

Ion-Regulatory Proteins in Neuronal Development and Communication

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List of original publications

This Thesis is based on the following publications, referred to in the text by their Roman numerals.

- I. Rivera C, Voipio J, Payne JA, ¹**Ruusuvuori E**, Lahtinen H, Lamsa K, Pirvola U, Saarma M, Kaila K. (1999) The K^+ - Cl^- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. *Nature* 397:251–255.
- II. ²**Ruusuvuori E**, Li H, Huttu K, Palva JM, Smirnov S, Rivera C, Kaila K, Voipio J. (2004) Carbonic anhydrase isoform VII acts as a molecular switch in the development of synchronous gamma-frequency firing of hippocampal CA1 pyramidal cells. *J Neurosci* 24:2699-26707.
- III. Jacobs S*, ³**Ruusuvuori E***, Sipilä ST*, Haapanen A, Damkier HH, Kurth I, Hentschke M, Schweizer M, Rudhard Y, Laatikainen L, Tyynelä J, Praetorius J, Voipio J, Hübner CA. (2008) Mice with targeted Slc4a10 gene disruption have small brain ventricles and show reduced neuronal excitability. *Proc Natl Acad Sci U S A*. 105:311-316.

*These authors contributed equally to this work.

¹The author contributed to designing the research, performing intracellular Cl^- measurements on hippocampal slices, analyzing data, and writing the manuscript.

²The author designed and performed all intracellular pH measurements from isolated pyramidal neurons and hippocampal slices, designed and performed extracellular field potential measurements, extracellular potassium measurements and intracellular sharp electrode recordings. The author organized and wrote the manuscript with K.K with input from co-authors.

³The author performed intracellular pH measurement from hippocampal pyramidal neurons and analyzed the data, was responsible for the breeding of the knockout animals, and performed and analyzed field potential measurements. The author organized and wrote the manuscript with S.T.S., J.P., J.V. and C.A.H.

Abbreviations

AE	anion exchanger
AIS	axon initial segment
AMPA	α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid
4-AP	4-aminopyridine
ASIC	acid sensing ion channel
ATP	adenosine triphosphate
AQP-1	aquaporin-1 water channel
BA	benzolamide
BBB	blood-brain barrier
BCECF	2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein
BT	bicarbonate transporter
CA1-3	cornu ammonis areas 1-3 of hippocampus
CAI-XV	carbonic anhydrase isoforms I-XV
CARP	carbonic anhydrase related protein
CCC	cation-chloride cotransporter
CNS	central nervous system
CSF	cerebrospinal fluid
DIA	depolarization-induced alkalization
DIV	days <i>in vitro</i>
DRG	dorsal-root ganglion
E	embryonic day
E_{Cl}	equilibrium potential of Cl^-
E_{GABA-A}	reversal potential of $GABA_A$ channel mediated response
ECF	extracellular fluid
EZA	ethoxzolamide
F	Faraday's constant
FRET	fluorescence-resonance-energy-transfer
g	conductance
GABA	γ -aminobutyric acid
GFAP	glial fibrillary acidic protein
GDP	giant depolarizing potential
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HFS	high-frequency stimulation
I	current
IPSP	inhibitory postsynaptic potential
ISME	ion-sensitive microelectrode
KCC	K^+ - Cl^- cotransporter
KO	knockout
MEQ	6-methoxy-N-ethylquinolinium iodide
mGluRs	metabotropic glutamate receptors
MQAE	N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide
MRI	magnetic resonance imaging

NBCe	electrogenic Na ⁺ -bicarbonate cotransporter
NBCn	electroneutral Na ⁺ -bicarbonate cotransporter
NCBE	<u>Na</u> ⁺ -driven <u>Cl</u> ⁻ -bicarbonate <u>ex</u> changer
NCC	Na ⁺ - Cl ⁻ cotransporter
NDCBE	<u>Na</u> ⁺ -driven <u>Cl</u> ⁻ -bicarbonate <u>ex</u> changer
NHE	Na ⁺ -H ⁺ exchanger
NKCC	Na ⁺ -K ⁺ -Cl ⁻ - cotransporter
NMDA	N-methyl-D-aspartate
P	postnatal day
P _{CO2}	partial pressure of CO ₂
PNS	peripheral nervous system
(P)ODN	(phosphorothionate-protected) oligonucleotides
<i>R</i>	gas constant
SLC	solute carrier
SPQ	6-methoxy- <i>N</i> -(3-sulfopropyl)quinolinium
<i>T</i>	absolute temperature
V _m	membrane potential
WT	wild-type
<i>z</i>	valence

1 Summary

Brain function is critically dependent on the ionic homeostasis in both the extra- and intracellular compartment. The regulation of brain extracellular ionic composition mainly relies on active transport at blood–brain and at blood–cerebrospinal fluid interfaces whereas intracellular ion regulation is based on plasmalemmal transporters of neurons and glia. In addition, the latter mechanisms can generate physiologically as well as pathophysiologically significant extracellular ion transients. In this work I have studied molecular mechanisms and development of ion regulation and how these factors alter neuronal excitability and affect synaptic and non-synaptic transmission with a particular emphasis on intracellular pH and chloride (Cl⁻) regulation.

Why is the regulation of acid-base equivalents (H⁺ and HCO₃⁻) and Cl⁻ of such interest and importance? First of all, GABA_A-receptors are permeable to both HCO₃⁻ and Cl⁻. In the adult mammalian central nervous system (CNS) fast postsynaptic inhibition relies on GABA_A-receptor mediated transmission. Today, excitatory effects of GABA_A-receptors, both in mature neurons and during the early development, have been recognized and the significance of the “dual actions” of GABA on neuronal communication has become an interesting field of research. The transmembrane gradients of Cl⁻ and HCO₃⁻ determine the reversal potential of GABA_A-receptor mediated postsynaptic potentials and hence, the function of pH and Cl⁻ regulatory proteins have profound consequences on GABAergic signaling and neuronal excitability. Secondly, perturbations in pH can cause a variety of changes in cellular function, many of them resulting from the interaction of protons with ionizable side chains of proteins. pH-mediated alterations of protein conformation in e.g. ion channels, transporters, and enzymes can powerfully modulate neurotransmission. In the context of pH homeostasis, the enzyme carbonic anhydrase (CA) needs to be taken into account in parallel with ion transporters: for CO₂/HCO₃⁻ buffering to act in a fast manner, CO₂ (de)hydration must be catalyzed by this enzyme. The acid-base equivalents that serve as substrates in the CO₂ dehydration-hydration reaction are also engaged in many carrier and channel mediated ion movements. In such processes, CA activity is in key position to modulate transmembrane solute fluxes and their consequences.

The bicarbonate transporters (BTs; SLC4) and the electroneutral cation-chloride cotransporters (CCCs; SLC12) belong to the large gene family of solute carriers (SLCs). In my work I have studied the physiological roles of the K⁺-Cl⁻ cotransporter KCC2 (*Slc12a5*) and the Na⁺-driven Cl⁻-HCO₃⁻ exchanger NCBE (*Slc4a10*) and the roles of these two ion transporters in the modulation of neuronal communication and excitability in the rodent hippocampus. I have also examined the cellular localization and molecular basis of intracellular CA that has been shown to be essential for the generation of prolonged GABAergic excitation in the mature hippocampus.

The results in my Thesis provide direct evidence for the view that the postnatal up-regulation of KCC2 accounts for the developmental shift from depolarizing to hyperpolarizing postsynaptic E_{GABA-A} responses in rat hippocampal pyramidal neurons. The results also indicate that after KCC2 expression the developmental onset of excitatory GABAergic transmission upon intense GABA_A-receptor stimulation depend on the expression of intrapyramidal CA, identified as the CA isoform VII. Studies on mice with targeted *Slc4a10* gene disruption revealed an important role for NCBE in neuronal pH regulation and in pH-dependent modulation of neuronal excitability. Furthermore, this ion transporter is involved in the basolateral Na⁺ and HCO₃⁻ uptake in choroid plexus epithelial cells, and is thus likely to contribute to cerebrospinal fluid production.

2 Review of the literature

2.1. Ion levels in the brain

2.1.1 Intra- and extracellular compartments of the brain

Mammalian brain tissue is composed of two types of cells, neurons and glia. Neurons, which are highly specialized for electrical signal transmission, are supported both structurally and functionally by glial cells. Most neurons in cortical structures can be further classified on the basis of their synaptic transmitters e.g. in glutamatergic principal neurons and in GABAergic interneurons. The two main types of CNS glial cells are oligodendrocytes and astrocytes. The former are responsible for axonal myelination and the latter contribute to the maintenance of a chemical environment suitable for neuronal signalling. Together neurons and glial cells form the physiologically relevant intracellular compartment of the brain (see Table 1 for intraneuronal ion levels). The densely packed cells are separated from each other by the extracellular space. The distance between brain cells varies but on average it is estimated to be no more than 20 nm (see Nicholson, 2001). Despite these tiny dimensions, this extracellular space constitutes roughly 20 % of the volume of brain tissue. In addition, ~10 % of the total brain volume is taken by the space of brain ventricles and subdural space. These compartments are filled with an aqueous solution; brain cells are bathed in the extracellular fluid (ECF) while the cerebrospinal fluid (CSF) fills the ventricular system and covers the external surfaces of the brain. The ependymal lining of cerebral ventricles (and of *pia mater*) permits a relatively free diffusion of ions between these two compartments. There is also a constant, but slow bulk flow from ECF to CSF, suggesting that the composition of these two fluids is

largely similar. Samples taken from CSF have shown that, in comparison to blood plasma, CSF is slightly hypertonic and contains less proteins, glucose and amino acids. Also the ionic composition differs from that of plasma (Table 1).

The intracellular compartment is separated from the extracellular space by a thin lipid bilayer, the plasma membrane. The tight regulation of cytoplasmic inorganic cations, such as sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), and protons (H^+), and anions chloride (Cl^-) and bicarbonate (HCO_3^-), mainly relies on a variety of plasmalemmal ion transporters. Organellar compartmentalization of ions e.g. into the endoplasmic reticulum, via organellar transporters, further contributes to the regulation of the cytoplasmic ion levels.

On larger scale, compartments with different ionic milieus are, in a similar manner, separated from each other by cell membranes. The blood-brain barrier (BBB), formed of brain endothelial cells lining the cerebral vasculature, protects the mammalian brain from fluctuations in blood plasma composition. Two additional selective barriers are formed between blood and CSF by the choroid plexus epithelium (between blood and ventricular CSF) and by the arachnoid epithelium (between blood and subarachnoid CSF) (see Abbott et al., 2006).

2.1.2 Basic mechanisms of cellular ion regulation

2.1.2.1 Thermodynamics of transmembrane ion distribution

The transmembrane distribution of an ion species is influenced by two forces acting across cell membrane: (1) The concentration gradient creates a chemical driving force, (2) since ions carry electric charge, an electrical driving force results from the membrane potential (V_m). When the concentration gradient of the ion and

Free ion concentrations in body fluids			
Ion (unit)	Arterial Plasma	CSF	CNS neurons
Na ⁺ (mM)	148	152	10
K ⁺ (mM)	5.3	3.4	125
Ca ²⁺ (mM)	1.5	1.0	0.00006
Mg ²⁺ (mM)	0.44	0.88	0.5
H ⁺ (nM)	40	50	80
pH	7.4	7.3	7.1
Cl ⁻ (mM)	121	132	6.6 (25-40)*
HCO ₃ ⁻ (mM)	31	28	18

Table 1. The free ion concentrations in mature rodent arterial plasma, cerebrospinal fluid (CSF), and central nervous system (CNS) neurons. *Reported Cl⁻ concentrations for immature CNS neurons is given in brackets. The concentrations shown represent typical values that can be found in references cited in the ‘Review of the literature’. H⁺ concentration was calculated using pH = -log[H⁺].

the electrical gradient balance each other exactly, the ion is at electrochemical equilibrium and there will be no net driving force (see below) for conductive fluxes of the ion. Consequently, the ion will exhibit no net flux in either direction across the membrane. The membrane potential at the equilibrium is obtained from the Nernst equation

$$E_S = \frac{RT}{z_S F} \ln \frac{[S]_o}{[S]_i}$$

which defines the relationship between the chemical gradient and the equilibrium potential (E_S) for an ion S, with extra- and intracellular concentrations $[S]_o$ and $[S]_i$, respectively, and a charge z_S . R, T, and F are the gas constant, absolute temperature, and Faraday’s constant, respectively.

If E_S does not equal the membrane potential, ion S is not at electrochemical equilibrium and there will exist an electrochemical driving

force that is commonly quantified as $V_m - E_S$. Provided that the membrane potential dependence of a channel-mediated current of ion S (I_S) is sufficiently linear, I_S driven by the electrochemical driving force may be given by a modified version of Ohm’s law

$$I_S = g_S(V_m - E_S)$$

where g_S is the membrane conductance of S. The reversal potential (E_{rev}) of a channel-mediated conductive current is defined as the membrane potential at which current is zero and changes its polarity. Measured E_{rev} values often differ from equilibrium potentials of ions, because of finite selectivity of conductive pathways.

Here it is worth noting that very small changes in the transmembrane charge distribution generate significant changes in the membrane potential. Therefore, very small net currents are required to generate shifts in V_m . However, in reality cells in neuronal

networks receive overlapping inhibitory- and excitatory inputs resulting in large current components in opposite directions mediated by simultaneous influx of anions through GABAergic channels and cations through glutamatergic channels. Under these circumstances the net fluxes of different ion species exceed the capacitive current by orders of magnitude (Buzsaki et al., 2007).

2.1.2.2 Regulation of steady state concentrations: transport mechanisms

Inorganic ions are virtually insoluble in lipid bilayers and must therefore move across the cell membrane through channels or transporters. The nomenclature of functionally distinct transporter subtypes reflects the direction of transported ions as well as the movement of charges. *Cotransporters* (also called symporters) move two or more ions in the same direction whereas *countertransporters* (also called antiporters or exchangers) move two or more ions in opposite directions. While co- and countertransporters move at least two different ions, *uniporters* (= facilitated diffusion carriers) enable the movement of a single substance down its concentration gradient. *Electrogenic* transport drives a net current across the membrane while transport is said to be *electroneutral* if the transport process does not produce any current.

At steady-state, the passive (“down-hill”) flux of ions in one direction is counteracted by active transport of ions in the opposite (“up-hill”) direction. Two types of active transport mechanisms mediate regulation of ion concentrations. Primary active transport is fuelled by hydrolysis of adenosine triphosphate (ATP). A considerable portion, 20-40%, of the energy turnover in mammalian brain is accounted for by the activity of a single type of ATPase pump, namely the $\text{Na}^+\text{-K}^+\text{-ATPase}$ (Møllergård and Siesjö, 1998; Attwell and Laughlin, 2001). By moving 3 Na^+ ions out in exchange to 2 inwardly transported K^+ ions the $\text{Na}^+\text{-K}^+\text{-ATPase}$ creates and

maintains the transmembrane gradients of Na^+ and K^+ . Other ATPases, like $\text{Ca}^{2+}\text{-H}^+\text{-ATPase}$ and $\text{H}^+\text{-ATPase}$, are also present in the brain and function in cytoplasmic control and/or organellar compartmentalization of ions (Bevensee and Boron, 1998; Rose and Ransom, 1998; Mata and Sepulveda, 2005). The energy stored in ion gradients created by the primary active, ATP-fuelled transporters is used by secondary active transporters. Most often it is the energetically “down-hill” influx of Na^+ that is used to transport other ions against their electrochemical gradients. Cellular pH regulation serves as a good example for a process that is largely dependent on Na^+ -coupled ion transport. Transport of acid-base equivalents is mediated by e.g. the $\text{Na}^+\text{-H}^+$ exchanger, the Na^+ -driven $\text{Cl}^- \text{-HCO}_3^-$ exchange and the $\text{Na}^+\text{-HCO}_3^-$ co-transporters. The outward gradient of K^+ , instead, serves as the driving force for Cl^- extrusion via $\text{K}^+\text{-Cl}^-$ cotransporters with a 1:1 stoichiometry. Secondary active transporters can also couple the energy derived from an electrochemical gradient of an ion to the transport of e.g. amino acids, sugars, and nucleotides. The present thesis will focus on transmembrane movements of inorganic ions in the brain, with a particular emphasis on Cl^- and HCO_3^- .

