) is the distance between adjacent recording sites (in our case, \(\Delta z = 0.1 \text{ mm}\), the distance between 2 recording sites), and \(n \Delta z\) represents the differentiation grid (in our case, \(n = 1\)).

The following equations were used for calculations. For calculating the thermodynamic driving force for \(\text{K}^+\text{-Cl}^-\) at equilibrium level of \([\text{Cl}^-]\_i\), the Henderson-Hasselbalch equation was used (Williams and Payne 2004)

\[
[\text{Cl}^-]_i = [\text{Cl}^-]_o [\text{K}^+]_o/[\text{K}^+]_i.
\]

For calculating \(E_{\text{Cl}}\), we employed the Nernst equation

\[
E_{\text{Cl}} = -61.5 \times \log\left(10\left([\text{Cl}^-]_o/[\text{Cl}^-]_i\right)\right)\]

(2)

To calculate the \(E_{\text{GABA}}\), which takes into account \(\text{HCO}_3^-\) movement through GABA receptors, we used the following equation

\[
E_{\text{GABA}} = -61.5 \times \log\left(10\left([\text{Cl}^-]_o + \alpha [\text{HCO}_3^-]_o/[\text{Cl}^-]_i + \alpha [\text{HCO}_3^-]_o\right)\right)\]

(3)

Where \(\alpha\) is the conversion factor for \(\text{HCO}_3^-\) and equals to 0.3.

Membrane potential was calculated using the Goldman–Hodgkin–Katz equation
\[ V_M = -61.5 \times \log 10 \left( \frac{P_K \times [K^+]_o + P_{Na} \times [Na^+]_o}{P_{Cl} \times [Cl^-]_o} \right) + P_{Cl} \times [Cl^-]_o \]

The equation used to calculate the driving force is expressed as the difference between the resting membrane potential \( V_M \) and the chloride equilibrium potential \( E_{Cl} \)

\[ \text{Driving force} = V_M - E_{Cl} \]

Parameters not specified in the figure legends are to be considered related to normal physiological conditions, as follows: \([HCO_3^-]_o = 16\) mM; \([HCO_3^-]_i = 25\) mM (i.e., pH \( = 7.2 \) and pH \( = 7.4, 5\% \) CO\(_2\)); temperature \( 37^\circ\)C. The relative permeability of GABA\(_A\) receptors to chloride equilibrium potential \( \left( E_{Cl} \right) \) was first calibrated in 10 and 100 mM Cl\(^-\) solution \( (A2) \) and then lowered to the hippocampus where measurements were obtained. In agreement with our hypothesis, differences in the \([Cl^-]_o\) were recorded during spontaneous brain activity at different hippocampal layers. In general, the \([Cl^-]_o\) gradually increased by depth (Fig. 2A1, \( n = 11 \)). In particular, \([Cl^-]_o\) was generally lower within the upper 0- to 700-\(\mu\)m layers \((112.9 \pm 1.3 \) mM\) as compared with deeper layers 1,200–1,700 \(\mu\)m \((117.7 \pm 1.2 \) mM\). A sharp reversal between a \( Cl^- \) increase and a \( Cl^- \) decrease was observed in layers located at 700 and 800 \(\mu\)m, corresponding to the s. molecular of the dentate gyrus. Our CSD analysis revealed a \( Cl^- \) source \( (Cl^- \) influx into cells) at the depth of 800 \(\mu\)m with a sink \( (Cl^- \) efflux from cells) were located directly above at 700 \(\mu\)m (Fig. 2A1).

