Activity dependent layer-specific changes in the extracellular chloride concentration and chloride driving force in the rat hippocampus

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Abstract

The transmembrane distribution of chloride anions (Cl-) determines the direction of the Cl- flux through GABA_A-receptors which 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±1.3mM) than the CA3/dentate gyrus areas (117.7±1.2mM). 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±51.1mM) and in the hilus (261±43.7mM) while in the granular cell layer it reached only 159.5±41mM. 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 ±0.7mM) 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 the Cl- clearance was similar between layers (83.4±15.9ms), but increased after carbenoxolone application (207.1± 44.4ms). Stimulation-induced changes in the [Cl-]_o significantly decreased the Cl- driving force and resulted in large fluctuations between layers (Δ=9.4mV). The lowest value was observed in the stratum radiatum of the CA1 and the hilar region (7.7mV) whilst the highest value was calculated for the granule cell layer (16.3mV). 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 gamma-amino-butyric-acid (GABA) A receptors. The gradient between the extra- and intracellular Cl⁻ concentrations establishes whether GABAₐ receptor-mediated responses are hyperpolarizing or depolarizing in neurons (Kaila 1994). Activity-dependent disinhibition is a reduction in the efficacy of synaptically activated GABAₐ 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 GABAₐ 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 homogenously (Hara et al. 1992; Kuner and Augustine 2000). Thus, if the extracellular Cl⁻ ([Cl⁻]₀) is to be considered homogeneous (Dietzel et al. 1982), the driving force for Cl⁻ flux, which is the difference between the membrane potential (Vₘ) and Cl⁻ equilibrium potential (Eₘ), varies from cell compartment to compartment. On the other hand, if the [Cl⁻]₀ is not as homogeneous as suggested by previous reports (Dietzel et al. 1982), then the driving force for Cl⁻ can vary considerably. Thus [Cl⁻]₀ is an important factor in the activity-dependent disinhibition which can vary from one neuronal compartment to another. Since 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 E₆GABA 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 (ClC-2 in neurons and astroglial cells) only passively distribute Cl⁻. Though 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; Kimelberg et al. 1990; Kimelberg and Kettenmann 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).

In order to address the questions whether the distribution of [Cl⁻]o is indeed heterogeneous and whether this distribution is activity dependent, we recorded [Cl⁻]o in the extracellular compartment using double-barrel micropipettes filled with a Cl⁻ sensitive solution. We provide evidence that a) the [Cl⁻]o indeed differs layer by layer, b) that this phenomenon is activity dependent, and c) 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 anaesthetized through i.p. injection of urethane (1.3-1.5 g/kg) and placed in a stereotaxic apparatus (Narishige, Japan). Anaesthesia 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 (centred at -3.6mm anterio-posterior, 2.5mm medio-lateral). 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 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 Institute 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 Cl-
sensitive barrels of the micropipettes were pre-treated with dimethylchlorosylane and the
pipettes were then baked at 120ºC for 2 h. The tip was filled with Cl⁻-sensitive ionophore I-
cocktail A (Fluka, Neu-Ulm, Germany), and backfilled with a solution of NaCl (0.1 M). The other non-selective barrel (blank) was filled with NaCl 0.2 M. The Cl⁻-sensitive barrel
was calibrated using the following solutions: A) 100 mM NaCl solution, B) 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 (LFP), the latter were measured with the pair electrode and subtracted from the
former. The resulting signal was then compared to the points taken from the calibration
solutions in order to assign Cl⁻ values to the signal. The relationship between concentration
and voltage was derived in accordance with the Nicolsky–Eisenmann equation (Ammann
1986). The Cl⁻ sensitivity was of 15-20 mV per decade. The logarithmic selectivity of the
electrode to HCO₃⁻ was -1.5 (Kondo et al. 1989) resulting in about 3% sensitivity. Thus at
least 97% of the signal recorded by the electrode was originated from Cl⁻.
Drug administration

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

Stimulation

In this study we recorded the extracellular chloride concentration throughout hippocampal layers every 100 µm using double-barrel Cl⁻-sensitive micropipettes. For each 100 µm step we recorded a 1 min control period prior to stimulation onset. Then each 5 seconds 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 [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 ksample/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 3900µm; 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):

$$\frac{\partial^2 \varphi}{\partial z^2} = \frac{\varphi(z + n \cdot \Delta z) - 2 \varphi(z) + \varphi(z - n \cdot \Delta z)}{(n \cdot \Delta z)^2},$$

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

The following equations were used for calculations.