2.1.2.3 Thermodynamics of secondary active, electroneutral transport

For an electroneutral ion transporter, the thermodynamic driving force is given by the sum of changes in the chemical potential of the transported ions. At equilibrium, the sum of the chemical potential differences is zero, i.e. the free energy change associated with the transport proteins is zero. Thus, the net influx and efflux of the transported ions are equal and, even though unidirectional ion fluxes may exist, there is no *net flux* of ions. Using these principles, it is straightforward to show that, for instance, a $\text{K}^+\text{-Cl}^-$ cotransporter operating with a 1:1

stoichiometry is at thermodynamic equilibrium when $E_K = E_{Cl}$, i.e. when

$$[K^+]_o [Cl^-]_o = [K^+]_i [Cl^-]_i$$

If such a transporter is constitutively active in the absence of significant conductive leaks, it would operate close to its thermodynamic equilibrium. Under such conditions, minor changes in the driving force will markedly affect the net fluxes, or even change their direction.

The functional activity of an ion transporter is not dictated only by the prevailing electrochemical gradients as transporters are not necessarily active even if they are facing a driving force that favours ion transport (Rocha-Gonzalez et al., 2008). The transporter can undergo fast allosteric modulation e.g. by phosphorylation or de-phosphorylation of the transporter protein. The rate of ion fluxes depends, in addition to the transporter kinetics, on the number of functional transporters located on the plasma membrane. Hence, on a longer time-scale, transport kinetics can be modulated by altering the trafficking of transporters to and from the plasma membrane or by changing the expression of transport protein genes.

2.1.2.4 Buffering

Buffering is a major determinant of pH changes when a solution is challenged by an acid or alkaline load. The chemical buffering power results from buffer pairs of conjugated weak acid(s)/base(s). These buffer pairs are capable of reversibly releasing/binding a proton, and thereby act to minimize and to slow the rates of pH changes. Hence, buffers determine the ability of the solution to resist pH transients without contribution of active transporters. Buffering power (β) is defined as

$$\beta = \frac{\Delta \text{Strong Base}}{\Delta pH} = - \frac{\Delta \text{Strong Acid}}{\Delta pH}$$

where $\Delta \text{Strong Base}$ (or Acid) is the amount of strong base (or acid) added (in mM) and ΔpH is the resulting

change in pH. The unit of β is mM or mmol/l. The total buffering capacity (β_T) of a cell's cytoplasm is due both to the intrinsic buffering capacity of the cell's cytoplasm (β_i) and to the buffering provided by the extrinsic, CO_2/HCO_3^- buffer system (β_{CO_2}).

$$\beta_T = \beta_i + \beta_{CO_2}$$

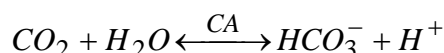
β_i mainly arises from the titratable imidazole groups of proteins and from phosphates. These buffers can not cross the plasma membrane and therefore they form a *closed buffer system* (Burton, 1978; Roos and Boron, 1981). The buffering power of the closed system buffers is maximal when pH equals pK_a . β_i is the sum of the contribution of individual intrinsic buffers and it can be determined experimentally by recording intracellular pH (pH_i) changes upon addition/removal of a strong acid or base in the absence of CO_2/HCO_3^- . Buffering provided by the CO_2/HCO_3^- buffer system is usually considered as an *open buffer system*, i.e. the cellular partial pressure of CO_2 (P_{CO_2}) is maintained constant because it equilibrates with the extracellular compartment that serves as a fixed, infinite source of CO_2 . However, with instantaneous acid/base loads, the immediate pH response is that of a closed system, since the equilibration of CO_2 is not immediate. In a system which is open with respect to P_{CO_2} , the β_{CO_2} is given by

$$\beta_{CO_2} = 2.3[HCO_3^-]$$

Because at a fixed P_{CO_2} $[HCO_3^-]$ rises with pH, also β_{CO_2} increases at higher pH.

Unless catalyzed by the enzyme carbonic anhydrase (CA) the hydration of CO_2 is slow, having a time constant of 20-30 seconds at room temperature (Maren, 1967). Thus, the ability of the CO_2/HCO_3^- -system to efficiently buffer fast H^+ fluxes depends on CA activity. Ignoring the intermediate step of carbonic acid, and further dissociation to carbonate, both of which are present at low concentrations at physiological pH

levels, the hydration-dehydration reaction of CO₂ is



The fast CA-dependent CO₂/HCO₃⁻ - buffer system attenuates pH changes that originate from net fluxes of protons. However, if the hydration-dehydration reaction of CO₂ is brought out of equilibrium by a substance other than H⁺, CA will *facilitate* rapid shifts in [H⁺]. The above reaction results in distinct pH responses in open and closed buffer systems depending on the type of the acid or base load as well as on the presence of CA and intrinsic buffers (Voipio, 1998). The large and constantly growing number of identified members of the CA gene family is described in chapter 2.2.3.

2.1.2.5 Intracellular pH

Without active regulation of pH_i, passive equilibration of protons driven by the resting membrane potential would make pH_i significantly lower than extracellular pH (pH_o). However, neuronal pH_i is typically only slightly more acidic than pH_o (pH_i = 7.1 vs. pH_o = 7.3). Provided that the hydration-dehydration reaction of CO₂ and the transmembrane distribution of CO₂ are at equilibrium, the transmembrane HCO₃⁻ distribution is set by the pH gradient

$$[HCO_3^-]_i = 10^{(pH_i - pH_o)} \times [HCO_3^-]_o$$

Because, under these conditions, the equilibrium potential of protons (E_{H⁺}, which has the value of -12 mV with the pH values given above) equals E_{HCO₃⁻} (see Kaila and Voipio, 1990) the electrochemical gradient tends to drive H⁺ ions into, and OH⁻ and HCO₃⁻ out of the cell. As a result, any conductive leaks of these ionic species impose an acid load on the cell.

2.1.3 Ionic homeostasis of brain extracellular fluids

On the systemic level arterial blood pH is maintained within very narrow limits (7.35-7.45). Respiratory and renal regulatory mechanisms stabilize the arterial pH by excretion or retention of acid/base equivalents. Abnormalities in maintaining blood pH result in systemic acid-base balance disorders. A respiratory acidosis results in a fall in pH_o that is caused by an increase in P_{CO₂} whereas in metabolic acidosis a fall in pH_o is caused by a decrease in [HCO₃⁻]_o. Likewise, respiratory or metabolic alkalosis causes a rise in pH_o due to a decrease in P_{CO₂} or to an increase in HCO₃⁻, respectively. Arterial P_{CO₂} is the most powerful stimulus for ventilation, acting through peripheral and central chemoreceptors (for review see Nattie, 1999).

In chronic acid-base disturbances pH_o is maintained as close to normal as possible by compensatory changes in ventilation (in response to metabolic disorders) and in secretory mechanisms of kidneys (in response to respiratory disorders). The ion transporters of brain barriers are responsible for the short-term CSF/ECF pH normalization, in case of an acute change in P_{CO₂}. Combined measurements of CSF and plasma pH, P_{CO₂} and [HCO₃⁻] have shown that an acute elevation of P_{CO₂} is paralleled by a pH_o decrease (Messeter and Siesjö, 1971; Pavlin and Hornbein, 1975; Nattie and Edwards, 1981). With time, the pH_o was restored close to normal by the ion transporters located in choroid plexus and BBB epithelia (Messeter and Siesjö, 1971). These transient CSF/ECF pH_o changes are reflected in pH_i.

A relevant question is how well neurons can maintain their pH_i when faced with extracellular acid-base disturbances. Sustained pH_o changes mimicking respiratory and metabolic acid-base disturbances resulted in persistent pH_i changes in non-chemosensitive neurons both *in vivo* (Katsura et al., 1994) and *in vitro* (Bouyer et al., 2004). These findings challenge the proposal that during the

pH_o manipulations described above, a sustained change in pH_i is specific for chemosensitive neurons (Ritucci et al., 1997; Ritucci et al., 1998).

2.1.3.1 Cerebrospinal fluid

The tight junctions between the choroid plexus epithelial cells form a diffusion barrier between blood and ventricular CSF (Zlokovic, 2008). The epithelial cells of the choroid plexus are remarkably efficient secretory cells that express a variety of solute carriers (Bouzinova et al., 2005; Praetorius and Nielsen, 2006; Praetorius, 2007). With the exception of aquaporin-1 water channels (AQP1), the transport proteins have a highly polarized expression pattern either on the luminal (ventricular) or on the basolateral membrane. The ionic composition of the secreted CSF is set by the function of these transporters.

The production rate of CSF is critically dependent on the rate of Na⁺ secretion. The high luminal extrusion of Na⁺ is mainly due to the Na⁺-K⁺-ATPase whereas the question what is the main mechanism of basolateral Na⁺ uptake remained to be identified until recently. Due to their basolateral localization, the Na⁺-proton exchanger (NHE) and Na⁺-driven HCO₃⁻ transporters (NCBE and the electroneutral Na⁺-bicarbonate cotransporter NBCn1) have been considered as possible contributors in the net transport of Na⁺ that drives the secretion of CSF (Bouzinova et al., 2005; Praetorius, 2007). The involvement of NCBE in basolateral solute uptake was examined in Study III.

2.1.3.2 Extracellular fluid

While CSF provides the macro-environment for the brain and acts both as a fluid cushion and as a drainage route for solutes (Davson and Segal, 1969), the ECF fills the extracellular space in the immediate vicinity of the brain cells. The brain capillary endothelial cells are involved in secretory mechanisms producing fluid across the BBB into the brain

interstitial space. Because of the bulk flow of ECF from the endothelial secretory site to the ventricular system, ECF is estimated to make a contribution of one third to the CSF production (Cserr, 1974; Abbott, 2004). The tight junctions between the neighbouring endothelial cells form a 'physical barrier' that prevents paracellular movement of most molecules. Instead, molecular trafficking across the BBB is carried out by active transport systems or channel-mediated passive diffusion via the transcellular route. An exception is made by the gaseous molecules (like O₂ and CO₂) and small lipophilic agents that can freely diffuse through the epithelial plasma membranes. The electrical potential difference of a few millivolts that prevails between the CSF/ECF and blood is sensitive to changes in P_{CO2} and pH (Woody et al., 1970; Voipio et al., 2003). This suggests that the transporters involved in the generation of the transendothelial potential difference have a marked sensitivity to pH and/or that the acid-base transporters make a considerable contribution to the transendothelial transport.

Even though under normal circumstances the regulatory mechanisms of BBB (together with the blood-CSF barrier) maintain the global ion homeostasis of brain extracellular fluids, local fluctuations in ECF ion concentrations occur as a result of neuronal activity (Somjen, 2002). Extracellular ion concentrations are restored in co-operation with extracellular buffering and diffusion, and by transmembrane movements of ions. Astrocytes make a significant contribution to extracellular ion regulation. These glial cells have been shown to mediate both spatial buffering/siphoning and net uptake of K⁺ (Newman, 1996; Kofuji and Newman, 2004), and a role in pH_o regulation has been suggested (Deitmer and Rose, 1996).

Under physiological conditions, neuronal activity elicits only modest changes in the ionic composition of ECF (Sykova et al., 1974; Singer and Lux, 1975). The available data might partially reflect the limitations of the

methods established so far. Hence, it is possible that transients with higher amplitude take place within spatially restricted microdomains in the extracellular space, allowing e.g. Ca^{2+} and protons to serve second messenger-like actions (Chesler and Kaila, 1992). Under pathological conditions, ion fluctuations can be large. For example, marked K^+ (Heinemann and Louvel, 1983; McNamara, 1994; Avoli, 1996) and proton (Urbanics et al., 1978; Kraig et al., 1983; Somjen, 1984; Silver and Erecinska, 1992) transients have been measured *in vivo* during seizures or after direct electrical stimulation. The ion transients evoked in many experiments performed *in vitro* are pronounced and can even exceed those seen *in vivo* under pathological conditions (Kaila and Chesler, 1998; Somjen, 2001; Avoli et al., 2005), but they provide a useful approach in studies of the molecular and cellular mechanisms underlying ion transients and ion-based signalling in the brain.

2.1.4 Ionic basis of GABA_A receptor-mediated synaptic inhibition

The special importance of neuronal Cl^- regulation in neurotransmission arises from the fact that GABA_A (and glycine) receptor channels are permeable to Cl^- . Together with glycine, GABA is the main neurotransmitter responsible for synaptic inhibition in the CNS.

Hyperpolarizing inhibition mediated by ionotropic GABA receptors in mature neurons is based on plasmalemmal transporters that extrude Cl^- and thereby maintain an E_{Cl} more negative than the resting V_m (Eccles, 1966; Deisz and Lux, 1982). This creates a driving force for an inward Cl^- flux, i.e. an outward current, which accounts for conventional hyperpolarizing postsynaptic inhibition. The GABA_A channel-mediated currents depend also on HCO_3^- because GABA_A channels are permeable to both HCO_3^- and Cl^- , with a $\text{HCO}_3^-:\text{Cl}^-$ permeability ratio of 0.2-

0.4 (Kaila and Voipio, 1987; Bormann et al., 1987; Kaila et al., 1993). In a cell with a typical negative resting membrane potential around -60 mV and an $E_{\text{HCO}_3^-}$ close to -12 mV there is a deep electrochemical gradient favoring HCO_3^- efflux. The HCO_3^- efflux creates an inwardly directed, depolarizing current, which in Cl^- extruding cells where the intracellular Cl^- concentration is kept low, can make a significant contribution to the net GABA_A ergic current. This HCO_3^- current keeps E_{GABA_A} more positive than E_{Cl} and in some cases can even lead to a HCO_3^- -dependent depolarization (Kaila et al., 1989b; Kaila et al., 1993; Gullledge and Stuart, 2003).

In addition to the GABA mediated effect on the membrane potential, the input conductance of a cell increases significantly upon GABA_A receptor channel activation. The consequent local decrease in the membrane time and space constant efficiently suppresses changes in V_m generated by simultaneous excitatory currents (Staley and Mody, 1992). This *shunting inhibition* is effective even at slightly depolarizing GABA_A channel-mediated potentials seen e.g. in adult rat dentate granule cells (Staley and Mody, 1992) and neocortical neurons (Kaila et al., 1993).

2.2 Molecular mechanisms of proton and anion regulation in the brain

The Human genome organisation nomenclature committee database provides a list of transporter families of the SLC gene series, which currently covers 43 families, most of which have several transporter subtypes (Hediger et al., 2004). The SLC series includes genes encoding passive and coupled ion transporters located both on cell and organellar membranes. The members of each SLC family share at least 20–25% amino acid sequence identity between each other.

Identified members of the Slc4 and Slc12 gene families in mice			
Mouse gene symbol	Protein name	Predominant substrates	Expression in the CNS
<i>Slc4</i>			
<i>Slc4a1</i>	AE1	Cl ⁻ , HCO ₃ ⁻	-
<i>Slc4a2</i>	AE2(a-c)	Cl ⁻ , HCO ₃ ⁻	b: Choroid plexus
<i>Slc4a3</i>	AE3	Cl ⁻ , HCO ₃ ⁻	Neurons
<i>Slc4c4</i>	NBCe1(a-c)	Na ⁺ , HCO ₃ ⁻	b&c: Widespread
<i>Slc4a5</i>	NBCe2, "NBC4"	Na ⁺ , HCO ₃ ⁻	Choroid plexus
<i>Slc4a6 (not used)</i>	-	-	-
<i>Slc4a7</i>	NBCn1(b-e), "NBC3"	Na ⁺ , HCO ₃ ⁻	Widespread
<i>Slc4a8</i>	NDCBE	Na ⁺ , HCO ₃ ⁻ , Cl ⁻	Widespread
<i>Slc4a9</i>	"AE4"	Inconclusive	-
<i>Slc4a10</i>	"NCBE" or "NBCn2"	Na ⁺ , HCO ₃ ⁻ , (Cl ⁻ ?)	Neurons, choroid plexus
<i>Slc4a11</i>	"BTR1"	*Unknown	-
<i>Slc12</i>			
<i>Slc12a1</i>	NKCC2 (a,b&f))	Na ⁺ , K ⁺ , Cl ⁻	-
<i>Slc12a2</i>	NKCC1(a&b)	Na ⁺ , K ⁺ , Cl ⁻	Widespread
<i>Slc12a3</i>	NCC	Na ⁺ , Cl ⁻	-
<i>Slc12a4</i>	KCC1(a&b)	K ⁺ , Cl ⁻	Widespread; glial
<i>Slc12a5</i>	KCC2(a&b)	K ⁺ , Cl ⁻	Neuron-specific
<i>Slc12a6</i>	KCC3(a-c)	K ⁺ , Cl ⁻	Widespread
<i>Slc12a7</i>	KCC4	K ⁺ , Cl ⁻	Widespread
<i>Slc12a8</i>	CIP	*Unknown	Widespread
<i>Slc12a9</i>	CCC9	*Unknown	Widespread
*no evidence for transport activity			

Table 2. The identified members of the bicarbonate transporter (Slc4) and cation-chloride cotransporter (Slc12) gene families. The expression of the Slc4 and Slc12 proteins and their known splice variants (given in brackets after the protein name) in the rodent central nervous system. The data in the table are from Gamba et al., 2004 and Romero et al., 2004.