**Results**

**Extracellular chloride concentration under normal condition**

The main purpose of our study was to measure the distribution of \([Cl^-]_o\) in hippocampal layers to assess the efficacy of GABA\(_A\)-mediated inhibition in various neuronal compartments. We started with the assumption that the \( Cl^- \) redistribution mechanism is operating in the hippocampus as hypothesized earlier (Sik et al. 2000). Double-barrel pipettes (Fig. 1A1) were first calibrated in 10 and 100 mM \( Cl^- \) solution \( (A2) \) and then lowered to the hippocampus where measurements were obtained. In agreement with our hypothesis, differences in the \([Cl^-]_o\) were recorded during spontaneous brain activity at different hippocampal layers. In general, the \([Cl^-]_o\) gradually increased by depth (Fig. 2A1, \( n = 11 \)). In particular, \([Cl^-]_o\) was generally lower within the upper 0- to 700-\(\mu\)m layers \((112.9 \pm 1.3 \) mM\) as compared with deeper layers 1,200–1,700 \(\mu\)m \((117.7 \pm 1.2 \) mM\). A sharp reversal between a \( Cl^- \) increase and a \( Cl^- \) decrease was observed in layers located at 700 and 800 \(\mu\)m, corresponding to the s. molecular of the dentate gyrus. Our CSD analysis revealed a \( Cl^- \) source \( (Cl^- \) influx into cells) at the depth of 800 \(\mu\)m with a sink \( (Cl^- \) efflux from cells) were located directly above at 700 \(\mu\)m (Fig. 2A1).

**Extracellular \( Cl^- \) concentration after stimulation**

Because our working model predicted that \( Cl^- \) redistribution is activity dependent (Sik et al. 2000), the pattern of \( Cl^- \) transport was also assessed during stimulation-induced activity. In general, both single stimulations and trains of stimulation sharply increased the \([Cl^-]_o\), which gradually decreased to baseline levels and temporarily even below the baseline (Fig. 1B). We called the period of the initial \([Cl^-]_o\) decrease to the baseline level early \( Cl^- \) clearance, and the decrease of \([Cl^-]_o\) below the baseline late \( Cl^- \) clearance period. The difference between the baseline and minimum \([Cl^-]_o\) was determined as \( Cl^- \) undershoot (Fig. 1B). As a pilot measurement, we used two stimulation intensities \((1.0 \) and \( 1.5 \) mA\) to determine the relationship between intensity of the stimulation and \([Cl^-]_o\) (Fig. 1C). These stimulations were carried out within the same hippocampal layer in a randomized fashion and show that 1.5 mA stimulation resulted in an augmented maximum \( Cl^- \) in-...
crease as well as a stronger Cl\textsuperscript{−} undershoot (maximum Cl\textsuperscript{−} increase for 1.5 mA was 195.8 ± 2.5 mM vs. 167.5 ± 3.0 mM for 1.0 mA; while Cl\textsuperscript{−} undershoot for 1.5 mA was 104.6 ± 1.0 mM vs. 109.6 ± 0.9 mM for 1.0 mA). By taking the Cl\textsuperscript{−} increase and Cl\textsuperscript{−} undershoot values, we calculated the regression point (118.5 mM, Fig. 1D) indicating a linear relationship between stimulus intensity and Cl\textsuperscript{−} increase. A similar relationship was found between the increase of LFP amplitude and the increase in [Cl\textsuperscript{−}]\textsubscript{o} (Fig. 1E). We therefore chose to stimulate at 1.5 mA intensity throughout the experiment to better assess the redistribution mechanisms between hippocampal layers. Our 1.5 mA stimulation of the CA3 region led to a
substantial increase in the $[\text{Cl}^-]_o$ on average to 221.6 ± 28.5 mM across layers, up from 120 mM baseline (Fig. 2A; $n = 60$). The maximum $[\text{Cl}^-]_o$ rise after stimulation reached two peaks, one at around 600 µM (253.4 ± 51.1 mM; s. lacunosum-moleculare), the other at 1,100 µM (261 ± 43.7 mM; s. radiatum), with a prominent decrease to 159.5 ± 41 mM at 900 µM, corresponding to the upper granular layer blade of the dentate gyrus. As illustrated in the inset of Fig. 2A, the amplitude difference between the maximum and minimum $[\text{Cl}^-]_o$ increase after stimulation is substantial (as much as 101.5 mM) between layers. The CSD analysis revealed three $\text{Cl}^-$ sinks at 100 µM (s. oriens), 600 µM (s. lacunosum-moleculare), and 1,100 µM (hilar) as well as a source at 0 µM (alveus/s. oriens) and at 900 µM (s. granulosum).