For calculating the thermodynamic driving force for $K^+-Cl^-$ at equilibrium level of $[Cl^-]_i$ the Henderson-Hasselbalch equation was used (Williams and Payne 2004):

$$[Cl^-]_i = [Cl^-]_o*[K^+]_o/[K^+]_i \quad [1]$$

For calculating $E_{Cl}$ we employed the Nernst equation:

$$E_{Cl} = -61.5*\text{Log10}([Cl^-]_o/[Cl^-]_i) \quad [2]$$

To calculate the $E_{GABA}$ which takes into account $HCO_3^-$ movement through GABA receptors, we used the following equation:

$$E_{GABA} = -61.5*\text{Log10}\{([Cl^-]_o + \alpha[HCO_3^-]_o)/([Cl^-]_i + \alpha[HCO_3^-]_i)\} \quad [3]$$

Where $\alpha$ is the conversion factor for $HCO_3^-$, and equals to 0.3.

Membrane potential was calculated using the Goldman–Hodgkin–Katz equation:

$$V_M = -61.5*\text{Log10}\{(P_K*[K^+]_i+P_{Na}*[^{Na^+}]_i)/P_{Cl^+}[Cl^-]_o(\alpha[HCO_3^-]_o+P_{Na}*[^{Na^+}]_o+P_{Cl^+}[Cl^-]_i)\} \quad [4]$$
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 ($E_{Cl}$):

$$\text{Driving force} = V_M - E_{Cl}$$  \[5\]

Parameters not specified in the figure legends are to be considered related to normal physiological conditions, as follows: $[\text{HCO}_3^-]_i = 16$ mM; $[\text{HCO}_3^-]_o = 25$ mM (i.e. $pH_i = 7.2$ and $pH_o = 7.4$, 5% CO$_2$); temperature 37°C. The relative permeability of GABA$_A$ receptors to HCO$_3^-$ versus Cl$^-$ is 0.3 (Kaila 1994). Ionic permeabilities: $P_K = 100$; $P_{Na} = 4$; $P_{Cl} = 45$; $[Na^+]_i = 15$ mM; $[Na^+]_o = 150$ mM.

**Results**

*Extracellular chloride concentration under normal condition*

The main purpose of our study was to measure the distribution of $[Cl^-]_o$ in hippocampal layers in order 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 (Fig. 1A2) 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 0-700 μm layers ($112.9 \pm 1.3$ mM) as compared to deeper layers 1200-1700 μm ($117.7 \pm 1.2$ mM). A sharp reversal between a Cl$^-$ increase and a Cl$^-$ decrease was observed in layers located at 700 and 800 μm, corresponding to the str. moleculare of the dentate gyrus. Our CSD analysis revealed a Cl$^-$ source (Cl$^-$ influx into cells) at the depth of 800 μm with a sink (Cl$^-$ efflux from cells) were located directly above at 700 μm (Fig. 2A1).
Extracellular Cl\textsuperscript{-} concentration after stimulation