2.2.1 Members of cation-chloride cotransporter gene family

Members of the CCC family are secondary active, electroneutral transporters that are largely responsible for neuronal Cl⁻ regulation (Mercado et al., 2004; Gamba, 2005). The CCC family (encoded by the genes *Slc12a1-9*, see Table 2) includes transporters that mediate either Na⁺-driven Cl⁻ uptake (Na⁺-K⁺-Cl⁻ cotransporters, NKCCs, isoforms NKCC1 and NKCC2, and a Na⁺-Cl⁻ cotransporter, NCC) or K⁺-driven Cl⁻ extrusion (K⁺-Cl⁻ cotransporters, KCCs; isoforms KCC1-4). The functions of the two most recent CCC family members (cation-chloride cotransporter interaction protein, CIP, and CCC9) have not yet been identified (Caron et al., 2000; Gamba, 2005).

2.2.1.1 Potassium-driven chloride cotransporters

In the adult brain transporter-mediated Cl⁻ extrusion is mainly carried out by the KCCs (Mercado et al., 2004; Gamba, 2005) (Fig. 1). From the four KCC isoforms (KCC1-4, encoded by four separate genes *Slc12a4-7*) KCC1, KCC3, and KCC4 have diverse and widespread expression patterns. KCC1 and KCC4 isoforms are both present in the brain, especially in the choroid plexus, but show little expression in mature CNS neurons (Study I of this Thesis; Mercado et al., 2004). Compared to KCC1 and KCC4, KCC3 is abundantly expressed in the brain, including the hippocampus, and it has been shown to contribute in neuronal volume and Cl⁻ regulation (Mount et al., 1999; Boettger et al., 2003; Le Rouzic et al., 2006). In addition to the neuronal expression, an association of KCC3 with myelin sheet has been reported (Pearson et al., 2001). In comparison to the three widespread KCC isoforms, KCC2 is found only in the CNS where its expression is strictly limited to neurons (Payne et al., 1996; Williams et al., 1999). In the adult rat hippocampus KCC2 immunoreactivity is observed in the somatic and

dendritic membranes but it is most prominent in dendritic spines of principal cells and of (parvalbumin positive) interneurons (Gulyas et al., 2001). KCC2 has two splice variants, KCC2a and KCC2b, with the KCC2b isoform prevailing over that of KCC2a in adult cortical neurons (Uvarov et al., 2007).

Even though KCCs under physiological conditions operate as net efflux pathways, in mature cortical principal neurons the process for Cl⁻ export is actually very near its thermodynamic equilibrium and even a small increase in [K⁺]_o may change the direction of net K⁺-Cl⁻ transport (Payne, 1997). Intense neuronal activity can result in elevation of [K⁺]_o, especially under pathological conditions (Traynelis and Dingledine, 1989; Avoli, 1996; Voipio and Kaila, 2000) and experimental work has, indeed, provided support for KCC2 mediated K⁺-Cl⁻ influx upon experimentally elevated [K⁺]_o (Jarolimek et al., 1999; DeFazio et al., 2000; Kakazu et al., 2000).

In addition to the Cl⁻ transporters and ligand-gated Cl⁻ channels (e.g. GABA_A- and glycine receptors), the members of the CLC family of Cl⁻ channels can also mediate Cl⁻ fluxes (Jentsch et al., 2005). These channels have been shown to have a variety of functions including stabilization of membrane potential, synaptic inhibition, cell volume regulation, and transepithelial transport. A channel-mediated outward net flux of Cl⁻ is possible only when the membrane potential is more negative than E_{Cl}.

Unlike the other K⁺-dependent Cl⁻ cotransporters, KCC2 is not activated by cellular swelling and it is not directly involved in cell volume regulation (Payne, 1997). KCC3 is a genuinely volume sensitive and its transport activity is sensitive to cellular swelling (Race et al., 1999; Boettger et al., 2003) and both KCC1 and KCC4 are activated by volume increase under hypotonic conditions (Race et al., 1999; Mercado et al., 2000). Neuronal volume regulation is further assisted by NHEs (Rotin and Grinstein, 1989) which also play a major role in HCO₃⁻

-independent pH regulation (Schwiening and Boron, 1994; Bevensee et al., 1996; Chesler, 2003).

2.2.1.2 Sodium-driven chloride cotransporters

Of the three Cl^- uptake-mediating CCCs, only NKCC1 is expressed in the brain, both by neurons and glia (Plotkin et al., 1997; Clayton et al., 1998; Mercado et al., 2004). There are two functional NKCC1 splice variants, NKCC1a and NKCC1b, whose relative proportions vary among different tissues (Randall et al., 1997; Vibat et al., 2001). Transcripts of both variants are present in the adult (human) brain (Vibat et al., 2001). In contrast to

KCCs, NKCC1 is driven by the Na^+ -gradient and only functions to accumulate Cl^- into cells. In all mammalian cells studied the stoichiometry of the cotransporter is $\text{Na}^+:\text{K}^+:2\text{Cl}^-$ (Russell, 2000).

NKCC1 and KCC1-4 are blocked by the 'loop' diuretics furosemide and bumetanide (Payne et al., 2003). The former drug inhibits the transporters with equal potency but the latter can be used as a selective blocker of NKCC1 at a concentration of 1-10 μM (Gillen et al., 1996, Williams et al., 1999). The somewhat contradictory data on NKCC1 expression in the brain is discussed in chapter 2.3.1.

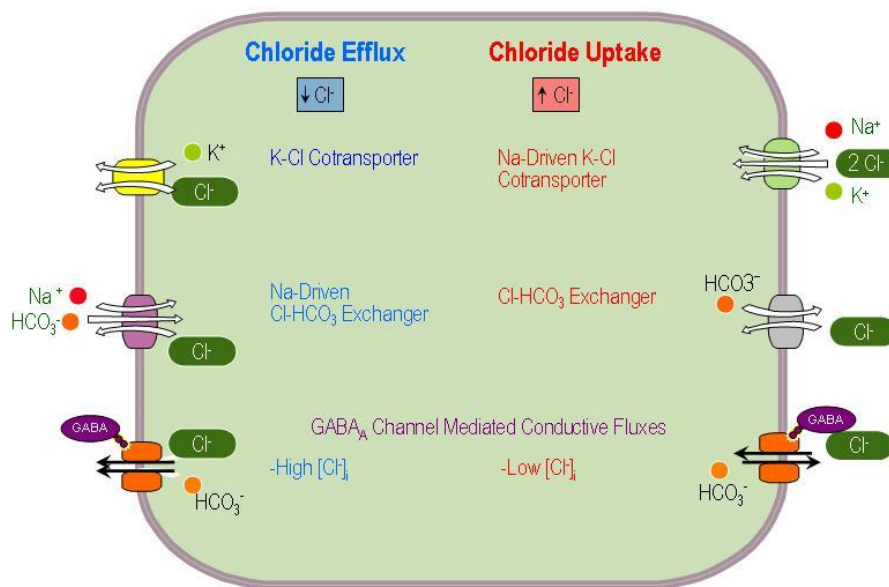


Figure 1. Transporter and GABA_A channel-mediated Cl^- movements. The electroneutral cation-chloride cotransporters KCCs and NKCC1 function as neuronal Cl^- extruders and loaders, respectively. The Na^+ -dependent and -independent anion-exchangers may contribute in the control of intracellular Cl^- levels in addition to their role as pH_i regulators. The electrochemical gradient of Cl^- determines the direction of GABA_A channel-mediated Cl^- fluxes. In (juvenile) neurons with high $[\text{Cl}^-]_i$, opening of GABA_A channels results in a net Cl^- efflux whereas in (mature) neurons with low $[\text{Cl}^-]_i$ there is a net influx of Cl^- .

2.2.2 Members of bicarbonate transporter gene family

The transporters involved in neuronal pH regulation can be divided into acid-extruding (transporters mediating efflux of H^+ or influx of HCO_3^- or CO_3^{2-}) and acid-loading transporters (transporters resulting in an influx of H^+ , or efflux of HCO_3^- or CO_3^{2-}). The secondary active BTs of the SLC4 gene family are, in co-operation with the NHEs (the SLC9 gene family), largely responsible for neuronal pH regulation (Romero et al., 2004; Orłowski and Grinstein, 2004). Out of the ten SLC4 gene family members, the physiological function of eight transporter subtypes has been established (Table 2). They can be divided into two major subfamilies: the anion exchangers (AEs) and the Na^+ -driven HCO_3^- transporters. The transport mode and the predominant substrate of the two remaining transporters, SLC4a9 and SLC4a11, respectively, are uncertain (Romero et al., 2004). Most of the members of the SLC4 gene family are inhibited by disulfonic stilbene derivatives such as DIDS and SITS. It should be mentioned that Cl^- is a substrate of some transporters that are typically classified as pH regulators. AEs and the Na^+ -driven Cl^- - HCO_3^- exchanger(s) mediate HCO_3^- coupled Cl^- fluxes. Future work will show how much these exchangers actually contribute to Cl^- regulation in various kinds of neurons.

Several other SLC gene families include members that, even though not considered as pH regulators, mediate transmembrane movements of acid-base equivalents (see Hediger et al., 2004). Primary active transporters are also involved in neuronal pH regulation. Neuronal acid extrusion in the nominal absence of Na^+ and CO_2/HCO_3^- is accomplished by a putative H^+ pump (Bevensee et al., 1996) whereas the Ca^{2+} - H^+ -ATPase functions as an acid loader while extruding Ca^{2+} (Paalasmaa et al., 1994; Smith et al., 1994; Trapp et al., 1996) (Fig. 2).

2.2.2.1 Sodium independent anion exchangers

AEs (isoforms AE1-3, Slc4a1-3) mediate electroneutral exchange of monovalent anions, mainly HCO_3^- and Cl^- . In adult neurons with a low $[Cl^-]_i$, it is the inward Cl^- chemical gradient that dominates and drives the exchange of extracellular Cl^- for intracellular HCO_3^- . The Cl^- - HCO_3^- exchange thus functions as Na^+ -independent acid (and Cl^-) loader that is assumed to largely be responsible for cellular recovery from an alkaline load (Chesler, 2003). Both AE3 and AE2 are detected in the brain: AE3 has a neuron-specific expression pattern (Hentschke et al., 2006) whereas AE2 localizes solely to the basolateral membrane of choroid plexus (Lindsey et al., 1990). AE1 expression is most pronounced in erythrocytes and kidney (Romero et al., 2004).

Na^+ -independent Cl^- - HCO_3^- exchanger activity has been demonstrated in adult rodent hippocampal neurons (Raley-Susman et al., 1993; Hentschke et al., 2006) and in cortical astrocytes (Shrode and Putnam, 1994). As AE3 is strictly neuronal in the CNS (Hentschke et al., 2006), the anion exchanger responsible for the glial recovery from alkalosis, described by Shrode and Putnam (1994) remains to be characterized. In experiments on AE3 knockout mice (AE3 KO), the hippocampal pyramidal cell recovery from alkalosis was impaired but not abolished (Ruusuvuori et al., 2007). These results imply that also some other acidifying mechanism(s) that is independent of Cl^- and HCO_3^- contributes to pyramidal cell pH regulation during intracellular alkalosis.

2.2.2.2 Sodium-driven bicarbonate transporters

The Na^+ -driven HCO_3^- transporters can be classified into electrogenic and electroneutral transporters.

The three electroneutral Na^+ -driven HCO_3^- transporters function as acid extruders. The NBCn1 (Slc4a7), the only stilbene-insensitive transporter in

the SLC4 gene family, mediates a 1:1 influx of Na^+ and HCO_3^- (Bevensee et al., 2000) whereas NDCBE (Slc4a8) functions as an Na^+ -driven Cl^- - HCO_3^- exchanger (Grichtchenko et al., 2001). Both transporters have recently been shown to localize to the soma and dendrites of hippocampal pyramidal

neurons (Cooper et al., 2005; Damkier et al., 2007; Boedtkjer et al., 2008; Chen et al., 2008a). NCBE (Slc4a10) was also initially thought to be a Na^+ -driven Cl^- - HCO_3^- exchanger (Wang et al., 2000) but the Cl^- dependence of the (rodent) transporter is still debated (Choi et al., 2002). It was

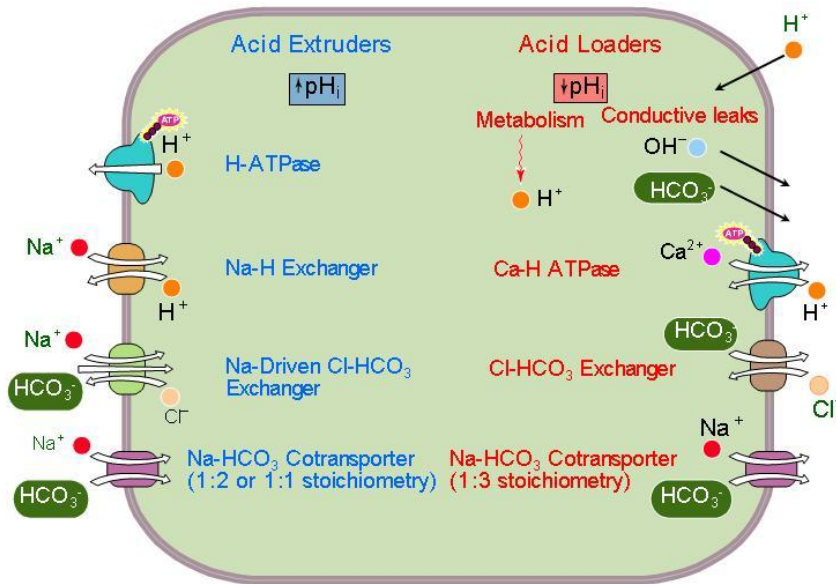


Figure 2. Acid extruders and loaders that contribute in pH_i regulation. The cellular steady-state pH_i depends on the balance of acid-base equivalent movements mediated by the transporters, and from the acid load generated by metabolism and by the passive entry/exit of H^+ , OH^- , and HCO_3^- . Modified from Boron (2004).

recently shown that the Na^+ - HCO_3^- transport activity of the human SLC4A10 is, under physiological conditions, independent of Cl^- countertransport and thus the transporter should rather be called NBCn2 (Parker et al., 2008). Two variants of the NCBE, rb1NCBE and rb2NCBE, have been identified in the adult rat brain (Giffard et al., 2003). The rb2NCBE terminates in a PDZ motif which is absent from the rb1NCBE. RT-PCR showed that both variants were present in RNA isolated from rat (and mouse) brain. In cultured

brain cells both variants were present in neurons but the rb2NCBE was more prominent in astrocytes. However, in the embryonic mouse brain NCBE expression was suggested to follow a mainly neuronal pattern (Hübner et al., 2004). In the rodent hippocampus NCBE mRNA has been detected (Wang et al., 2000; Giffard et al., 2003; Hübner et al., 2004) but the protein distribution has not been previously assessed. Functional studies have shown that under physiological conditions acid extrusion in adult rat hippocampal

CA1 pyramidal neurons is governed by an (amiloride insensitive) $\text{Na}^+\text{-H}^+$ exchanger and by Na^+ -driven Cl^- - HCO_3^- exchanger(s) (Schwiening and Boron, 1994; Bevensee et al., 1996). These findings suggest that, in addition to the NHEs, NCBE and/or NDCBE are critically involved in pyramidal cell pH regulation. The localization of NCBE at the protein level and its contribution to pyramidal cell pH regulation and neuronal excitability are assessed in Study III.