**Chloride clearing mechanism**

The increase in $[\text{Cl}^-]_o$ after stimulation was followed by a period of decreasing $[\text{Cl}^-]_o$, probably as a result of clearing mechanisms setting in. However, we observed an actual undershoot of $[\text{Cl}^-]_o$ in the sense that extracellular $\text{Cl}^-$ level fell below the control values, and only gradually reached equilibrium (Fig. 1B). The undershoot of $\text{Cl}^-$ ($\text{Cl}^-$ clearance) reached a maximum amplitude of 21.6 ± 0.7 mM at a depth of 400 µM (s. radius) and remained constant in levels below this depth (average of 20.9 ± 1.5 mM; Fig. 2A). Thus for most of the hippocampus (82%), we observed an undershoot of $\text{Cl}^-$ clearance to (99.1 ± 1.5 mM) before an equilibrium could be restored at 120 mM. The CSD analysis indicated no substantial sinks or sources: $\text{Cl}^-$ influx and efflux were not spatially segregated.

**Effect of gap junction blocker on $[\text{Cl}^-]_o$ distribution**

Because clearing and redistribution mechanisms likely rely on spatial buffering through astroglial syncytial networks, we applied the wide-spectrum gap junction blocker carbenoxolone (CBX) in three experiments. Systemic application of CBX (100 mg/kg) slightly increased the baseline $[\text{Cl}^-]_o$ (128.3 ± 6.7 mM) and enhanced a source/sink compartment in the deep granule cell layer of the dentate gyrus (Fig. 2B1). CBX administration also decreased the maximum $\text{Cl}^-$ extrusion after stimulation to an average of 218 ± 15 mM. Importantly, the $\text{Cl}^-$ movement pattern between layers was significantly affected by CBX application in that the maximum peak shifted upward to 400 µM with 245.7 ± 21.7 mM, followed by a minimum peak at 600 µM with 212 ± 16.1 mM. In general, $[\text{Cl}^-]_o$ was decreased in the dentate gyrus and CA3 area (Fig. 2B2; $P = 0.5421$, Wilcoxon matched pairs $t$-test, $n = 60$). The CSD analysis displayed a source of ions at 600 µM (s. lacunosum-moleculare), and one prominent sink at 400 µM depth (s. radius), implying an overall interruption of $\text{Cl}^-$ distribution and clearance mechanisms caused by CBX (Fig. 2B2). CBX also significantly reduced the $\text{Cl}^-$ undershoot ($[\text{Cl}^-]_o$ clearing) by an average of 97% from 19 to 0.5 mM (Fig. 2B3; $P = 0.0002$, Wilcoxon matched pairs $t$-test, $n = 60$). Last, CBX influenced the occurrence of the $\text{Cl}^-$ undershoot phenomenon in different layers. From 0 to 500 µM, we observed a lack of $\text{Cl}^-$ clearance undershoot, while a marginal undershoot persisted within the layers 600 to 1,300 µM (average of 4 mM; Fig. 2B3).

**Time constant of $\text{Cl}^-$ clearing**

To evaluate the efficacy of the $\text{Cl}^-$ clearance process between layers, we fitted the decaying $\text{Cl}^-$ increase after stimulation with an exponential curve and calculated the time constants of the decay (Fig. 3A). On average, the time constant was similar between layers (average of 83.4 ± 15.9 ms) with a notable peak increase to 137 ms at a depth of 900 µM (granule cell layer of the dentate gyrus). After CBX application, time constants increased on average by 148% to 207.1 ± 44.4 ms and were particularly increased for the upper layers 0 to 600 µM (average of 258.8 ± 19.2 ms; Fig. 3B). We separated the early and late clearing mechanisms as described in the preceding text. Our analysis shows that the time constants for the late clearance did not vary between layers under control conditions (Fig. 3A). Thus this result indicates that by blocking glial gap-junction pathways with CBX, the mechanisms of $\text{Cl}^-$ clearance within the hippocampus were drastically slowed down ($P = 0.0002$, Wilcoxon matched pairs $t$-test, $n = 60$).