Because our working model predicted that Cl\textsuperscript{-} redistribution is activity dependent (Sik et al. 2000), the pattern of Cl\textsuperscript{-} transport was also assessed during stimulation-induced activity. In general, both single stimulations and trains of stimulation sharply increased the [Cl\textsuperscript{-}]\textsubscript{o} which gradually decreased to baseline levels and temporarily even below the baseline (Fig. 1B). We called the period of the initial [Cl\textsuperscript{-}]\textsubscript{o} decrease to the baseline level \textit{early Cl\textsuperscript{-} clearance}, and the decrease of [Cl\textsuperscript{-}]\textsubscript{o} below the baseline \textit{late Cl\textsuperscript{-} clearance} period. The difference between the baseline and minimum [Cl\textsuperscript{-}]\textsubscript{o} was determined as \textit{Cl\textsuperscript{-} undershoot} (Fig. 1B). As a pilot measurement we used two stimulation intensities (1.0 and 1.5mA) to determine the relationship between intensity of the stimulation and [Cl\textsuperscript{-}]\textsubscript{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\textsuperscript{-} increase as well as a stronger Cl\textsuperscript{-} undershoot (max. Cl\textsuperscript{-} increase for 1.5mA was 195.8 ± 2.5 mM vs. 167.5 ± 3.0 mM for 1.0mA; whilst Cl\textsuperscript{-} undershoot for 1.5mA was 104.6 ± 1.0 mM vs. 109.6 ± 0.9 mM for 1.0mA). 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 [Cl\textsuperscript{-}]\textsubscript{o} on average to 221.6 ± 28.5 mM across layers, up from 120 mM baseline (Fig. 2A2; n=60). The maximum [Cl\textsuperscript{-}]\textsubscript{o} rise after stimulation reached two peaks, one at around 600 μm (253.4 ± 51.1 mM; str. lacunosum-moleculare), the other at 1100 μm (261 ± 43.7 mM; hilar region), 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 Figure 2A2, the amplitude difference between the maximum and minimum [Cl\textsuperscript{-}]\textsubscript{o} increase after stimulation is substantial (as much as 101.5 mM) between layers. The CSD analysis revealed three Cl\textsuperscript{-} sinks at 100 μm (str. oriens), 600 μm (str. lacunosum-moleculare, and
1100 μm (hilus), as well as a source at 0 μm (alveus/str. oriens) and at 900 μm (str. granulorum).

Chloride clearing mechanism

The increase in [Cl\(^-\)]\(_o\) after stimulation was followed by a period of decreasing [Cl\(^-\)]\(_o\), probably as a result of clearing mechanisms setting in. However, we observed an actual undershoot of [Cl\(^-\)]\(_o\), in the sense that extracellular Cl\(^-\) level fell below the control values, and only gradually reached equilibrium (Fig. 1B). The undershoot of Cl\(^-\) (Cl\(^-\) clearance) reached a maximum amplitude of 21.6 ± 0.7 mM at a depth of 400 μm (str. radiatum) and remained constant in levels below this depth (average of 20.9 ± 1.5 mM) (Fig. 2A3). Thus, for most of the hippocampus (82 %), we observed an undershoot of 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: Cl\(^-\) influx and efflux were not spatially segregated.

Effect of gap junction blocker on [Cl\(^-\)]\(_o\) distribution

Since 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 (100mg/kg) slightly increased the baseline [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 Cl\(^-\) extrusion after stimulation to an average of 218 ± 15 mM. Importantly, the Cl\(^-\) movement pattern between layers was significantly affected by CBX application in that the maximum peak shifted upwards to 400 μm with 245.7 ± 21.7 mM, followed by a minimum peak at 600 μm with 212 ± 16.1 mM. In general, [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. radiatum), implying an overall interruption of Cl\(^-\) distribution and clearance mechanisms caused by CBX (Fig.
CBX also significantly reduced the Cl\textsuperscript{−} undershoot ([Cl\textsuperscript{−}]\textsubscript{o} clearing) by an average of 97% from 19 mM to 0.5 mM (Fig. 2B3; p=0.0002, Wilcoxon matched pairs t-test, n=60).

Lastly, CBX influenced the occurrence of the Cl\textsuperscript{−} undershoot phenomenon in different layers. From 0 to 500 μm, we observed a lack of Cl\textsuperscript{−} clearance undershoot, whilst a marginal undershoot persisted within the layers 600 to 1300 μm (average of 4 mM) (Fig. 2B3).

**Time constant of Cl\textsuperscript{−} clearing**

In order to evaluate the efficacy of the Cl\textsuperscript{−} clearance process between layers, we fitted the decaying Cl\textsuperscript{−} 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 mechanism as described above. 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 Cl\textsuperscript{−} clearance within the hippocampus were drastically slowed down (p=0.0002, Wilcoxon matched pairs t-test, n=60).