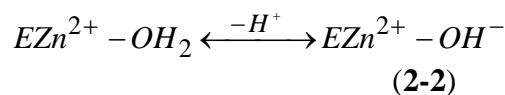
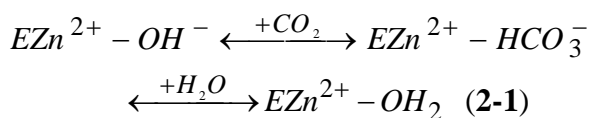
The last subgroup of the BTs consists of the electrogenic $\text{Na}^+\text{-HCO}_3^-$ cotransporters. In brain cells and choroid plexus epithelial cells (NBCe1 and NBCe2; Slc4a4 and Slc4a5) these transporters most likely have a stoichiometry of 1:2 and hence import HCO_3^- (Romero et al., 2004, but see Boussouf et al., 1997) but the number and the direction of transported HCO_3^- ions appear to be cell-type specific. In the renal proximal tubule where NBCe1-A is involved in the HCO_3^- reabsorption the transporter operates with a stoichiometry of 1:3 and extrudes HCO_3^- into the interstitial space (Gross et al., 2001), whereas if expressed in *Xenopus* oocytes the stoichiometry is 1:2 (Heyer et al., 1999).

The NBCe expressed in brain cells (NBCe1; Giffard et al., 2000; Schmitt et al., 2000; Rickmann et al., 2007) has classically been considered as a 'glial transporter' that has a prominent role in intragial pH regulation (Deitmer and Schlue, 1989; Brune et al., 1994; O'Connor et al., 1994; Shrode and Putnam, 1994; Giffard et al., 2000). NBCe-mediated transport of HCO_3^- is involved in the generation of depolarization-induced alkalization (DIA) of glial cells, a form of neuron-glia signalling where changes in glial membrane potential caused by neuronal activity are converted into glial pH_i changes (Chesler and Kraig, 1987; Chesler and Kraig, 1989; Deitmer and Schlue, 1989; Pappas and Ransom, 1994). The transmembrane movements of HCO_3^- that give rise to DIA produce a simultaneous acid transient in the brain extracellular space (Chesler and Kraig, 1989;

Deitmer and Szatkowski, 1990; Grichtchenko and Chesler, 1994) and thus contribute to activity-induced pH_o changes (Deitmer, 1992; Deitmer and Rose, 1996). Today there is accumulating molecular biological data suggesting that in addition to glial cells some neuronal subpopulations, including dentate granule cells and hippocampal pyramidal neurons, express NBCe1 (Bevensee et al., 2000, Schmitt et al., 2000, Rickmann et al., 2007; Majumdar et al., 2008). The expression of NBCe2 in the brain is limited to the luminal membrane of choroid plexus epithelial cells (Bouzinova et al., 2005).

2.2.3 Carbonic anhydrase isoforms

CAs are zinc-metalloenzymes that catalyze the reversible hydration of CO_2 (Maren, 1967; Sly and Hu, 1995; Supuran et al., 2004). The rate-limiting step in the process, schematically represented by Equations (2-1) and (2-2), is the regeneration of the catalytically active, basic form of the enzyme ($\text{EZn}^{2+}\text{-OH}^-$). It requires a proton-transfer reaction from the active site to the environment (Equation 2-2), a process that is in the isoforms with the highest turn-over rates (CAII, CAIV, CAV, CAVII, CAIX) assisted by several histidine residues.



So far, 15 distinct isozymes or CA-related proteins (CARP) with diverse subcellular localization have been characterized (Supuran et al., 2003; Hilvo et al., 2005) (see Table 3). From the twelve enzymatically active isoforms, five are cytosolic (CAI-III, VII, and XIII), five are extracellular (CAIV, IX, XII, XIV, and XV), one is mitochondrial (CAVa,b), and one is

secreted (CAVI). CAs are among the fastest enzymes: the substrate turnover goes up to $1.4 \times 10^6 \text{ s}^{-1}$, (at $+25 \text{ }^\circ\text{C}$); thus approaching the $5 \times 10^6 \text{ s}^{-1}$ (at $+20 \text{ }^\circ\text{C}$) of catalase, the fastest enzyme known (Hille, 2001).

The three CARPs (CAVIII, X, and XI) are evolutionarily conserved cytoplasmic proteins that lack catalytic

activity because at least one of the three zinc-binding histidine residues is replaced with other amino acids (Supuran et al., 2004). Even though roles in e.g. protein complex formation and cell proliferation have been suggested for CARPs, there is not yet much knowledge about their involvement in biological functions.

Identified (α -)carbonic anhydrase isoforms			
Isoform	Catalytic activity	Subcellular localization	Expression in the CNS
CAI	Low	Cytosolic	-
CAII	High	Cytosolic	Widespread
CAIII	Very Low	Cytosolic	Some glia, choroid plexus
CAIV	High	Membrane bound	Widespread
CAVA	Moderate-high	Mitochondrial	Some neurons and astrocytes
CAVB	Low	Mitochondrial	Spinal cord
CAVI	Moderate	Secreted	-
CAVII	High	Cytosolic	Widespread, mostly neurons
CAVIII-CARP	Acatalytic	Cytosolic	Widespread
CAIX	High	Transmembrane	Low expression*
CAX-CARP	Acatalytic	Cytosolic	Some expression
CAXI-CARP	Acatalytic	Cytosolic	Moderate expression
CAXII	Low	Transmembrane	Low expression*
CAXIII	Moderate	Cytosolic	Some glia
CAXIV	High	Transmembrane	Widespread
CAXV	Low	Membrane bound	Some expression

Table 3. The (α -)carbonic anhydrase isoforms and their catalytic activity, subcellular localization, and expression in the rodent central nervous system (CNS).* Isoform present in normal brain at low levels but is over-expressed in certain carcinomas. The data in the table are from Supuran et al., 2003 and Hilvo et al., 2005.

2.2.3.1 Intracellular carbonic anhydrases and their expression in hippocampus

It is not long ago when intracellular carbonic anhydrase (CA_i) activity in the rodent CNS was thought to be restricted to glial cells (Cammer and Tansey, 1988; Agnati et al., 1995; Nogradi et al., 1997) and CAII expression was considered a reliable marker for oligodendrocytes (Ghandour et al., 1980; Ghandour et al., 1992). Even though CA is highly expressed in glial cells and in the myelin compartment, there is by now both functional (Pasternack et al., 1993; Munsch and Pape, 1999; Schwiening and Willoughby, 2002) and molecular biological evidence (Nogradi et al., 1989; Lakkis et al., 1997; Nogradi et al., 1997; Wang et al., 2002b; Kida et al., 2006) for the presence of intraneuronal CA.

In addition to the well-described localization of CAII in glial cells, Halmi et al. (2006) reported a diffuse expression of CAII mRNA in *stratum pyramidale* and dentate gyrus. At the protein level, staining with a CAII-specific antibody suggested a widespread neuronal expression (Wang et al., 2002b; Kida et al., 2006). In the hippocampus, CAII immunoreactivity was most prominent within the somata and proximal dendrites of pyramidal neurones. Furthermore, there is a strong transcriptional signal for CAVII in the juvenile and adult rodent hippocampal pyramidal cell layer (Lakkis et al., 1997; Halmi et al., 2006). Besides these two cytosolic isoforms, adult mouse hippocampal neurons show a strong positive immunostaining for CAV (Ghandour et al., 2000). However, CAV is a mitochondrial CA isoform (Nagao et al., 1994) which is unlikely to play a direct role in reactions associated with transmembrane movements of H⁺, CO₂, and HCO₃⁻.

2.2.3.2 Extracellular carbonic anhydrases and their expression in hippocampus

In the adult rodent CNS the extracellular CA (CA_o) activity is associated with the transmembrane (CAXIV, IX and XII) and membrane-attached (CAIV and XV) CA isoforms that have their active site oriented to the extracellular side. CAIV and CAXV are attached to the plasma membrane with a glycosyl-phosphatidylinositol anchor (Zhu and Sly, 1990; Hilvo et al., 2005) whereas CAIX, XII, and XIV have a transmembrane segment (Pastorek et al., 1994; Tureci et al., 1998; Mori et al., 1999).

By using in situ hybridization and RT-PCR Hilvo et al. (2005) showed that CAXV is expressed, among other tissues, in the mouse brain. However, based on the sequence data, it seems that in humans and chimpanzees this isoform had become a non-processed pseudogene. The lack of CAXV expression in several human tissues, including the brain, was confirmed with RT-PCR. CAIX and XII are expressed at low levels in the normal rodent brain, CAXII being most abundant in the choroid plexus (Ivanov et al., 2001; Hilvo et al., 2004; Kallio et al., 2006). These two isoforms have an exceptional expression pattern as they are markedly up-regulated in certain human carcinoma cells in e.g. kidney, lung, and CNS (Tureci et al., 1998; Ivanov et al., 2001). CAIV (Carter et al., 1990; Ghandour et al., 1992; Tong et al., 2000; Wang et al., 2002b) and CAXIV (Parkkila et al., 2001) are both present in rodent and human brain at the mRNA and protein level. At least in the rodent hippocampus the CAIX, XII, and XV make a minor contribution to the total CA_o activity. Direct monitoring of extracellular pH transients with H⁺ sensitive microelectrodes in hippocampal slices from rats (Tong et al., 2000) and from CAIV and XIV double knock-out mice (Shah et al., 2005) demonstrated that these two CA isoforms are largely responsible for CA_o activity in the hippocampus.

2.3 Developmental changes in ion regulation

2.3.1 Developmental changes in neuronal chloride regulation

The ion whose intracellular concentration changes the most during development of the mammalian CNS is Cl^- . No other ion undergoes such a dramatic ontogenic alteration in its intraneuronal concentration (Luhmann and Prince, 1991, Zhang et al., 1991; Owens et al., 1996; Tyzio et al., 2008; Blaesse et al., 2008). In immature CNS neurons Cl^- concentration is typically in the range of 25-40 mM (see e.g. Owens et al., 1996; Balakrishnan et al., 2003; Yamada et al., 2004; Sipilä et al., 2006b; Achilles et al., 2007) whilst in the soma of mature neurons the Cl^- concentration is kept around 5-7 mM (for recent reviews see Farrant and Kaila, 2007; Blaesse et al., 2008). This change reflects major differences in the functional expression of Cl^- transporters during neuronal maturation.

KCCs show distinct spatiotemporal expression patterns in the embryonic brain. Li et al. (2002) have shown that all four KCC isoforms are expressed in the embryonic brain but their regional expression was not overlapping. Rather, their expression reflected the developmental stage of the individual brain region with KCC4 being expressed in undifferentiated regions with proliferating cells, KCC3 in recently differentiated regions, and KCC2 in differentiating regions following the functional maturation of neurons. Because of the strictly neuronal localization of KCC2 and its vast expression in the adult rodent brain (Payne et al., 1996, Williams et al., 1999 but see Gulacsi et al., 2003; Bartho et al., 2004), increased KCC2 mediated cellular Cl^- extrusion is an obvious candidate to contribute to the maintenance of low intracellular Cl^- , characteristic for most mature neurons. Before Study I of this Thesis the expression pattern of KCC2 in different brain regions had been studied both in the embryonic and

adult brain but no such studies had been performed during the postnatal development of the rodent brain. Some indication of increased postnatal expression of KCC2, occurring in parallel with the decrease in $[\text{Cl}^-]_i$ seen in cortical neurons (Owens et al., 1996; Tyzio et al., 2008) had been provided by Lu et al. (1999). Using RT-PCR, immunoblotting, and immunofluorescence Lu et al. (1999) showed that KCC2 transcript and protein levels increased during the two first postnatal weeks. However, the specificity of the KCC2 antibody and cDNA probe used in these experiments might be questioned because the authors report a strong KCC2 expression in the dorsal root ganglion (DRG). According to Study I of this Thesis, the DRG can be used as a negative control for KCC2.

NKCC1 mRNA is detected in the hippocampus at birth (Plotkin et al., 1997; Li et al., 2002; Wang et al., 2002a). Indeed, electrophysiological recordings from juvenile hippocampal neurons suggest that NKCC1 acts as the main Cl^- uptake mechanism responsible for GABA_A -mediated depolarizing currents (Yamada et al., 2004; Sipilä et al., 2006b; Achilles et al., 2007). Some groups have reported a decline in NKCC1 expression during later developmental stages (Plotkin et al., 1997; Hübner et al., 2001a; Yamada et al., 2004) but there is also contrasting data showing NKCC1 expression in adult animals (Wang et al., 2002a; Clayton et al., 1998). If there is no consensus on the postnatal NKCC1 mRNA expression, the overall picture of the developmental expression of NKCC1 protein is not much clearer. The conflicting data on the protein expression can be at least partially explained by the use of an NKCC antibody (T4) that seems to be reliable in immunoblots (Zhang et al., 2006) but not in immunohistochemistry (see Kaila et al., 2008). Electrophysiological data provide support for the finding that NKCC1 expression continues into the adulthood. Szabadics et al. (2006) reported that in the axon initial segment (AIS) of mature neocortical layer 2/3 neurons $E_{\text{GABA-A}}$ is at a much more depolarized level than the resting

membrane potential. By using immunoelectronmicroscopy, the authors also demonstrated an uneven subcellular distribution of KCC2 between the soma and AIS. However, impaired Cl⁻ extrusion alone cannot explain the highly (20 mV) depolarizing reversal potential of GABA. To study the role of Cl⁻ uptake mechanisms in setting the E_{GABA-A}, Khirug et al. (2008) performed experiments on NKCC1 KO animals and in neurons in which NKCC1 was inhibited by bumetanide. The depolarizing GABA_A responses seen at the AIS of mature neocortical pyramids was shown to be caused by an axo-somatic Cl⁻ gradient that is explained by distinct subcellular expression patterns of NKCC1 and KCC2.

It can be concluded that the interplay between KCCs and NKCC1 dominates neuronal Cl⁻ regulation both during development and in adulthood. The functional importance of the postnatal up-regulation of KCC2 on GABAergic signalling is the subject of Study I.

2.3.2 Developmental changes in neuronal pH regulation

2.3.2.1 Changes in acid-base transporter expression and function

pH_i values measured from hippocampal neurons with the fluorescent pH indicator 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) are distributed over a broad range and can vary even by one pH unit in a single study (Schwiening and Boron, 1994; Bevensee et al., 1996; Baxter and Church, 1996; Kelly and Church, 2006). The postnatal development of steady-state pH_i values in hippocampal neurons has been followed in two separate studies. In both cases the mean initial pH_i of acutely isolated adult and embryonic/juvenile hippocampal pyramids did not differ significantly between the two age groups studied (Raley-Susman et al., 1993; Bevensee et al., 1996). Bevensee et al. (1996) also reported that the rate of pH_i recovery from an acute acid

load, in the nominal absence of CO₂/HCO₃⁻, was similar in adult and juvenile neurons.

There is little molecular biological evidence for postnatal up- (or down) regulation of BTs or other transporters involved in pH regulation in rodent hippocampal neurons. Douglas et al. (2001) used immunoblotting to detect various transporters involved in pH_i regulation at several different postnatal ages. In protein lysates from the whole rat cerebral cortex NBCe expression increased significantly from embryonic day 16 (E16) to adulthood (postnatal day 105; P105). NBCe mRNA has also been shown to be up-regulated during the two first postnatal weeks in the rat brain (Giffard et al., 2000). The latter result, however, does not permit conclusions on neuronal NBCe up-regulation because the expression of the transporter was reported to be restricted to astrocytes. Other groups have reported NBCe expression in several neuronal subpopulations in the adult brain, including hippocampal granule cells and pyramidal neurons (Bevensee et al., 2000; Schmitt et al., 2000; Rickmann et al., 2007; Majumdar et al., 2008). In the work by Douglas et al. (2001) the expression of the NHE isoforms 1, 2, and 4 was assessed in addition to NBCe expression. As with the NBCe expression, the levels of all three NHE isoforms increased in the cerebral cortex during the postnatal development. These results, both on NBCe and NHE expression patterns, should be considered with some reservation because no reference proteins were used in the densitometry analyses.

The studies on the developmental expression of other BTs have mainly been limited to individual time points. The NCBE mRNA is present in the hippocampal pyramidal cell layer already at prenatal stages (Hübner et al., 2004; Chen et al., 2008a) and the transcriptional signal remained fairly stable during the early postnatal development (Giffard et al., 2003). The expression of NDCBE and NBCn1 have not been thoroughly followed but immunohistochemical and single-cell PCR data show that both transporters

are present in embryonic and adult mouse hippocampal pyramidal neurons (Cooper et al., 2005; Chen et al., 2008b). Experiments using *in situ* hybridization have revealed that the Na^+ -independent $\text{Cl}^-/\text{HCO}_3^-$ exchanger AE3 is present in the embryonic rat (Raley-Susman et al., 1993) and mouse brain (Hentschke et al., 2006). However, the results from studies addressing the functional activity of the exchanger in embryonic neurons are somewhat contradictory. Intracellular pH recordings from acutely isolated and cultured (10 days *in vitro*, DIV) fetal hippocampal pyramidal neurons were performed using a pH-sensitive fluorescent dye (Raley-Susman et al., 1993). These authors did not detect functional activity of $\text{CO}_2/\text{HCO}_3^-$ exchange upon removal of Cl^- from the extracellular solution whereas according to (Baxter and Church, 1996) $\text{Cl}^-/\text{HCO}_3^-$ exchange is functionally active in cultured rat fetal neurons (6-14 DIV). In adult CA1 pyramidal neurons washout of Cl^- results in an intracellular alkalization indicating reversal of $\text{Cl}^-/\text{HCO}_3^-$ exchange (Raley-Susman et al., 1993; Hentschke et al., 2006; Ruusuvaori et al., 2007).