**Layer-specific differences of $E_{\text{Cl}}$ and $E_{\text{GABA}}$ and $\text{Cl}^-$ driving force**

The significant increase in the $[\text{Cl}^-]_o$ has important consequences for the functioning of neurons. To estimate these effects, we calculated $E_{\text{Cl}}$, $E_{\text{GABA}}$, and the $\text{Cl}^-$ driving force during the control condition as well as after stimulation-induced strong neuronal activity and during the disruption of glial gap junction communication by CBX. These calculations were based on the above-mentioned values.

The driving force of a given ion is the difference between the membrane potential ($V_m$) and the equilibrium potential ($E_i$). The $V_m$ is minimally affected by the $[\text{Cl}^-]_o$ ($\Delta V_m = 5.7$ mV, Fig. 4A) but exerts a prominent effect on the driving force (Goldman 1943): the increase of $[\text{Cl}^-]_o$ augments the $\text{Cl}^-$ driving force if the intracellular $[\text{Cl}^-]_i$ is constant (Fig. 4B).
[Cl\textsuperscript{−}]\textsubscript{i} is around 10 mM, then the [Cl\textsuperscript{−}]\textsubscript{o} will determine the direction of the Cl\textsuperscript{−} movement. It is important to note that in this calculation the concentration of K\textsuperscript{+} is kept constant. However, [Cl\textsuperscript{−}]\textsubscript{o} values determine [Cl\textsuperscript{−}]\textsubscript{i} levels (Eq. 1), and these are also dependent on [K\textsuperscript{+}]\textsubscript{o}; at high [K\textsuperscript{+}]\textsubscript{o} (8 mM, reflecting values in epileptic activity), the [Cl\textsuperscript{−}]\textsubscript{o} increase results in larger [Cl\textsuperscript{−}]\textsubscript{i} augmentations than at low [K\textsuperscript{+}]\textsubscript{o} levels (3 mM; Fig. 4C). In addition, [K\textsuperscript{+}]\textsubscript{o} represents a major factor in determining E\textsubscript{Cl} and E\textsubscript{GABA} in that the increase of [K\textsuperscript{+}]\textsubscript{o} significantly alters E\textsubscript{Cl} and E\textsubscript{GABA}. At high [Cl\textsuperscript{−}]\textsubscript{o} concentrations (260 mM), E\textsubscript{Cl} is slightly shifted to more depolarized values than at low [Cl\textsuperscript{−}]\textsubscript{o} values (135 mM), while E\textsubscript{GABA} displays a shift toward hyperpolarization (Fig. 4D). The E\textsubscript{Cl} and E\textsubscript{GABA} are also influenced by [Cl\textsuperscript{−}]\textsubscript{o}. Therefore we employed Eq. 2 (see preceding text) and plotted the change of E\textsubscript{Cl} at increasing [Cl\textsuperscript{−}]\textsubscript{o} concentrations (100–260 mM) at three different [Cl\textsuperscript{−}]\textsubscript{i} values. E\textsubscript{Cl} reached more hyperpolarized values (\Delta E\textsubscript{Cl} = 24.5 mV) with increasing [Cl\textsuperscript{−}]\textsubscript{o} values and was lowest when [Cl\textsuperscript{−}]\textsubscript{i} reached a level of 15 mM (Fig. 4E). Because the movement of Cl\textsuperscript{−} ions is accompanied by HCO\textsubscript{3}\textsuperscript{−}
changes through GABA<sub>A</sub> receptors, we also calculated the \( E_{GABA} \) by employing Eq. 3. The resulting curves display shapes identical to the \( E_{Cl} \) but are shifted to more depolarized values (Fig. 4E). The combination of Eqs. 1, 2, 4, and 5, which takes into account the \( Cl^- \) mediated K<sup>+</sup> change, shows that the increase of \([Cl^-]_o\) actually decreases the \( Cl^- \) driving force (Fig. 4F).