**Layer specific differences of E\textsubscript{Cl} and E\textsubscript{GABA} and Cl\textsuperscript{−} driving force**

The significant increase in the [Cl\textsuperscript{−}]\textsubscript{o} has important consequences for the functioning of neurons. To estimate these effects we calculated 1) E\textsubscript{Cl}, 2) E\textsubscript{GABA} and 3) the Cl\textsuperscript{−} 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 \( (E_{CI}) \). The \( V_m \) is minimally affected by the \( [CI^-]_o \) \( (\Delta V_m = 5.7 \, mV, \text{Fig. 4A}) \) but exerts a prominent effect on the driving force \( (Goldman, 1943) \): the increase of \( [CI^-]_o \) augments the \( CI^- \) driving force if the intracellular \( [CI^-]_i \) is constant \( (\text{Fig. 4B}) \). If \( [CI^-]_i \) is around 10 mM, then the \( [CI^-]_o \) will determine the direction of the \( CI^- \) movement. It is important to note that in this calculation the concentration of \( K^+ \) is kept constant. However, \( [CI^-]_o \) values determine \( [CI^-]_i \) levels \( (\text{equation 1}) \) and these are also dependent on \( [K^+]_o \): at high \( [K^+]_o \) (8 mM, reflecting values in epileptic activity) the \( [CI^-]_o \) increase results in larger \( [CI^-]_i \) augmentations than at low \( [K^+]_o \) levels (3 mM; \text{Fig. 4C}). In addition, \( [K^+]_o \) represents a major factor in determining \( E_{CI} \) and \( E_{GABA} \) in that the increase of \( [K^+]_o \) significantly alters \( E_{CI} \) and \( E_{GABA} \). At high \( [CI^-]_o \) concentrations (260 mM), \( E_{CI} \) is slightly shifted to more depolarized values than at low \( [CI^-]_o \) values (135 mM), while \( E_{GABA} \) displays a shift towards hyperpolarization \( (\text{Fig. 4D}) \). The \( E_{CI} \) and \( E_{GABA} \) are also influenced by \( [CI^-]_o \).

Therefore, we employed equation 2 \( (\text{see above}) \) and plotted the change of \( E_{CI} \) at increasing \( [CI^-]_o \) concentrations \( (100-260 \, mM) \) at three different \( [CI^-]_i \) values. \( E_{CI} \) reached more hyperpolarized values \( (\Delta E_{CI}= 24.5 \, mV) \) with increasing \( [CI^-]_o \) values and was lowest when \( [CI^-]_i \) reached a level of 15 mM \( (\text{Fig. 4E}) \). Because the movement of \( CI^- \) ions is accompanied by \( HCO_3^- \) changes through \( GABA_A \) receptors, we also calculated the \( E_{GABA} \) by employing equation 3. The resulting curves display shapes identical to the \( E_{CI} \), but are shifted to more depolarized values \( (\text{Fig. 4E}) \). The combination of equations 1, 2, 4 and 5, which takes into account the \( CI^- \) mediated \( K^+ \) change, shows that the increase of \( [CI^-]_o \) actually decreases the \( CI^- \) driving force \( (\text{Fig. 4F}) \).

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

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

Since the [Cl⁻]₀ increased in a non-homogeneous way after strong stimulation, the Cl⁻ driving force should also differ between the various hippocampal layers. Using [Cl⁻]₀ values obtained from our experiments and employing the combined equations illustrated above (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 str. radiatum, increased in the outer molecular layer and sharply decreased in the inner molecular layer. Though a change in driving force was calculated, the overall difference between layers were minimal (Δ= 1.8mV). However, stimulation-induced changes in the [Cl⁻]₀ significantly decreased the driving force and resulted in large fluctuations between layers (Δ= 9.4mV). The lowest value was observed in the str. radiatum of the CA1 and the hilar region (7.7mV) whilst the highest value was calculated for the granule cell layer of the dentate gyrus (16.3mV, Fig. 5B). Lastly, 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 to the control condition, however, the layer dependent fluctuations were drastically decreased (Δ= 3.5mV).

**Discussion**

In the present study we report that 1) [Cl⁻]₀ values differ in various hippocampal layers during normal brain activity, 2) [Cl⁻]₀ levels significantly increase after stimulation, 3) the stimulation-induced increase in [Cl⁻]₀ levels can be modified by gap junction blocking agents, and 4) the driving force for Cl⁻ significantly decreases after stimulation and shows large layer-specific variations.