2.3.2.2 Changes in intracellular buffering

Even though steady state cytoplasmic pH doesn't seem to undergo marked changes during development, a change in the intrinsic buffering capacity or in the activity of CA could significantly modulate the amplitude, and thus the functional impact, of activity-induced pH transients. The pH_i dependence of intrinsic buffering power of CA1 pyramidal neurons is kept relatively stable during postnatal development (Bevensee et al., 1996). This result implies that significant qualitative changes in β_i capacity are also unlikely to take place during this time window. Hence, developmental changes in CA expression would be highly relevant as such a change would be in a key position to modulate β_{CO_2} with a consequent, age-dependent, effect on the kinetics of pH_i transients.

In the rodent CNS CAII mRNA is expressed at a very low level at birth (Cammer and Zhang, 1996) but the levels increase several-fold during the first postnatal months (Nogradi et al., 1997; see Kida et al., 2006 for CAII expression in humans). Because of the vast expression of CAII in oligodendrocytes, this increase might largely reflect myelin formation which takes place within the same time window (Suzuki and Raisman, 1994; Savaskan et al., 1999). Developmental changes in the expression of other intracellular CA isoforms have not been studied before Study II in which we assessed the postnatal development of intrapyramidal CA activity and the expression profiles of both CAII and CAVII, the two putative intraneuronal isoforms in adult rodent hippocampal pyramidal neurons.

Here it is tempting to speculate that the developmental decrease in Cl^- concentration is a specific adjustment of adult neurons to GABA- (and glycine-) mediated fast synaptic inhibition (see Blaesse et al., 2008) whereas the relatively constant resting level of somatic pH values reflect the pH sensitivity of several elementary cytoplasmic processes and proteins that are kept unchanged during postnatal development. Only modulation of pH transients, during development or within subcellular compartments, would permit the cell to modify proton-mediated signaling.

An intriguing question is also how the electroneutrality within a cell is maintained when intracellular anion concentration is reduced as the intracellular Cl^- concentration decreases. Assuming that the osmolarity is kept constant, the amount of intracellular charges could be maintained if (1) the concentration of other anions is accordingly increased, (2) the net loss of Cl^- is accompanied by an equal loss of a cation (the likely candidate being K^+), which would require that the osmolarity is maintained by an increase in some neutral macromolecules or low-weight organic molecules, or (3) the charge carried by intracellular proteins is changed.

Because of the shortage of data on the two latter options, only the first will be discussed further. It is obvious that a corresponding increase in HCO_3^- concentration does not occur as, at fixed P_{CO_2} , such a change would disturb cellular pH balance. A ~30 mM elevation in phosphate concentration is also unlikely to take place as this would result in a concomitant increase in the intrinsic buffering capacity. As already discussed, the results of Bevensee et al. (1996) did not suggest changes in the intrinsic buffering capacity in hippocampal neurons during postnatal development, although the age window of the animals may not be optimal for the present examination (P2-10 vs. P21-30). Hence, the question what mechanisms compensate for the loss of Cl⁻ under constant osmolarity remains unanswered.

2.3.3 Developmental changes in extracellular ion homeostasis

Whether the fetal mammalian BBB is leaky is still a matter of debate (Saunders et al., 2000; Engelhardt, 2003). Tight junctions, the morphological basis of brain barriers, are present from the very early stages of development both between endothelial cells of blood vessels and between epithelial cells of the choroid plexus (Mollgård et al., 1979). The junctions are impermeable to small molecules from early development and are thus able to restrict diffusion already in fetal and juvenile animals (Johansson et al., 2006; Ek et al., 2006). Actually, there are additional morphological barriers unique to the fetal brain at the interface between CSF and brain tissue (Fossan et al., 1985). The transient expression of the specific intercellular junctions, “the strap junctions”, prevents the movement of macromolecules at the inner neuroependymal surface of ventricles (Mollgård et al., 1987; Saunders et al., 2000). However, indications of developmental tightening of the BBB have been presented. The transendothelial resistance of pial microvessels,

reflecting the permeability of the BBB to ions, was measured in fetal and adult rats (Butt et al., 1990; Keep et al., 1995). A marked increase in the resistance was found to take place just before birth.

The distribution of ions between CSF and plasma in prenatal (Jones and Keep, 1987) and juvenile rat pups (Amtorp and Sorensen, 1974) is similar to what is seen in older animals. This indicates that the transport systems responsible for maintenance of concentration differences of ions across the blood-CSF barrier are functional in newborn mammals (Ferguson and Woodbury, 1969; Bradbury et al., 1972; Amtorp and Sorensen, 1974). The most striking developmental change in CSF composition is the considerably higher concentration of proteins present in the fetal and newborn CSF (Amtorp and Sorensen, 1974; Dziegielewska et al., 2000). As the leakiness of the immature BBB can be excluded (Ek et al., 2006; Johansson et al., 2006) the developmental changes in the protein concentration likely reflect changes in the transepithelial transport (Habgood et al., 1992; Johansson et al., 2008). The significance of the finding is not clear but the high protein concentration of CSF appears to be maintained during the period of ventricular expansion thus contributing to specific features of CNS development.

Like the intracellular pH of brain cells, also the CSF pH is maintained constant during postnatal development (Pavlin and Hornbein, 1975; Nattie and Edwards, 1981; Johanson et al., 1992). Because the buffering of pH in brain fluids is dominated by β_{CO_2} , CA activity has a central role in modulating activity-dependent pH transients (Chesler and Kaila, 1992). In the developing rat hippocampus measurements of ECF CA_o activity with OH⁻ injections and pH sensitive microelectrodes have demonstrated that CA_o activity commenced at the end of the first postnatal week (Voipio et al., 1999) most likely reflecting the expression of CAIV and/or CAXIV (Shah et al., 2005).

2.4 Functional significance of ion regulation

2.4.1 Neuronal ion regulation modulates GABA_A receptor-mediated signaling

2.4.1.1 GABA_A receptor-mediated transmission in developing hippocampal neurons

The high intracellular Cl⁻ of the immature neurons results in an E_{Cl} more positive than resting V_m (Payne et al., 2003) and GABA_A receptor-mediated responses are often considered to be depolarizing enough to trigger neuronal spiking, open voltage-gated Ca²⁺ channels, and facilitate the activation of NMDA channels (Janigro and Schwartzkroin, 1988; Ben-Ari et al., 1989; Cherubini et al., 1990; Yuste and Katz 1991; Leinekugel et al., 1997; Fukuda et al., 1998). The depolarizing GABA_A receptor-mediated currents have been suggested to represent a general mechanism of the developing brain that controls different aspects of neuronal development e.g. migration, differentiation, synaptogenesis, and refinement of the emerging neuronal networks (LoTurco et al., 1995; Owens and Kriegstein, 2002; Demarque et al., 2002; Chudotvorova et al., 2005; Akerman and Cline, 2006).

GABA_A receptor-mediated signaling begins before synaptic connections emerge (Valeyev et al., 1993; Tyzio et al., 1999; Demarque et al., 2002). At this early developmental stage the anion currents evoked by GABA are mediated by tonic activation of non-synaptic GABA_A receptors. Only after neuronal migration is completed, synaptic transmission starts to contribute to GABA_A-receptor activation. The establishment of GABAergic and glutamatergic synapses in developing rat hippocampus is sequential, with GABAergic transmission preceding that of glutamatergic neurons (Tyzio et al., 1999; Hennou et al., 2002).

In the developing nervous system spontaneous network activity is

characteristic for different brain regions, including the hippocampus (Yuste et al., 1992; Katz, 1993; Ben-Ari et al., 1989; O'Donovan, 1999). Although the CA3 subregion of hippocampus often functions as a pacemaker, spontaneous network events can be generated in other areas (i.e. CA1 and dentate gyrus) even when isolated from other regions (Khazipov et al., 1997; Garaschuk et al., 1998; Ben-Ari, 2001). Experiments using Ca²⁺ imaging have revealed that spontaneous network activity in the immature hippocampus is associated with synchronous intracellular Ca²⁺ oscillations mediated by voltage-dependent Ca²⁺- and NMDA-channels (Leinekugel et al., 1997; Garaschuk et al., 1998; Garaschuk et al., 2000). This joint presynaptic activity and postsynaptic [Ca²⁺]_i influx could mediate Hebbian type of activity-dependent plasticity of developing synapses and be involved in activity-dependent synaptogenesis and network formation (Feller, 1999; Spitzer, 2006).

When Ben-Ari et al. (1989) first described spontaneous oscillations in the neonatal rat hippocampal CA3 pyramidal cells (named as giant depolarizing potentials, GDPs) the events were described as network-driven synaptic events mediated by GABA. The GABAergic nature of GDPs was suggested by intracellular measurements which showed that GDPs were blocked by the GABA_A receptor antagonist bicuculline and reversed at the same membrane potential as exogenously applied GABA. The contribution of pyramidal neurons, i.e. glutamatergic transmission, in GDP generation was also observed and the importance of the synergistic action of GABA_A and ionotropic glutamatergic transmission in synchronizing neuronal network discharges was underlined in later studies (Khazipov et al., 1997; Leinekugel et al., 1997). However, the central role of depolarizing GABA_A-channel mediated postsynaptic currents in providing the excitatory drive for GDP generation was still strongly emphasized (Ben-Ari, 2002; Ben-Ari

et al., 2007; see also Strata et al., 1997).

Recently Sipilä et al., 2005, 2006a) have presented a different mechanistic explanation for GDP generation. The authors demonstrated that immature CA3 pyramidal neurons are able to produce bursts of action potentials in the absence of ionotropic glutamate and GABA mediated transmission. The intrinsic bursting was triggered by a slow regenerative depolarization mediated by a persistent Na^+ current and terminated by a slow, Ca^{2+} -activated K^+ -current (Sipilä et al., 2006a). In line with previous observations (Ben-Ari et al., 1989, Bolea et al., 1999; Lamsa et al., 2000) CA3 network activity was fully blocked by ionotropic glutamate-receptor antagonists. According to Sipilä et al. (2005, 2006a) this endogenous bursting activity of immature CA3 pyramids acts to generate GDPs while the depolarizing GABA_A mediated transmission has a permissive role facilitating the spontaneous activity of the immature neurons.

The gradual disappearance of GDPs during the second postnatal week (Ben-Ari et al., 1989; Khazipov et al., 2004) occurs in parallel with the transition to more negative E_{Cl} values and to hyperpolarizing GABA_A responses. This developmental increase in the Cl^- gradient is readily explained by a major reorganization of intracellular Cl^- regulation (Study I of this Thesis).

2.4.1.2 Depolarizing GABA_A ergic transmission in the mature hippocampus

It has been known for a long time that activation of dendritic GABA_A receptors using exogenous agonists can result in depolarizing responses in mature hippocampal pyramidal neurons (Alger and Nicoll, 1982a; Alger and Nicoll, 1982b; Huguenard and Alger, 1986; Kaila et al., 1993; Voipio and Kaila, 2000). Previous results from our laboratory have shown that large-scale activation of GABA_A channels, by using high-frequency

stimulation (HFS), can evoke a HCO_3^- -dependent depolarization of adult hippocampal CA1 pyramidal (Kaila et al., 1997; see also Grover et al., 1993). The GABA_A ergic response is biphasic with an early hyperpolarization (representing fused individual hyperpolarizing inhibitory postsynaptic potentials) followed by a prolonged depolarization that is often associated with pronounced spiking (Perreault and Avoli, 1988; Grover et al., 1993; Staley et al., 1995; Kaila et al., 1997; Kaila et al., 1997; Smirnov et al., 1999). The depolarization is generated by a fast initial anionic redistribution and of a long-lasting increase in $[\text{K}^+]_o$ (Voipio and Kaila, 2000). The anion redistribution is caused by the depolarizing HCO_3^- current that leads to accumulation of intraneuronal Cl^- and consequently, to a large positive shift in $E_{\text{GABA-A}}$ (Kaila et al., 1987; Kaila et al., 1989a; Voipio et al., 1991). Inhibition of intra- but not extracellular CA has been shown to attenuate the post-tetanic depolarization (Kaila et al., 1997) but before Study II of this Thesis the cellular subtype expressing the intracellular CA activity that plays a crucial role here had not been identified.

Synchronous firing of principal neurons is seen under both physiological and pathophysiological conditions: it plays a central role in neuronal plasticity (Traub et al., 1998; Linden, 1999) as well as in the genesis and maintenance of epileptic activity (McCormick and Contreras, 2001). Therefore it is intriguing that the HFS-induced neuronal firing can be locally synchronized into gamma frequency range oscillations (afterdischarges) of confined pyramidal-cell and interneuronal networks (e.g. Traub et al., 1996a; Colling et al., 1998; Bracci et al., 1999). The HFS-induced gamma oscillations are characterized by massive, synchronous firing of principle cells, a property that more likely represents epileptiform activity than the small-amplitude, theta-modulated gamma oscillations measured *in vivo*, which are suggested to be involved in cognition and perception (Ylinen et al., 1995;

Penttonen et al., 1998; Gray, 1999). In addition to the intrinsic properties of neurons (Connors and Gutnick, 1990; Stanford et al., 1998; Hille, 2001), the mechanisms that have been shown to contribute to the temporal patterns of neuronal firing include GABA_A receptor-mediated inhibition (Whittington et al., 1995), ionotropic glutamatergic transmission (Traub et al., 1996a), gap junctions (Draguhn et al., 1998; Schmitz et al., 2001), and ephaptic effects (Bracci et al., 1999). Intracellular recordings from CA1 pyramidal neurons have shown that the HFS-induced prolonged excitation of the principal cells depends on the GABA_A channel-mediated depolarization described above (Kaila et al., 1997; Bracci et al., 1999; Smirnov et al., 1999). Furthermore, HFS-induced population activity in the gamma frequency range can be evoked in the absence of ionotropic glutamate receptor activity and blocked by GABA_A antagonist bicuculline (Colling et al., 1998; Bracci et al., 1999). In light of the important role of the GABA_A-channel mediated, K⁺_o-dependent depolarization of pyramidal cells (Perreault and Avoli, 1988; Grover et al., 1993; Kaila et al., 1997) it is interesting that oscillations at gamma frequencies can also be evoked by brief K⁺ application in the CA1 (and CA3) region of rat hippocampus (LeBeau et al., 2002). The activation of interneuronal metabotropic glutamate receptors (mGluRs) has also been suggested to be involved in the HFS-induced gamma oscillations (Whittington et al., 1995; Whittington et al., 1997; Traub et al., 1996a; Traub et al., 1996b; Whittington et al., 1997; but see Bracci et al., 1999) although the role of mGluRs has been shown to be most significant in the 'pure' interneuronal network oscillations (Whittington et al., 1995). A shift to depolarizing GABAergic responses is also detected in several trauma models of adult neurons e.g. after injury of neurites or anoxia/ischemia (Katchman et al., 1994; van den Pol et al., 1996; Nabekura et al., 2002). There is increasing evidence suggesting that the depolarizing E_{GABA-A} seen after

neuronal trauma depends on changes in the expression of the CCCs (Nabekura et al., 2002; Fukuda et al., 1998; Pond et al., 2006). It has been proposed that these expressional changes might function to "turn back the time" of the injured neurons in order to restore their developmental flexibility, which would be able to rearrange the neuronal connectivity by e.g. axonal sprouting and retargeting (see Payne et al., 2003).

The study by Cohen et al., 2002) assessed the role of GABA_A receptor-mediated transmission in hippocampal preparations from human epilepsy patients. Depolarizing GABAergic transmission was shown to underlie the interictal activity in a subpopulation of subicular neurons. In a follow-up paper, this finding was explained by a down regulation of KCC2 (Huberfeld et al., 2007). A pronounced change in neuronal E_{GABA-A}, with a concomitant reduction in KCC2 levels, has also been shown to take place in animal models of epilepsy both *in vitro* and *in vivo* (Rivera et al., 2002; Rivera et al., 2004; Pathak et al., 2007). Hence it might be that expressional changes of CCCs are a general response of neurons to different kind of trauma.