Concentration of \( Cl^- \) and driving force changes across hippocampal layers

When both \( Cl^- \) clearance measurements of maximum \( Cl^- \) extrusion and \( Cl^- \) undershoot are added together, the result is a value of total \( Cl^- \) clearance after a stimulation-induced activity—from the maximum \( Cl^- \) increase to the low point of the undershoot (Fig. 5A). On average, 120.1 ± 30.5 mM of \( Cl^- \) are cleared from the extracellular space after the stimulation. The pattern of \( Cl^- \) movement follows closely the outline of the maximum \( Cl^- \) extrusion points with a sharp decrease within the granuleus layer (57.2 mM \( Cl^- \) clearance) and two high points in the s. lacunosum-moleculare (154.7 mM) and hilus (154 mM), respectively.

Because the \([Cl^-]_o\) increased in a nonhomogeneous way after strong stimulation, the \( Cl^- \) driving force should also differ between the various hippocampal layers. Using \([Cl^-]_o\) values obtained from our experiments and employing the combined equations illustrated in the preceding text (Fig. 4F), we estimated the \( Cl^- \) driving force in different hippocampal layers. During control conditions, the \( Cl^- \) driving force was the lowest in the CA1 s. radiatum, increased in the outer molecular layer, and sharply decreased in the inner molecular layer. Although a change in driving force was calculated, the overall difference between layers were minimal (\( \Delta = 1.8 \) mV). However, stimulation-induced changes in the \([Cl^-]_o\) significantly decreased the driving force and resulted in large fluctuations between layers (\( \Delta = 9.4 \) mV). The lowest value was observed in the s. radiatum of the CA1 and the hilar region (7.7 mV) while the highest value was calculated for the granule cell layer of the dentate gyrus (16.3 mV, Fig. 5B). Last we determined the \( Cl^- \) driving force after CBX administration. Our results indicate that overall CBX did not significantly alter the \( Cl^- \) driving force as compared with the control condition; however, the layer dependent fluctuations were drastically decreased (\( \Delta = 3.5 \) mV).

**DISCUSSION**

In the present study, we report that \([Cl^-]_o\) values differ in various hippocampal layers during normal brain activity, \([Cl^-]_o\) levels significantly increase after stimulation, the stimulation-induced increase in \([Cl^-]_o\) levels can be modified by gap junction blocking agents, and the driving force for \( Cl^- \) significantly decreases after stimulation and shows large layer-specific variations.

Based on previous anatomical observations, we predicted different \([Cl^-]_o\) in various hippocampal layers and that this variation would be activity dependent and mediated by the gap junction coupled astrocytic network (Sik et al. 2000). Astroglial cells play a role in controlling K<sup>+</sup> levels (potassium siphoning: Newman et al. 1984) and spatial buffering (Orkand et al. 1966) and are also suggested to engage in the redistribution of anions such as \( Cl^- \). These processes are believed to be coupled in that a cationic influx into glial cells is accompanied by an anionic influx balancing the positive charge of the entering K<sup>+</sup> (Bormann and Kettenmann 1988; Hodgkin and Horowicz 1959; Kimelberg and Kettenmann 1990; Kimelberg et al. 1990). Previously, we proposed that CIC-2 in astrocytes might be part of a \( Cl^- \) buffering/redistribution mechanism set in place to collect \( Cl^- \) from areas of lower requirement for this anion (i.e., where glutamatergic synaptic activity occurs) and redistribute it to layers in which the demand for \( Cl^- \) is higher (where appropriate GABA<sub>A</sub> receptor-mediated inhibition is required).