Based on previous anatomical observations, we predicted different [Cl⁻]₀ 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\(^+\) levels (potassium siphoning: Newman et al. 1984), 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\(^+\) (Bormann and Kettenmann 1988; Hodgkin and Horowicz 1959; Kimelberg et al. 1990; Kimelberg and Kettenmann 1990). Previously, we proposed that ClC-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\(_A\) 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 str. pyramidale and lower in str. radiatum. The high level of [Cl\(^-\)]\(_o\) in the hilar region, as well as the observed low [Cl\(^-\)]\(_o\) in str. oriens and lacunosum-moleculare, are difficult to explain considering the distribution of ClC-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 hippocampal 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 str. oriens and lacunosum-moleculare in the CA1, but also the molecular layer and hilus of the dentate gyrus. Increased vascularization results in faster ion transport into the blood flow ([Cl\(^-\)] in plasma: \(\sim 102\) mM, (Kandel et al. 2000). Therefore the [Cl\(^-\)]\(_o\) in str. oriens and lacunosum-moleculare should be lower than
expected. Though it is likely that vascularization plays an important role in ion distribution, the unexpected very fast $[\text{Cl}^-]_o$ increase cannot be attributed to hemodynamic differences.

As we predicted, our results show $[\text{Cl}^-]_o$ elevation during strong stimulation. In contrast to an earlier study (Dietzel et al. 1982) we measured substantial $[\text{Cl}^-]_o$ increases. This previous report indicated a small $[\text{Cl}^-]_o$ 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 b) the measured $[\text{Cl}^-]_o$ increase. Several authors, who employed various methods, have shown a ~30% decrease of the ECS during excessive neuronal activity (Dietzel et al. 1980; Freygang and Landau 1955; McBain et al. 1990; Van Harreveld and Khattab 1967). Based on calculations, the observed ECS reduction should have caused $[\text{Cl}^-]_o$ to increase up to 210 mM, but Dietzel and colleagues observed only a 7 mM increase. This significant underestimation of the reported $[\text{Cl}^-]_o$, 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 $[\text{HCO}_3^-]_o$-change could have affected the measurement of $[\text{Cl}^-]_o$, resulting in a reduced electrode response to $[\text{Cl}^-]_o$ increases (Deisz and Lux 1978). In contrast, our electrodes are highly selective for Cl$^-$ and virtually unaffected by $\text{HCO}_3^-$ (see Materials and 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 ~210 mM $[\text{Cl}^-]_o$ increase, and we conclude that our measurements likely reflect the true $[\text{Cl}^-]_o$ increase.

Augmentations of $[\text{Cl}^-]_o$ 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 non-stimulated 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 $[\text{Cl}^-]_i$ is
the main cause for the observed decrease in inhibition. Although the increase in $[\text{Cl}^-]_i$ is a critical factor in activity-dependent disinhibition, we propose that the measured \textit{in vitro} values are underestimations of this effect (Fig. 4F) since the $[\text{Cl}^-]_o$ was kept at a constant low level in the \textit{in vitro} bath solution (~135 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 $[\text{Cl}^-]_i$ values (Hara et al. 1992; Kuner and Augustine 2000), but instead is founded on calculated concentrations. These studies showed an uneven $[\text{Cl}^-]_i$ distribution in cultured neurons, in which conditions the expression of numerous proteins, including various ion channels, does not reflect \textit{in vivo} patterns.

One of the most interesting observations in the present study is the high level of $[\text{Cl}^-]_o$ measured after stimulation. Because the $[\text{Cl}^-]$ level in the blood is about 102 mmol, lower than in the brain (Kandel et al. 2000), other sources of $\text{Cl}^-$ were considered to explain the surge of $[\text{Cl}^-]_o$. Intracellularly, $[\text{Cl}^-]_i$, 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 $\text{Cl}^-$ 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 $[\text{Cl}^-]_o$. 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 $[\text{K}^+]_o$ level through stimulation can lead to a $\sim30\%$ shrinkage of ECS and augment neuronal activity, resulting in increasing $[\text{Cl}^-]_o$ levels above 210 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 $[\text{K}^+]_o$. One explanation for why this fast and high level of ion increase has not been reported previously may be due to the kinetics of $\text{K}^+$ 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⁺]₀ increase occur after intense network activity, which is quickly dissipated by the [K⁺]₀ spatial buffering.