2.4.2 Neuronal excitability is modulated by pH

A common observation both *in vitro* and *in vivo* has been that a fall in pH leads to a decrease in neuronal activity. Manipulations that aim to reduce tissue pH decrease hippocampal neuronal excitability and suppress evoked seizure activity *in vitro* (Aram and Lodge 1987; Jarolimek et al., 1989; Lee et al., 1996; de Curtis et al., 1998; Bonnet et al., 2000; Xiong et al., 2000; Dulla et al., 2005) and *in vivo* (Balestrino and Somjen, 1988; Ziemann et al., 2008). From a teleological point of view, such pH dependence provides a negative feedback control of network activity that would limit the generation and propagation of seizure activity. Furthermore, such a feedback loop would assist in seizure termination (Velisek et al., 1994; de Curtis et al.,

1998; Ziemann et al., 2008). In most neurons protons suppress electrical activity. However, the pH dependence of intrinsic excitability appears to be opposite in central chemosensory neurons. These cells detect changes in CO_2/H^+ and respond to a raise in plasma CO_2/H^+ (i.e. to respiratory acidosis) by increased activity (Nattie, 2001, Putnam et al., 2004). Via these cells, an increase in CO_2/H^+ serves as the major stimulus for increased ventilation in response to respiratory acidosis. This forms a “higher level of excitability control” where the brain regulates breathing, and thus its own CO_2 levels, thereby affecting its own excitability.

In contrast to acidification, a rise in pH leads to increased neuronal excitability that may even lead to epileptiform activity *in vitro* (Aram and Lodge, 1987; Jarolimek et al., 1989; Lee et al., 1996; de Curtis et al., 1998; Dulla et al., 2005). *In vivo* experiments provide support for the findings done *in vitro*. Balestrino and Somjen, 1988) showed that rat hippocampal neurons were highly sensitive to small deviations from normal pH. Reduction in P_{CO_2} concentration enhanced neuronal firing (and increased P_{CO_2} depressed it). Recently, hyperthermia-induced respiratory alkalosis was demonstrated to trigger ictal activity in a febrile seizure model (Schuchmann et al., 2006).

The effect of pH on neuronal excitability is commonly believed to reflect changes in the activity of ion channels (voltage-, ligand-, or proton-gated) and gap junctions. The intrinsic excitability of a neuron is suppressed by a decline in pH probably because the currents through voltage-gated Na^+ and especially Ca^{2+} channels are often depressed by protons (Tombaugh and Somjen, 1996; Tombaugh and Somjen, 1997). Notably, the proton-mediated suppression of currents flowing through voltage gated K^+ channels tend to mitigate the stabilizing effect of K^+ currents on resting membrane potential and to reduce after-hyperpolarization and thus to enhance neuronal excitability.

The acid sensing ion channels (ASICs) are non-selective cation channels that

are directly gated by extracellular protons (Waldmann et al., 1997). ASICs are widely expressed in the CNS (Waldmann et al., 1997; de la Rosa et al., 2003) where they modulate neuronal excitability (Vukicevic and Kellenberger, 2004). Recently, ASICs were shown to be involved in seizure termination in an animal model where the gene encoding ASIC1a protein was disrupted (Ziemann et al., 2008).

The high sensitivity of ligand-gated ion channels to extracellular pH further supports the important modulatory role of protons in synaptic transmission. NMDA receptor channels are often inhibited (Tang et al., 1990; Traynelis and Cull-Candy, 1990) whereas GABA_A responses are increased upon a fall in pH_o (Takeuchi and Takeuchi, 1967; Pasternack et al., 1996). The pH sensitivity of glutamate- and GABA -mediated transmission can differ markedly because different subunits of postsynaptic ligand-gated channels show varying sensitivity to pH (Traynelis et al., 1995; Krishek et al., 1996; Wegelius et al., 1996). Even though the steep pH_o dependency of GABA_A and NMDA channels has been recognized for several decades, the amount of data supporting the functional impact of e.g. activity-evoked pH_o transients on synaptic transmission is strikingly sparse (Taira et al., 1993; Gottfried and Chesler, 1994; Tong et al., 2006; Fedirko et al., 2007; Makani and Chesler, 2007). Nevertheless, because the pH_o dependence of synaptic transmission takes place at proton concentrations close to the physiological pH range, the ability of endogenous pH transients to modulate GABA_A and NMDA receptor mediated synaptic transmission is of much potential impact (Chesler and Kaila, 1992; Gottfried and Chesler, 1993; Makani and Chesler, 2007).

Cellular communication via electrical synapses, gap junctions, is affected by intracellular pH with cytoplasmic acidification closing and alkalinization opening the channels (Spray et al., 1981; Spray and Scemes, 1998; Church and Baimbridge, 1991). During early development principal neurons in e.g. rat neocortex are extensively coupled

via gap junctions (LoTurco and Kriegstein, 1991). However, experiments on the adult rat hippocampus have repeatedly revealed electrical synapses among inhibitory interneurons but not between excitatory cells (Michelson and Wong, 1994; Hormuzdi et al., 2001; Connors and Long, 2004 but see Schmitz et al., 2001).

2.4.2.1 Activity-induced pH transients in neurons and in the extracellular space

In addition to the metabolic activity and the constant tendency of conductive leaks to impose a chronic intracellular acid load, electrical activity of neurons has been shown to induce rapid and robust pH changes. The underlying mechanisms vary, but rapid pH shifts are largely generated by transmembrane fluxes of acid equivalents.

Intracellular pH measurements from cultured mammalian neurons have shown that glutamate-induced membrane depolarization results in an intracellular acidification (Hartley and Dubinsky, 1993). In experiments with prolonged-glutamate exposure it was suggested that the glutamate-induced pH_i decline may, in concert with the increased calcium levels, mediate excitotoxicity. Furthermore, bursts of action potentials result in an intracellular acidification in neurons of rat brain stem slices (Trapp et al., 1996). The effect of membrane depolarization on pH_i occurs in the absence of $\text{CO}_2/\text{HCO}_3^-$ and is probably directly linked to the depolarization-induced entry of Ca^{2+} ions. The subsequent extrusion of Ca^{2+} by Ca^{2+} - H^+ -ATPase has been suggested to explain the activity-induced decrease of pH_i (Paalasmaa et al., 1994; Smith et al., 1994; Trapp et al., 1996).

The pH_i shifts associated with inhibitory GABA-mediated neurotransmission are caused by an entirely different mechanism. As discussed earlier on, GABA_A and glycine receptor channels are permeable to HCO_3^- , and it is the efflux of HCO_3^- and influx of CO_2 that

produce the intracellular acidification (and extracellular alkalization) upon GABA_A receptor channel opening (Kaila and Voipio, 1987; Chen and Chesler, 1990, Kaila et al., 1990; Chen and Chesler, 1991; Voipio et al., 1991; Pasternack et al., 1993; Luckermann et al., 1997).

The pH shifts evoked by synaptic transmission and neuronal activity are not limited to the intracellular space. A pronounced extracellular alkalization is the immediate pH response upon hippocampal Schaffer collateral stimulation (Jarolimek et al., 1989; Walz, 1989; Chen and Chesler, 1992a; Chesler and Kaila, 1992). The initial, fast alkalization evoked by neuronal activity is frequently followed by a slower and longer lasting acidification (Jarolimek et al., 1989; Chen and Chesler, 1992a; Kaila et al., 1992) that can be, at least under certain experimental conditions, accounted for by a rise in tissue P_{CO_2} (Voipio and Kaila, 1993).

Both excitatory and inhibitory neurotransmission are involved in the generation of the extracellular alkaline transients, the relative contribution of glutamatergic and GABAergic components depending on the pattern of stimulation (Taira et al., 1995). In the rat hippocampus exogenously applied GABA or direct stimulation of pharmacologically-isolated inhibitory pathways produced a fast, HCO_3^- -dependent alkalosis (Chen and Chesler, 1992a; Kaila et al., 1992; Voipio et al., 1995). The pH_o transients were abolished with GABA_A -channel blockers and after inhibition of extracellular CA activity. Thus, the GABAergic extracellular alkaline shift is strictly dependent on the presence of $\text{CO}_2/\text{HCO}_3^-$ and on interstitial CA activity (Kaila and Voipio, 1987; Saarikoski and Kaila, 1992), and it is caused by a transmembrane GABA_A receptor channel mediated $\text{CO}_2/\text{HCO}_3^-$ shuttle (Kaila et al., 1990; Chen and Chesler, 1991; Kaila et al., 1992).

Extracellular alkalization evoked by excitatory synaptic transmission is fundamentally different from the GABA-mediated pH_o increases. Repetitive stimulation of Schaffer collaterals or pressure injection of

glutamate (or its agonists) in hippocampal CA1 area evoked a $\text{CO}_2/\text{HCO}_3^-$ independent pH_o increase that was amplified upon CA_o inhibition (Chen and Chesler, 1992b; Voipio et al., 1995; Taira et al., 1995). In this HCO_3^- -independent, glutamate-mediated alkaline shift the role of glutamate uptake (Amato et al., 1994), Ca^{2+} - H^+ -ATPase (Paalasmaa et al., 1994; Smith et al., 1994; Grichtchenko and Chesler, 1996) and H^+ channel (Thomas and Meech, 1982; Thomas, 1988) activity have been studied, but the mechanism behind this alkalization has not been firmly established.

2.4.2.2 CA activity facilitates transmembrane ion fluxes

Proton diffusion can be rate limiting in processes involving a molecular sink or source of protons and in movements of other acid-base equivalents (Geers et al., 1985; Wetzel and Gros, 1998; Voipio, 1998). In line with this, an important role of CA_o activity in lactic acid transport was reported in rat skeletal muscle cells (Wetzel et al., 2001) and later in hippocampal neurons and astrocytes (Svichar and Chesler, 2003). In view of the postulated astrocyte-neuron lactate-shuttle (Magistretti and Pellerin, 1999), brain CA_o s may turn out to be of great importance for neuronal energy supply.

CA_o activity affects the function of HCO_3^- -transport proteins (Seki and Fromter, 1992; Alvarez et al., 2005; Becker and Deitmer, 2007; Svichar et al., 2007). The coupling of CA activity to BTs is suggested to result in more efficient HCO_3^- transport. The closely associated CA and BT are thought to form a complex, a transport metabolon, where the sequential pathway provides an efficient mechanism for BT mediated HCO_3^- fluxes (Sterling et al., 2001; Sterling et al., 2002; but see Lu et al., 2006; Piermarini et al., 2007).

2.4.3 Studies on mouse models with genetically impaired chloride and pH regulation

KO mice have provided useful models in studies of the specific roles of different ion regulatory proteins in the CNS. Notably, abnormalities in either the function and/or membrane targeting of certain members of the SLC4, as well from the SLC12A and CA, gene families cause genetic diseases in humans (see below).

The outcomes of the CCC KO mice are in most cases far more deleterious than those of the BTs or CAs, which in general have an apparently normal phenotype. At the time when Hübner et al. (2001) and Woo et al. (2002) generated their KCC2 KO mouse strains, only one KCC2 isoform (now termed KCC2b) had been characterized. The recent finding of the novel KCC2a isoform (Uvarov et al., 2007) provides an explanation to the different outcome of the two KO strains and indicate distinct functions of the two isoforms. Full KCC2 KO mice (i.e. KCC2a and KCC2b KO) die immediately after birth due to respiratory failure (Hübner et al., 2001b) while the selective KCC2b KO has a life span of about two weeks and dies possibly because of spontaneous generalized seizures (Woo et al., 2002). Comparison of these two KCC2 KO mouse strains implies that KCC2a expression is vital for motor control while KCC2b is responsible for the hyperpolarizing GABAergic responses seen in adult cortical neurons (but see also Balakrishnan et al., 2003). KCC2 hypomorphic mice that have 15-20% of the normal KCC2a and KCC2b protein levels are viable but show reduced seizure susceptibility and altered behaviour in tests measuring learning, anxiety, and nociception (Vilen et al., 2001, Tornberg et al., 2005).

The two KCC3 KO mouse lines developed by Howard et al (2002) and Boettger et al. (2003) both show motor dysfunction caused by the progressive degeneration of peripheral nervous system (PNS). The KCC3 KO mice generated by Boettger et al.

(2003) also show degeneration of CNS, inner ear defects, abnormal electrocortico-gram, and reduced seizure threshold. The degeneration of cells is likely due to the impairment of cellular volume and Cl⁻ regulation as well as to the changes in the relative extra- and intracellular ion levels (Boettger et al., 2003). Apart from the lack of corpus callosum degeneration, the KCC3 KO phenotype replicates the symptoms of the human Anderman's syndrome (Howard et al., 2002). KCC4 (Boettger et al., 2002) and NKCC1 (Delpire et al., 1999; Dixon et al., 1999; Flagella et al., 1999; Pace et al., 2000) KO mice both have non-neuronal inner ear defects and suffer from distinct physiological malfunctions but the gross morphology of the CNS is surprisingly normal.

The relevance of AE3 in seizure susceptibility was addressed in experiments where the *Slc4a3* gene was disrupted (Hentschke et al., 2006). Adult AE3 KO mouse had an apparently normal phenotype but showed a reduced susceptibility to pharmacologically-induced seizures. However, in the febrile-seizure model (Schuchmann et al., 2006) the time of tonic-clonic seizure onset in juvenile, P14-15, AE3 KO pups was similar to that seen in the wild type littermates (E.Tolner, J.Voipio, and K.Kaila, unpublished observations). AE3 is activated at high pH_i values and is commonly suggested to mediate cellular recovery from alkaline load (Chesler, 2003). Since the predominant pH_i response to neuronal activity is an intracellular acidosis, the degree to which the reduced seizure susceptibility seen in adults is due to impaired, AE3-mediated pH_i regulation is not known. Deletion of another BT, the NBCn1, resulted in a specific visual and auditory phenotype of the animal model (Bok et al., 2003). The selective degeneration of sensory receptors in the eye and inner ear in the NBCn1 KO mice are characteristic for Usher syndrome in humans (Petit, 2001).

Many cells express more than one of the 12 enzymatically active CA

isozymes (Supuran et al., 2004). To clarify their individual contributions, five selective KOs and one double KO have been developed. Because CAIII and CAIX are mainly expressed in skeletal muscle (Kim et al., 2004) cells and gastrointestinal tract (Hilvo et al., 2004), respectively, these KO mice will not be discussed.

CAIV and CAXIV have been suggested to be the two predominant CA_o isoforms expressed in the brain (Tong et al., 2000; Parkkila et al., 2001). The development of CAIV and CAXIV KO mice, and by intercrossing, a CAIV/CAXIV double-KO, provided further support for the previous findings (Shah et al., 2005). The double KO animals had no obvious phenotype and appeared normal except for growth retardation and slightly distorted sex ratio of the offspring. Knowing the high pH sensitivity of ligand-gated ion channels, an interesting subject for future studies is to examine whether the impaired ECF buffering of these animals has significant effects on e.g. NMDA- and GABA_A-channel mediated transmission.

A mouse mutant lacking CA II was developed by Lewis et al., 1988). In this animal model for human CAII-deficiency syndrome, the symptoms include systemic and renal tubular acidosis, and growth retardation. No signs of osteopetrosis or brain calcification, as seen in CAII-deficient patients, were noticed (Sly et al., 1983). This probably reflects species differences in the physiological roles of CAII in osteoclast function and in the maintenance of the acid-base balance in the brain. It also points to limitations of modelling human disorders using transgenic mice.

The antiepileptic effect of CA inhibitors has been proposed to depend on a P_{CO2} increase that results in extra- and intracellular acidification (e.g. Millichap et al., 1955; Bickler et al., 1988; Leniger et al., 2002). Thus, the possible effect of the systemic acidosis observed in the CAII KO animals on seizure susceptibility was tested (Velisek et al., 1993). Mice from heterozygous breeding pairs were used in experiments in which the time of

seizure onset was measured, and the severity of the pharmacologically-induced and audiogenic seizures were rated. In comparison to the wild type and heterozygote mice, CAII KO animals had an increased latency to the onset of pharmacologically induced seizures and showed reduced incidence of both clonic and tonic-clonic seizures. A decrease in the incidence of audiogenic seizures in KO animals was seen only if animals were primed at an earlier developmental stage. Surprisingly, a similar seizure resistance was not observed in *in vitro* experiments, where spontaneous epileptiform activity was induced in brain slices by withdrawal of extracellular Mg^{2+} (Velisek et al., 1995). In contrast, slices from CAII KO mice had a shorter latency to the onset of epileptiform activity which also developed faster into sustained, status-like epileptiform activity that was not inhibited by increasing the CO_2 concentration.