Our current results are, however, only partly in line with this prediction showing that during spontaneous activity, \([Cl^-]_o\) is higher in s. pyramidalde and lower in s. radiatum. The high level of \([Cl^-]_o\) in the hilar region as well as the observed low \([Cl^-]_o\) in s. oriens and lacunosum-moleculare are difficult to explain considering the distribution of CIC-2-expressing astrocytic endfeet alone. Thus other mechanisms might also be operational here: glial cells could morphologically, electrophysiologically, and thus functionally differ in various hippocampal regions as also suggested by previous reports (D’Ambrosio et al. 1998). Another possible explanation is that additional pumping mechanisms play a role in \([Cl^-]_o\) regulation. Indeed the expression pattern of several proteins important in \( Cl^- \) regulation has been shown to be uneven within the hippocam-
pal formation. The relative distribution of NKCC1 (which pumps Cl\(^-\) into neurons), and KCC2 (which causes Cl\(^-\) efflux) show a complementary pattern (Gulyas et al. 2001; Kang et al. 2002). Importantly, ion movement has been shown to be regulated by blood flow through the neural tissue and that vascularization in the hippocampus is not uniform (Coyle 1978, 1976). The main vascular pathway is through the longitudinal hippocampal artery which follows the hippocampal fissure, vascularizing mostly the s. oriens and lacunosum-moleculare in the CA1 but also the molecular layer and hilus of the dentate gyrius. Increased vascularization results in faster ion transport into the blood flow ([Cl\(^-\)] in plasma: \(\sim 102 \text{ mM}\) (Kandel et al. 2000). Therefore the [Cl\(^-\)]\(_{o}\) in s. oriens and lacunosum-moleculare should be lower than expected. Although it is likely that vascularization plays an important role in ion distribution, the unexpected very fast [Cl\(^-\)]\(_{o}\) increase cannot be attributed to hemodynamic differences.

As we predicted, our results show [Cl\(^-\)]\(_{o}\) elevation during strong stimulation. In contrast to an earlier study (Dietzel et al. 1982), we measured substantial [Cl\(^-\)]\(_{o}\) increases. This previous report indicated a small [Cl\(^-\)]\(_{o}\) increase induced by strong network activity in the cat cortex (Dietzel et al. 1982). As the authors noted, there was a discrepancy between a) the observed shrinkage of the extracellular space (ECS) (which would induce an increasing osmolarity), and the measured [Cl\(^-\)]\(_{o}\) increase. Several authors, who employed various methods, have shown a \(\sim 30\%\) decrease of the ECS during excessive neuronal activity (Dietzel et al. 1980; Freygang and Landau 1955; McBurney et al. 1990; Van Harreveld and Khattab 1967). Based on calculations, the observed ECS reduction should have caused [Cl\(^-\)]\(_{o}\) to increase \(\approx 210 \text{ mM}\), but Dietzel and colleagues observed only a 7 mM increase. This significant underestimation of the reported [Cl\(^-\)]\(_{o}\), which was acknowledged by the authors, could be explained by various mechanisms: first, the Cl\(^-\) exchangers in the ion sensitive electrode utilized in the study had low sensitivity for Cl\(^-\) (Corning 477315 and Orion exchangers). Second, the concomitant [HCO\(_3\)]\(_{o}\) change could have affected the measurement of [Cl\(^-\)]\(_{o}\), resulting in a reduced electrode response to [Cl\(^-\)]\(_{o}\) increases (Deisz and Lux 1978). In contrast, our electrodes are highly selective for Cl\(^-\) and virtually unaffected by HCO\(_3\)\(_{o}\) (see METHODS). Third, the authors used halothane anesthesia, a gap junction blocker which disrupts the glial communication and thus the buffering and redistribution mechanism. Therefore our observations are in line with the theoretical value of a \(\sim 210 \text{ mM}\) [Cl\(^-\)]\(_{o}\) increase, and we conclude that our measurements likely reflect the true [Cl\(^-\)]\(_{o}\) increase.