The rapid increase and subsequent slower decrease of [Cl⁻]₀ indicate that several mechanisms are responsible for [Cl⁻]₀ regulation. The very short time constant of the [Cl⁻]₀ 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 (Badaut 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 increased neuronal activity increases blood flow, circulation also likely plays a part in the restoration of normal ion homeostasis.

Although the observed undershoot in [Cl⁻]₀ is similar to other compensatory mechanisms such as the O₂ concentration change after neuronal activity (overshoot in this case) (Viswanathan and Freeman 2007), blood-flow mediated overcompensation is unlikely in our study since CBX abolished the [Cl⁻]₀ undershoot. Instead, the effect of the gap junction blocker suggests that glial redistribution mechanisms are responsible for the observed [Cl⁻]₀ 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; Gareri et al. 2004b; Gigout et al. 2006). Though high concentrations (400mg/kg) of carbenoxolone can have unspecific effects (Hosseinzadeh 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), whilst 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 (at least 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 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). Since the effect of CBX on blood circulation is somewhat ambiguous, the possibility of a CBX-mediated vascular component of Cl^- clearing mechanisms may require further investigations.

Our findings clearly demonstrate that [Cl^-]o is neither evenly distributed throughout hippocampal layers under control conditions, nor that there is a uniform increase of [Cl^-]o after increased neuronal activity. We therefore suggest that future research should take these [Cl^-]o changes into consideration for their measurements, especially if studies are conducted in in vitro preparations and investigate 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 Université Laval Robert-Giffard, Quebec, Canada.

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**Figure legends**

**Figure 1.** Measurement of \([\text{Cl}^\text{-}]_o\) in the hippocampus. **A1** shows the Cl\(^{-}\) sensitive double-barrel glass pipette for recordings of both \([\text{Cl}^\text{-}]_o\) as well as local field potentials. **A2**, Calibration sample measuring 10 mM Cl\(^{-}\) and 100 mM Cl\(^{-}\). **B**, Sample trace of Cl\(^{-}\) signal after hippocampal CA3 stimulation. We observed a sharply increasing Cl\(^{-}\) concentration after single (inset) or train stimulation (upwards deflection), which was followed by an undershoot of Cl\(^{-}\) below the control level. Two possible clearing mechanisms (early and late) are indicated. **C**, Increase of stimulus intensity results in larger LFP responses as well as augmented maximum \([\text{Cl}^\text{-}]_o\) increase and larger Cl\(^{-}\) undershoot. **D**, Augmentation of stimulation intensity linearly increases \([\text{Cl}^\text{-}]_o\) and Cl\(^{-}\) undershoot. **E**, Similarly, the increase of stimulation intensity linearly changes LFP and \([\text{Cl}^\text{-}]_o\). Diamond: 1mA stimulation, rectangular 1.5mA stimulation. In both cases the regression point is close to the unstimulated \([\text{Cl}^\text{-}]_o\) value (117-118 mM).

**Figure 2.** Depth profiles of \([\text{Cl}^\text{-}]_o\) in control condition and following hippocampal stimulation. **A1**, Depth profile of \([\text{Cl}^\text{-}]_o\) throughout the hippocampus under control conditions (spontaneous activity preceding stimulation), and corresponding current source density (CSD) analysis, indicating sources (Cl\(^{-}\) influx to the cell) and sinks of Cl\(^{-}\) ion flow (Cl\(^{-}\) efflux from the cell). A schematic drawing of the hippocampus was superimposed onto the dept profile figure to indicate hippocampal structures CA1, CA3, dentate gyrus (DG) and all corresponding layers. Error bars represent SD. **A2**, \([\text{Cl}^\text{-}]_o\) increases after stimulation vary between hippocampal layers. The inset depicts two sample traces indicating extreme points of Cl\(^{-}\) increase. A CSD analysis reveals sinks and sources of Cl\(^{-}\) ions in different layers. Sources of Cl\(^{-}\) were revealed in the alveus/str. oriens and upper granule cell layer while sinks were located in the str. oriens, lacunosum-moleculare and the hilus. **A3**, Maximum Cl\(^{-}\) undershoot, indicating a Cl\(^{-}\) clearance beyond the control level, mainly present in medium and deep layers (str. radiatum-dentate gyrus). **B**, Measurements of \([\text{Cl}^\text{-}]_o\)
and Cl⁻ undershoot after systemic administration of CBX indicating shifts in the Cl⁻
distribution in different layers as well as an overall change in the movement pattern of Cl⁻
following stimulation. B1, CBX slightly increases the overall baseline [Cl⁻]₀ concentration
and strengthens a source/sink component in deep layers. B2, CBX administration
drastically changes the Cl⁻ distribution and the sink-source pattern during stimulation-
induced activity and diminishes the Cl⁻ clearing effect (B3). Abbreviations: O: str. oriens;
P; str. pyramidale; R: str. radiatum; LM: str. lacunsum-moleculare; M: molecular layer; G:
granule cell layer; H: hilus