2.5 Ion measurements in the mammalian brain

There are several means for monitoring of ion concentrations in live preparations (i.e. radio-isotopic tracer and atomic absorption methods) but only two techniques enable direct, *continuous* recordings of ionic changes at the cellular level. These methods are ion-sensitive microelectrodes (ISMEs) and optical indicators.

The technical advancement of both of the methods during the last decades has enabled researchers to perform measurements with higher selectivity and with better temporal and spatial resolution and hence, to examine ion homeostasis in the brain more accurately. Especially fluorescent indicators (e.g. the genetically encoded Cl⁻ indicator Clomeleon; Künér and Augustine, 2000) and the equipment for detection of optical signals (e.g. two-photon imaging; Williams et al., 1994) have been a rapidly developing field. Despite the great advances, both techniques have their limitations and recordings of ion transients from e.g.

extracellular microdomains are a challenge for the future.

2.5.1 Ion-sensitive microelectrodes

The commonly used ISMEs are glass microelectrodes with an organic liquid-membrane solution forming a selectivity barrier at the very tip of the electrode (Voipio, 1998). The liquid-membrane at tip of the microelectrode behaves as an ion-selective barrier so that the voltage-difference across it depends only on the permeant ion. Today there are commercially available, ready-to-use membrane solutions for many different ions, including H⁺, K⁺, and Cl⁻. Ion-sensitive electrodes can also be made using glass that has ion-sensitive properties. However, ion-selective glass is only available for measurements of H⁺ and Na⁺. The use of ion-sensitive glass is also limited because these electrodes have considerably large tip-diameter or, if the recessed tip configuration is used (Thomas, 1974), the resistance of the electrodes is high and the response time long.

Because of the small dimensions of mammalian neurons and glial cells, the use of liquid-membrane microelectrodes is under *in vitro* conditions mainly limited to extracellular ion measurements in brain tissue preparations. Neurons and glial cells seem to tolerate ion measurements with ISMEs somewhat better under *in vivo* conditions, and brief recordings of intracellular Ca²⁺ and H⁺ concentrations have been performed in rats (Chesler and Kraig, 1987; Chesler and Kraig, 1989; Silver and Erecinska, 1990) and cats (Ballanyi et al., 1994). However, e.g. many invertebrate nerve cells and the giant axon of the squid are so large and easily accessible that ISMEs can be used for long-lasting intracellular ion measurements in them (Voipio, 1998). ISMEs provide a valuable tool for intracellular ion measurements as they are highly sensitive and selective for the permeant ion. The calibration of ISMEs is usually fairly simple and the ion activity is measured strictly from

the cytoplasm with no “contamination” from possible organellar ion compartments. The major limitations of the ISMEs include the possible interference of lipophilic pharmacological substances with the organic liquid-membrane (and hence with the calibration), the susceptibility of the electrodes to noise due to the high electrical resistance of the selectively permeable membrane, and their slow response time. Recently, Fedirko et al., (2006) modified the previously established low impedance coaxial liquid ion-exchanger microelectrodes to prepare concentric Ca^{2+} and H^+ sensitive microelectrodes. These electrodes had significantly shorter time constants than the conventional ISMEs and hence provide a better accuracy for detection of ion transients with rapid kinetics. The ability of the conventional K^+ sensitive, liquid-membrane microelectrodes, used for instance in the Study II in this Thesis, to detect fast K^+ transients has been previously verified. The response time of the K^+ sensitive microelectrodes has been evaluated by Santhakumar et al., (2003). These authors used a fast calibration system and showed that the K^+ sensitive microelectrodes could detect changes in $[\text{K}^+]_o$ lasting less than 500 ms. Kaila et al. (1997) measured glial membrane potential, known to selectively reflect changes in $[\text{K}^+]_o$ (Lothman and Somjen, 1975), to confirm the temporal resolution of ISMEs used in HFS-induced K^+ transient measurements. ISMEs will inevitably cause some damage when placed into the preparation. A dead space surrounding the tip of the electrode will lead to a diffusion delay and false averaging of the signal. Reduction of the tip diameter to, or below, 2-4 μm effectively minimizes the damage and prevents the distortion of rapid K^+ signals (Ransom et al., 1987; K Lamsa, K Kaila and J Voipio, unpublished observations).

2.5.2 Fluorescent indicators

The ability of fluorescent indicators to visualize changes in ion activities is

based on the specific sensitivity of their fluorescence excitation or emission-spectra to a certain ion. Since the development of modern fluorescent indicators in the early 1980s (Rink et al., 1982), the use of ion-sensitive dyes has become a more and more popular method for continuous measurements of ions in small cells and in cell populations. The response time of this recording technique is usually not limited by the properties of the indicators but rather by the properties of the hardware. Thus, fluorescent dyes provide a selective and very fast (down to a millisecond time-scale) method for intracellular ion measurements. Because fluorescent indicators are chemical substances that are introduced into the cell(s), there are several aspects that are essential for a good ion-sensitive fluorescent dye. The properties of the widely used pH indicator BCECF, introduced by Roger Tsien (Rink et al., 1982), will be taken as an example.

2.5.2.1 Sensitivity and ratiometric quantitation of the indicator

Ion-induced spectral shifts are more informative for detection and quantification of ion transients than what are the changes in magnitude of peaks (Yuste et al., 2000). In the case of BCECF fluorescence emission is steeply dependent on pH at wavelengths around 500 nm but is practically insensitive at one wavelength (at 440 nm). The ion-insensitive wavelength is called the isosbestic point. The ratio of these two intensities (I) is sensitive to pH but, because $I_{440\text{ nm}}$ depends only on the concentration of the dye, other parameters e.g. volume changes, leakage or photobleaching of the dye, are excluded from the ratio. In some dyes the ratio can also be taken from two intensities with opposite ion-sensitive responses (e.g. the Ca^{2+} sensitive indicator FURA) as this gives a better signal-to-noise ratio. If an isosbestic point can not be used, pseudoratiometric measurements are sometimes possible. This protocol combines two fluorescent indicators,

one of which is sensitive to the ion and one only to the dye concentration as such.

Here it should be emphasized that there is a clear need for a selective and ratiometric Cl⁻ dye. The problems with the quinoline-based, non-ratiometric Cl⁻ dyes available at the moment are their instability (Lucigenin, MQAE), sensitivity to other anions (SPQ), required processing before non-invasive loading (MEQ), and most of all, the movement of water associated with Cl⁻ fluxes. The movement of water unavoidably results in cellular volume changes that will affect fluorescence signal due to changes in ion concentrations and movement of the cell with respect to the region of interest in the fluorescent measurement (E.Ruusuvuori, K.Kaila, and J.Voipio unpublished results). The recently developed approach using genetically encoded fluorescent proteins sensitive to Cl⁻ have so far not provided the desired resolution to the problem. The yellow fluorescent protein (Wachter and Remington, 1999) and its derivatives (Jayaraman et al., 2000; Galletta et al., 2001) have been used for intraneuronal Cl⁻ monitoring (Slemmer et al., 2004; Kruger et al., 2005) but they are markedly sensitive to other anions and protons, and they are non-ratiometric. The construction of a fusion protein (Clomeleon), the yellow fluorescent protein with the Cl⁻ insensitive cyan fluorescent protein by Kuner and Augustine (2000) has allowed fluorescence-resonance-energy-transfer (FRET)-based ratiometric measurements of [Cl⁻]_i in neurons and retinal cells of the transgenic animals (Kuner and Augustine, 2000; Duebel et al., 2006). Markova et al., 2008) have modified the fusion protein in order to improve the low Cl⁻-sensitivity of the indicator. However, the construct still exhibits a significant pH-sensitivity over a broad range of pH values (see also Kuner and Augustine, 2000). This property should be recognized, especially if the indicator is used in measurements of GABA_A-channel or AE mediated Cl⁻ fluxes, both of which are associated with pH_i changes.

2.5.2.2 Fluorescent indicators within the cell

One of the major advantages of some fluorescent indicators is that they can be loaded into cells *non-invasively*. Converting the indicator into its electrically neutral ester derivative and bathing the cells in this solution enables the compound to cross the cell membrane. Intracellular esterases cleave the ester groups and release the dye (with its charges now exposed) within the cell. In this way it is possible to measure ionic changes not only in one cell but even in a large population of cells. If ester derivatives are not available the dye can be introduced into the cell via a patch-pipette or using a single-cell electroporation technique (Khirug et al., 2008). The retention of the charged form of the indicator within the cell (detected on the basis of fluorescence intensity at the isosbestic wavelength) can be used as an indicator of membrane integrity if the possible leakage and photobleaching are taken into account (Bevensee et al., 1995).

There are some clear weaknesses of fluorescent indicators, in comparison to ISMEs. The cell membrane permeable ester form of the indicator can also move through membranes of cellular organelles and hence, the measured signal will be a combination of the cytoplasmic and organellar ion concentrations. Furthermore, even though the high selectivity of the indicator is its fundamental property, the selective binding of the measured ion to the indicator can result in buffering of the measured ion (Neher, 2000). With ions with low absolute concentrations (like Ca²⁺ and protons) fluorescent indicators can function as mobile buffers and facilitate the diffusion of the measured ion. The calibration of ratiometric fluorescent indicators is not as straightforward as the calibration of ISMEs and with the non-ratiometric indicators a reliable calibration is often impossible to obtain (Thomas et al., 1979; Eisner et al., 1989). Finally, the use of high-intensity excitation light in a live preparation (e.g. in confocal laser

microscopy) can induce photodamage due to phototoxic products, such as the highly reactive singlet oxygen and other free radicals.

3 Aims of the study

The present work aimed in specific:

- 1) To characterize the molecular mechanism that renders GABA_A receptor-mediated responses hyperpolarizing during maturation in rat hippocampal pyramidal neurons. First we studied the developmental expression pattern of the putative Cl⁻ extruder KCC2 on both mRNA and protein level. After this primary finding we aimed to show that the ontogenetic change in E_{GABA-A} from depolarizing to hyperpolarizing has a causal link to the developmental up-regulation of KCC2 (I)
- 2) To identify the cell type expressing the intracellular CA activity that is essential for the HFS-induced, depolarizing GABA_A-receptor mediated responses in mature hippocampus. Thereafter, the aim was to examine the postnatal development of intrapyramidal CA activity, to identify the cytosolic CA isoform(s), and to study its (their) role as key molecule(s) in the generation of the tonic GABAergic excitation and the underlying [K⁺]_o transients that provide the drive for the HFS-induced afterdischarges (II)
- 3) To examine the localization and function of the Na⁺-driven Cl⁻-HCO₃⁻ exchanger NCBE in the mouse CNS. In order to address the role of NCBE in physiological and pathophysiological processes we generated mice with targeted *Slc4a10* gene disruption (III)

4 Experimental procedures

Methods			
Northern blot analysis	I		III
RT-PCR	I		III
(Anti)sense oligodeoxynucleotide-exposed hippocampal slice cultures	I		
Immunoblotting	I		III
<i>In situ</i> hybridization	I	II	III
Intracellular recordings with sharp electrodes	I	II	
Extracellular field potential measurements		II	III
Extracellular K ⁺ measurements with ion-selective microelectrodes		II	
Intracellular ion measurements with fluorescent indicators	I	II	III
Generation of Slc4a10 knockout mice			III
Generation of Slc4a10 antibody			III
Immunohistochemistry			III
Behavioral testing			III
Epileptiform seizure induction			III
MRI analysis			III

Table 4. The methods used in the Studies I-III of this Thesis. The methods are listed here in order of appearance, and described in detail in the original publications. The contribution of the author in the experimental work is described on the page where the original publications are listed.

All experiments were approved by the Ethics Committee for Animal Research at the University of Helsinki. In the three original publications a total number of 356 rats (Studies I and II), 24 guinea pigs (Study I), and 305 mice (Study III) were used. The functioning and development of ion-regulatory proteins of hippocampal CA1 and CA3 pyramidal neurons was the main focus of this Thesis (see Fig 3.).

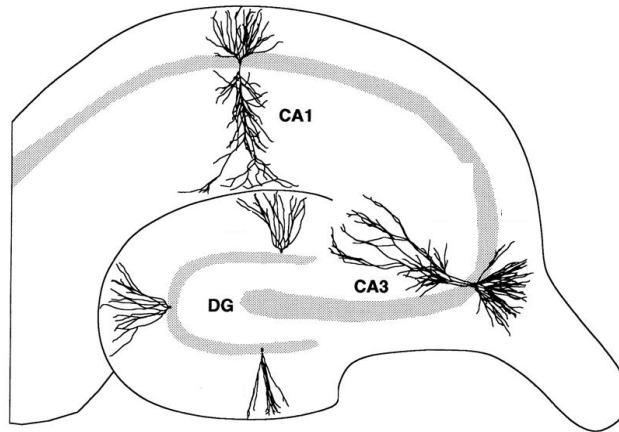


Figure 3. Line drawing of a transverse section of the hippocampus illustrating the orientation of granule cells in the dentate gyrus (DG) and pyramidal cells in the CA3 and CA1 fields of the hippocampus. Modified from Fig. 6 in Witter and Amaral, 2004, courtesy of Elsevier Academic Press, San Diego.

The evaluation of the applicability of fluorescent chloride indicators for the present study

In Study I of this Thesis we aimed to measure the intracellular Cl^- concentration before and after KCC2 down regulation. However, because of the calibration-limitations of the single-wavelength Cl^- dyes such experiments were not possible to perform reliably. Hence, we tried to carry out measurements to demonstrate that the intracellular Cl^- fluxes in pyramidal neurons were affected by the down-regulation of KCC2 in phosphorothionate-protected oligonucleotides A (PODN A) treated hippocampal slices. We took advantage of the fact that KCC2 operates close to its thermodynamic equilibrium; by raising the $[\text{K}^+]_o$ we could reverse the transporter and measure the change in the $[\text{Cl}^-]_i$. For this purpose we loaded the pyramidal cells with the Cl^- sensitive dye MEQ (and in some experiments with MQAE) (see e.g. Galeffi et al., 2004; Chub et al., 2006). The detection of Cl^- in both of these dyes is based collisional quenching and thus, an

increase in $[\text{Cl}^-]_i$ will be seen as a decrease in the fluorescence.

Raising $[\text{K}^+]_o$ from the 3 mM concentration used in the control solution to 9 mM resulted in a decrease in the fluorescence that was larger in the control slices than in the slices treated with PODN A (Figure 4a). These results suggested that an KCC2 mediated increase in $[\text{Cl}^-]_i$ could be evoked by raising extracellular K^+ concentration.

Unfortunately measurements using the pH sensitive dye BCECF showed that this approach caused significant volume changes in pyramidal neurons (Figure 4b). The fluorescence detected at the isosbestic point (I440 nm) decreased markedly upon increase in $[\text{K}^+]_o$. It is likely, that the change in the direction of KCC2 transport results in an influx of Cl^- that is accompanied by an influx of water. Thus, the signal from the Cl^- sensitive indicator is reduced both due to cellular swelling and increased $[\text{Cl}^-]_i$.

As already mentioned, cellular swelling will affect the fluorescence signal due to changes in ion concentrations and because of the movement of the cell with respect to the region of interest used in analysis

of fluorescence intensity in the measurement. Quenching of the quinoline-based Cl^- indicators is not accompanied by a spectral shift, excluding the possibility of direct ratiometric measurements. Pseudoratiometric measurements with an ion/pH insensitive indicator like calcein (see e.g. Yamada et al., 2001) could be an option, but the instability of many Cl^- dyes (Bowers and Verkman, 1991; Chub et al., 2006) complicates reliable estimation on the relative contributions of volume and Cl^- changes to the signal obtained,

especially in long-lasting recordings. Furthermore, because the relative change in the fluorescence intensity was roughly similar in amplitude with Cl^- sensitive dye and BCECF (I440) (Fig. 4), the signal-to-noise ratio in the pseudoratiometric signal was too low for detection of a change in $[\text{Cl}^-]_i$. For these reasons the Cl^- indicators turned out to be unsuitable for the present study.