Augmentations of [Cl\(^-\)]\(_{o}\) of such magnitude have substantial effects on the Cl\(^-\) driving force as this force is coupled to the transmembrane distribution of Cl\(^-\). Our calculations clearly show layer-specific differences in the driving force for Cl\(^-\). Under nonstimulated control conditions, the driving force is high and shows small but measurable amounts of layer specific fluctuations. Several studies previously reported a stimulation-induced decrease of the Cl\(^-\) driving force in in vitro preparations (Barker and McBurney 1979; Gallagher et al. 1978; Thompson and Gahwiler 1989a). The authors concluded that [Cl\(^-\)]\(_{i}\) is the main cause for the observed decrease in inhibition. Although the increase in [Cl\(^-\)]\(_{o}\) is a critical factor in activity-dependent disinhibition, we propose that the measured in vitro values are underestimations of this effect (Fig. 4F) because the [Cl\(^-\)]\(_{o}\) was kept at a constant low level in the in vitro bath solution (\(~ 135 \text{ mM}\)). Our data and calculations indicate that the driving force is decreased in the CA1-3 layers, whereas in the dentate granule cell somata and dendrites the driving force is significantly higher. In this context it needs to be noted that the \(E_{\text{GABA}}\) might be different in subclasses of inhibitory cells due to differences in the expression of KCC2 (Gulyas et al. 2001). In addition our calculation is not based on measured [Cl\(^-\)]\(_{i}\) values (Hara et al. 1992; Kuner and Augustine 2000) but instead is founded on calculated concentrations. These studies showed an uneven [Cl\(^-\)]\(_{o}\) distribution in cultured neurons, in which conditions the expression of numerous proteins, including various ion channels, does not reflect in vivo patterns.

One of the most interesting observations in the present study is the high level of [Cl\(^-\)]\(_{o}\) measured after stimulation. Because the Cl\(^-\) level in the blood is \(\sim 102 \text{ mmol}, lower than in the brain (Kandel et al. 2000), other sources of Cl\(^-\) were considered to explain the surge of [Cl\(^-\)]\(_{o}\). Intracellularly, [Cl\(^-\)]\(_{i}\) may vary between cell types—neurons display a lower concentration (5 mM) than astrocytes (6 mM) (Ballanyi et al. 1987) or oligodendrocytes (40 mM) (Kettenmann 1987). Although it is probable that Cl\(^-\) efflux from glial cells plays an important role, it is likely that a change in the ECS volume is also essential in contributing to the large increases in [Cl\(^-\)]\(_{o}\). It is known that activity-dependent ECS shrinkage results from the movement of water and electrolytes into glial cells (Ransom et al. 1985). Moreover, the ECS in the CA1 pyramidal layer of the hippocampus is \(>30\%\) smaller than in the CA3 pyramidal layer (McBain et al. 1990). Therefore a high [K\(^+\)]\(_{o}\) level through stimulation can lead to a \(\sim 30\%\) shrinkage of ECS and augment neuronal activity, resulting in increasing [Cl\(^-\)]\(_{o}\) levels \(>210 \text{ mM}\)—mainly caused by rapid water movement into both glial cells and neurons (also see Dietzel et al. 1980). Therefore swift changes in the ECS volume should also result in rapid increases of [K\(^+\)]\(_{o}\). One explanation for why this fast and high level of ion increase has not been reported previously may be due to the kinetics of K\(^+\) sensitive electrodes. The sensitivity is in the range of seconds and thus prevents the measurement of fast ion movement. We thus propose that similarly sudden [K\(^+\)]\(_{o}\) increase occur after intense network activity, which is quickly dissipated by the [K\(^+\)]\(_{o}\) spatial buffering.