Figure 3. Time constants of Cl⁻ clearance after CA3 stimulation. A, The trace of Cl⁻
clearance following stimulation is fitted with a double exponential curve indicating that in
most layers Cl⁻ clearance decays with a time constant of around 70 ms, however, a peak
was noted at a depth of 900 μm. B, Systemic administration of CBX resulting in a more
than doubled average time constant of Cl⁻ clearance overall, with a prominent increase
within the upper layers (CA1 area). Error bars represent SD. The insets show raw recording
data before (left) and after (right) application of CBX.

Figure 4. Relationship between driving force, [Cl⁻]₀, [K⁺]₀, [Cl⁻]ᵢ, Vₘ, Eₐ₉ and Eₐ₈₉ₐ.B.
mM; Square: [K⁺]₀ = 8 mM. B, Change in the driving force by [Cl⁻]₀ at different [Cl⁻]ᵢ.
Diamond: [Cl⁻]ᵢ= 4 mM; Square: [Cl⁻]ᵢ= 10 mM; Triangle: [Cl⁻]ᵢ= 15 mM. C, Change in the
[Cl⁻]ᵢ to the change of [Cl⁻]₀ at different [K⁺]₀. Diamond: [K⁺]₀= 3 mM; Square: [K⁺]₀= 8
mM. D, Change of Eₐ₉ and Eₐ₈₉ₐ by [K⁺]₀. Diamond: Eₐ₉ calculated using [Cl⁻]₀= 135 mM;
Square: Eₐ₈₉ₐ calculated using [Cl⁻]₀= 135 mM. Triangle: Eₐ₉ calculated using [Cl⁻]₀=
221.6 mM; Cross: Eₐ₈₉ₐ calculated using [Cl⁻]₀= 221.6 mM. Eₐ₉ and Eₐ₈₉ₐ have been
calculated assuming that KCC2 is at thermodynamic equilibrium: [Cl⁻]ᵢ= [Cl⁻]₀*[K⁺]₀/[K⁺]ᵢ,
(i.e. Eₐ₉ = E₉). E, Change in the Eₐ₈₉ₐ and Eₐ₉ to the change of [Cl⁻]₀ at different [Cl⁻]ᵢ.
Diamond: [Cl\(^{-}\)]\(_{i}\) = 4 mM; Triangle: [Cl\(^{-}\)]\(_{i}\) = 10 mM; Square: [Cl\(^{-}\)]\(_{i}\) = 15 mM. F, Change of Cl\(^{-}\) driving force by [Cl\(^{-}\)]\(_{o}\) when K\(^{+}\) movement was taken into consideration.

Figure 5. Summary of [Cl\(^{-}\)]\(_{o}\) concentration and driving force changes across hippocampal layers in different conditions. A, Cl\(^{-}\) movement throughout hippocampal layers. Lines depict the maximum [Cl\(^{-}\)]\(_{o}\) increase and Cl\(^{-}\) undershoot after stimulation, the total Cl\(^{-}\) clearance (summation of both, describing the total value of Cl\(^{-}\) which is cleared from the extracellular space), and values of [Cl\(^{-}\)]\(_{o}\) prior to stimulation. Note that the calibration bar applies to all traces but absolute values are not indicated here, only in the text. B, Driving force of Cl\(^{-}\) changes across the hippocampus in different conditions. Abbreviations: O: striatum oriens; P: stratum pyramidale; R: stratum radiatum; LM: stratum lacunosum-moleculare; M: stratum moleculare; G: granule cell layer; H: hilus.
References


A - control

1 - baseline Cl\(^-\) levels

B - CBX

2 - max. Cl\(^-\) extrusion

3 - Cl\(^-\) undershoot