The rapid increase and subsequent slower decrease of [Cl\(^-\)]\(_{o}\) indicate that several mechanisms are responsible for [Cl\(^-\)]\(_{o}\) regulation. The very short time constant of the [Cl\(^-\)]\(_{o}\) increase indicates that cation-anion transporters or passive channel-mediated mechanisms are only partially responsible for the effect due to their slower kinetics (Payne 1997; Williams and Payne 2004). Therefore we hypothesize that the initial Cl\(^-\) increase and decrease is due to a combined effect of Cl\(^-\) efflux/influx from glial cells and a rapid shrinkage in the ECS caused by intense neuronal activity. Regional variations in the extracellular space of the hippocampus have already been reported (McBain et al. 1990). Potassium-induced shrinkage was investigated only in somatic layers showing similar (\(~ 30\%) shrinkage in both CA3 and CA1 pyramidal layers. Considering the different composition of dendritic versus cellular layers (i.e.: astroglia and neuron content), the initial difference in the extracellular space, and difference in aquaporin 4 expression in hippocampal layers (Badau et al. 2002), it is feasible that hippocampal layers shrink differently. The second phase of the Cl\(^-\) clearing process could be related to astroglial clearance and redistribution mechanisms. Because in-
creased neuronal activity increases blood flow, circulation also likely plays a part in the restoration of normal ion homeostasis. Although the observed undershoot in $[\text{Cl}^-]$ is similar to other compensatory mechanisms such as the $O_2$ concentration change after neuronal activity (overshoot in this case) (Viswanathan and Freeman 2007), blood-flow mediated overcompensation is unlikely in our study because CBX abolished the $[\text{Cl}^-]$ undershoot. Instead the effect of the gap junction blocker suggests that glial redistribution mechanisms are responsible for the observed $[\text{Cl}^-]$ undershoot.

Several lines of evidence indicate that the lipophilic CBX easily crosses the blood-brain barrier (Dobbins and Saul 2000; Jellinck et al. 1993; Traub et al. 2002) exerting anticonvulsant effects after systemic administration (Bostanci and Bagirici 2007; Gareri et al. 2004a,b; Gigout et al. 2006). Although high concentrations (400 mg/kg) of carbenoxolone can have unspecific effects (Hosseinza-deh and Nassiri Asl 2003), several reports demonstrate that CBX has no significant effect on intrinsic neuronal properties (Draguhn et al. 1998; Kohling et al. 2001; Middleton et al. 2008; Pais et al. 2003; Schmitz et al. 2001; Yang and Michelson 2001) or GABA currents (Yang and Michelson 2001; Zsiros et al. 2007), while other reports claim to demonstrate unspecific actions of CBX in cell cultures (Rouach et al. 2003) and in the retina (Vessey et al. 2004). Most recently, a study demonstrated direct actions of CBX on synaptic transmission in mouse neuronal cultures (Tovar et al. 2009). Although the main effect was observed on glutamate transmission, a rapidly and fully reversible inhibition of GABA$_A$ IPSCs was observed. Considering that glial gap junctions vastly outnumber neuronal coupling (≥10–100:1), even if CBX has unspecific effects on neuronal excitability, the CBX mediated effect most likely reflects the action on the glial network. Until the extent and precise unspecific action of CBX on neuronal transmission is fully established in vivo, caution needs to be taken when gap junction blocking effect of CBX is considered. However, it needs to be noted that CBX also exerts an effect on peripheral blood pressure, which might influence the layer specific $\text{Cl}^-$ clearing mechanisms in the hippocampus. Most of the research reports show increases of blood pressure after CBX administration (Ullian et al. 1996; Walker et al. 1992), but there are also results indicating NO-mediated blood pressure decreases (Chaytor et al. 2000; Dembinska-Kiec et al. 1991). Because the effect of CBX on blood circulation is somewhat ambiguous, the possibility of a CBX-mediated vascular component of $\text{Cl}^-$ clearing mechanisms may require further investigations.

Our findings clearly demonstrate that $[\text{Cl}^-]_o$ is neither evenly distributed throughout hippocampal layers under control conditions nor that there is a uniform increase of $[\text{Cl}^-]_o$ after increased neuronal activity. We therefore suggest that future research should take these $[\text{Cl}^-]_o$ changes into consideration for their measurements, especially if studies are conducted in vitro preparations and investigate $\text{Cl}^-$ driving forces.

Author contributions

All authors contributed either to the conception and design of the experiments or analysis and interpretation of data as well as drafting the article and revising it critically for final approval of the version to be published. All experiments were conducted in the Centre de recherche Universite Laval Robert-Giffard, Quebec.

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