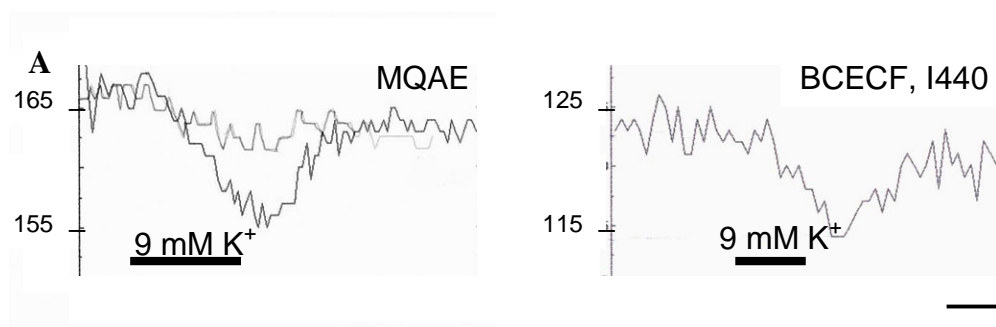


Figure 3. Intracellular fluorescence measurements from hippocampal pyramidal cells with the Cl^- and pH indicators MQAE and BCECF, respectively. **A)** Raising $[\text{K}^+]_o$ to 9 mM induced a more prominent change in MQAE fluorescence (apparent increase in $[\text{Cl}^-]_i$, excitation at 350 nm, emission at 460 nm) in semi-organotypic control slices (dark grey trace) than in slices exposed to PODN A (light grey trace). **B)** A recording from acute hippocampal slice with the isosbestic wavelength of BCECF (excitation at 440 nm, emission at 520 nm) revealed a response upon an increase in $[\text{K}^+]_o$ that was strikingly similar to that shown in (A) and most likely reflects a change in cellular volume (E. Ruusuvuori, unpublished results). The values on the y-axis are the absolute emission intensities. Time calibration 500 s (A) and 250 s (B).

5 Results and Discussion

5.1 The K^+ - Cl^- cotransporter KCC2 renders GABA hyperpolarizing during neuronal maturation (I)

From previous studies it was known that the K^+ - Cl^- cotransporter KCC1 was ubiquitously expressed in different mammalian tissues (Gillen et al., 1996) while KCC2 expression was restricted to brain (Payne et al., 1996). In their article Payne et al. (1996) also analyzed the expression of KCC1 and KCC2 mRNAs in cultured glial cells as well as in tissue samples from whole rat brain. KCC1 was present in pure glial samples whereas co-expression of the two transporters was seen in whole brain samples. This result indicated that KCC2 expression was most likely absent from glia.

The spatiotemporal expression pattern of KCC2 was studied in the postnatal rat brain and the role of this transporter in setting the hyperpolarizing GABA_A-receptor mediated responses in hippocampal pyramidal neurons was assessed. The expression of KCC1 and KCC2 mRNA in postnatal rat hippocampus was followed using Northern blot analysis. There was hardly any KCC2 present in the P0 rat hippocampus but a marked increase in the KCC2 mRNA levels occurred between P0 and P5. The KCC2 expression continued to increase, and by the end of the second postnatal week it had attained a level similar to that seen in the mature animals. In contrast to the steep postnatal upregulation of the KCC2, a considerable KCC1 expression was detected already at birth. There was no marked increase in the brain's KCC1 expression levels during the postnatal development.

Southern blot analysis of single-cell RT-PCR was used to examine the expression of KCC1 and KCC2 mRNA in 16 neurons, identified by their expression of light neurofilament. All

16 neurons were strongly positive for KCC2. Notably, in the two cells a weak KCC1 signal was evident only after increasing the exposure time of the film from 4 hours to 48 hours. Later studies have confirmed the glial expression of KCC1 and in accordance with our results, suggested that there is little, if any, KCC1 in mature neurons (Kanaka et al., 2001; Li et al., 2002). From the two remaining KCC isoforms, KCC3 and KCC4 (Mount et al., 1999), only KCC3 has been shown to be abundantly present in the postnatal rodent CNS (Pearson et al., 2001; Boettger et al., 2003). Studies on KCC3 KO mice have pointed to a role of this KCC isoform in the maintenance of Cl^- homeostasis and cellular volume in some adult neurons (Boettger et al., 2003).

The GABA_A-receptor responses in adult hippocampal pyramidal neurons are predominantly hyperpolarizing (Kaila, 1994; Ben-Ari et al., 1989; Khazipov et al., 2004). The suggested role of a KCC mediated Cl^- extrusion in the maintenance of low $[Cl^-]_i$ in these cells (Misgeld et al., 1986; Thompson et al., 1988a; Thompson et al., 1988b) is in good agreement with our results showing a high expression of KCC2 in adult rat hippocampus. It should be noticed however, that a low intraneuronal Cl^- level is not necessarily associated with hyperpolarizing IPSPs. First, the HCO_3^- permeability of GABA_A-receptors makes E_{GABA-A} less negative than E_{Cl^-} , and second, the polarity of IPSPs is dependent not only on the level of E_{GABA-A} but also on the level of resting V_m (see e.g. Kaila et al., 1993).

The DRG cells are a classical example of PNS neurons that maintain a high $[Cl^-]_i$ concentration and depolarizing GABA responses into adulthood (Deschenes and Feltz, 1976). The Cl^- accumulation in these cells is at least partially mediated by NKCC1 (Plotkin et al., 1997; Price and Trussell, 2006; Rocha-Gonzalez et al., 2008). To see if the KCC2 mediated intracellular Cl^- extrusion is strictly characteristic to adult neurons with hyperpolarizing GABA_A-receptor response we

examined KCC2 expression in DRG. Indeed, the RT-PCR results demonstrated that KCC2 expression was non-existing in DRG but the “house-keeping” isoform KCC1 was strongly expressed in DRG cells. The reversal potential of GABA_A-mediated synaptic responses has later been shown to reflect the presence of functional KCC2 also in the mature CNS. Gulacsi et al. (2003) demonstrated that within the rat substantia nigra, the KCC2 expressing neurons had significantly more hyperpolarizing GABA_A-mediated synaptic responses than the KCC2-negative neurons. In this context it should be noted that the evidence of KCC2 expression does not necessarily imply functional KCl extrusion (Khirug et al., 2005).

From a developmental point of view it was of interest to see if KCC2 expression was a more general indicator of neuronal maturation (see Li et al., 2002). To test this we used guinea pig pups because they are, if compared to rat pups, born at a much more developed stage. The newborn guinea pigs have physical features and behavior resembling those of a roughly two week old rat pups. Interestingly, in Northern blot analysis hippocampal KCC2 mRNA showed high levels already in E42 (full-term gestation day 68) guinea pig hippocampi and the levels did not further increase during the early postnatal life.

To study in more detail the localization of KCC2 mRNA in the developing rat brain we performed *in situ* hybridization with ³⁵S-labelled KCC2 antisense RNA probes in sagittal brain sections from E20 to P16 animals. KCC2 mRNA was absent from E20 rat hippocampus. At P0 there were some individual labeled neurons but at P5 a clear signal was seen in the whole hippocampal pyramidal cell layer. There was still a more gradual increase in the expression during the second postnatal week after which a steady level of expression was attained. KCC2 was expressed in different brain areas, or within a given area, strictly according to the state of maturation, e.g. with KCC2 expression in the brain stem preceding that of cortical

structures and within the dentate gyrus KCC2 expression in the dorsal blade preceding that of the ventral blade. Later, Li et al. (2002) have provided evidence that in prenatal rats and mice the spatiotemporal expression pattern of KCC2 mRNA likewise follows neuronal maturation. During embryogenesis, KCC2 was shown to be undetectable in areas where neurogenesis or neuronal migration took place but co-localized with cells that were positive to a marker of neuronal differentiation.

The results of Study I discussed so far indicated that KCC2 is the main K⁺-Cl⁻ cotransporter in mature neurons. Consequently, KCC2 is likely to mediate the postnatally occurring reduction in neuronal [Cl⁻]_i and hence, to be responsible for fast hyperpolarizing inhibition seen in mature hippocampal neurons. To gain further support to this idea we compared the voltage responses evoked by the GABA_A-receptor agonist muscimol in acute hippocampal slices during development as well as after a specific down-regulation of KCC2 in short-term organotypic slice cultures. Voltage responses were measured with sharp microelectrodes filled with a solution that did not impose a Cl⁻ load on the neurons.

In P12-30 rat pyramidal neurons iontophoretic application of GABA_A-receptor agonist muscimol resulted in a hyperpolarization with a mean driving force (DF_{GABA-A} , defined here as $E_{GABA-A} - V_m$) of -9.6 ± 0.9 mV. In P0-4 rat pyramidal neurons, which according to the results described above do not express KCC2, the muscimol-evoked responses were clearly depolarizing (7.0 ± 3.3 mV). In sharp contrast, pyramidal neurons in P0-4 guinea pig slices had hyperpolarizing responses (-10.6 ± 2.4 mV) similar to those seen in more mature animals (P17-40; -9.0 ± 3.3 mV). While these developmental studies clearly suggested a causal link between the KCC2 mRNA expression and the transition from depolarizing to hyperpolarizing GABA_A-receptor responses, we still aimed at obtaining causal evidence for this by

manipulating the KCC2 mRNA, and thus the protein, levels in juvenile hippocampal slices *in vitro* (P11-13, 2-5 DIV). For this purpose, we generated three distinct antisense oligonucleotides (ODNs A, B, and C) against KCC2 mRNA. Immunoblot analysis of KCC2 levels in the short-term cultured hippocampal slices treated with the phosphorothionate-protected antisenses (PODNs A-C) and the unprotected ODN A (8-15 hours) revealed that treatment with PODN A resulted in the largest down-regulation of KCC2 levels. This down-regulation of KCC2 was accompanied by a shift to only slightly hyperpolarizing DF_{GABA-A} values, confirming the strict dependency of hyperpolarizing postsynaptic inhibition on functional activity of KCC2. In slices exposed to PODN A the mean DF_{GABA-A} was $(-2.8 \pm 0.8 \text{ mV})$ whereas in control slices and in slices treated with sense counterparts of PODN A the mean values were significantly more hyperpolarizing, $-10.9 \pm 0.8 \text{ mV}$ and $-10.3 \pm 1.7 \text{ mV}$, respectively.

Taken together, the results of Study I provided direct evidence for the long-postulated, critical role of K^+-Cl^- cotransporter in intraneuronal Cl^- homeostasis. The increase in KCC2 mRNA in the rat hippocampal pyramidal neurons during the first two postnatal weeks reflects neuronal maturation and is necessary for the fast $GABA_A$ -receptor mediated hyperpolarizing inhibition.

5.2. CA isoform VII acts as a molecular switch in the development of synchronous gamma-frequency firing of hippocampal CA1 pyramidal cells (II)

Our group had previously shown that in mature rat hippocampus the tonic excitation driving HFS induced synchronous firing of CA1 principal cells is caused by a $GABA_A$ ergic, CO_2/HCO_3^- -dependent increase in $[K^+]_o$ that is critically dependent on the functional activity of CA_i (Kaila et al.,

1997; Taira et al., 1997; Smirnov et al., 1999). However, it was not known if the CA_i activity that plays a crucial role here originates from a certain cellular subtype. An intriguing question was whether the expression of brain cytosolic CA isoforms is developmentally regulated; and if so, would this shape the ontogeny of HFS-induced tonic $GABA_A$ ergic excitation and synchronous neuronal firing.

Characterization of intrapyramidal carbonic anhydrase expression and functional activity during postnatal development

Preliminary experiments based on pH_i measurements suggested an upregulation of CA activity in pyramidal neurons at around the end of the second postnatal week (Ruusuvaori et al., 1999). Therefore, the postnatal development of intracellular carbonic anhydrase activity in CA1 and CA3 pyramidal cells was followed. pH_i measurements from hippocampal slices and from acutely isolated rat hippocampal CA1 and CA3 pyramidal neurons were performed with fluorescence imaging using the pH sensitive indicator BCECF. Intracellular alkalization was induced by withdrawal of CO_2/HCO_3^- in the absence and presence of the membrane permeable CA inhibitor ethoxzolamide (EZA; 30-100 μM ; Fig. 3 in the original publication). At P0-8 the initial rate of change in intracellular pH (dpH_i/dt) was always slow and showed no sensitivity to EZA. These results were taken as evidence for the absence of cytosolic CA activity. During the second postnatal week the initial slope of the pH_i change became faster and, in parallel with the faster kinetics of the evoked alkalization, EZA started to cause a decrease in the dpH_i/dt . The appearance of functional CA activity did not change steady-state pH_i values of the pyramidal neurons. However, it is expected that pH_i shifts arising from CO_2/HCO_3^- fluxes, e.g. upon $GABA_A$ receptor activation are more prominent in cells with functional CA activity (Pasternack et al., 1993). The lack of cytosolic CA could indeed explain the

miniscule pH_i changes (0.0016 ± 0.002 pH) evoked in hippocampal neurons by 1 mM GABA in the experiments performed by Kuner and Augustine (2000). These authors used cultured neurons from E16 to P4 rat hippocampi after 7-14 DIV.

Two cytoplasmic, catalytically active CA isoforms had been proposed to localize to the hippocampal pyramidal cell layer; CAII and CAVII (Lakkis et al., 1997; Wang et al., 2002b). Notably, the nucleotide sequence that was used in the design of the probe used by Lakkis et al. (1997) has not been published. These authors also reported CAVII expression in the choroid plexus, a finding that has not been confirmed in latter publications (unpublished data of the authors of the Study II, see also Halmi et al., 2006).

The results from both radioactive and fluorescence *in situ* hybridizations indicated that the appearance of the cytoplasmic CA activity in hippocampal pyramidal neurons, detected with the pH_i measurements, coincided with the postnatal up-regulation of CAVII mRNA, but not with that of CAII. The expression of CAVII mRNA in all brain regions, including the hippocampus, was low at birth and increased significantly during the second postnatal week. In juvenile animals the most prominent CAVII expression was observed, in addition to the neurons of cornu ammonis and dentate gyrus, in cerebellar Purkinje neurons, olfactory bulb, cerebral cortex (with an especially intense labelling in the piriform cortex), and some thalamic nuclei (E.Ruusuvuori, C.Rivera K. Kaila, and J.Voipio, unpublished observations).

In contrast to the CAVII expression, we could not detect CAII mRNA expression in hippocampal principal (or any other) neurons during the first two postnatal weeks. At this developmental stage, data from radioactive *in situ* hybridizations indicate that CAII was strongly expressed only in choroid plexus epithelial cells. In the white matter, e.g. in the corpus callosum and in the white matter of cerebellum, CAII expression started after the second postnatal week. This up-regulation of

CAII coincides with the maturation of oligodendrocytes and myelin formation (Suzuki and Raisman, 1994; Savaskan et al., 1999). In line with the previous reports, there was a diffuse CAII signal in the P15-22 hippocampal pyramidal cell layer (mainly in the CA1; see also Halmi et al., 2006; Wang et al., 2002b) and a more punctuate labelling of the cerebellar Purkinje neurons (see also Nogradi et al., 1997; Wang et al., 2002b). Together the results from fluorescence imaging and *in situ* hybridizations indicate a strong postnatal up-regulation of functionally active CAVII expression in hippocampal pyramids at around P12.

Developmental expression of the HFS-induced GABAergic depolarization and extracellular potassium transient

Previous studies on the GABA_A receptor-mediated tonic excitation had been done on hippocampal slices from adult rats. As our results pointed to a simultaneous up-regulation of intrapyramidal CA expression and functional activity, we next studied the postnatal development of the HFS induced tonic GABAergic excitation and the underlying $[K^+]_o$ transients. In all experiments, HFS evoked an initial hyperpolarization of CA1 pyramidal cells but only after P12 it was followed by a prominent depolarization, often associated with action potential firing. The depolarization and the neuronal firing were markedly suppressed by the membrane permeable CA inhibitor EZA. Taken together, these results suggested that the rapid replenishment of HCO_3^- catalyzed by cytosolic CA activity (Kaila et al., 1990; Pasternack et al., 1993) in pyramidal neurons is involved in the generation of the HFS induced GABAergic depolarization. In full agreement with the idea that an activity-induced increase of $[K^+]_o$ mediates the tonic GABAergic excitation, the HFS-induced K^+ transients developed from small and EZA-insensitive shifts to large, over 7 mM in amplitude, transients which were decreased upon CA_i inhibition. Both the K^+ transients and the associated membrane depolarization of CA1 pyramidal neurons were

unaffected by bath application of the CA_o inhibitor benzolamide (BA). Stimulation-induced changes in the extracellular space, due to cellular swelling, can result in local interstitial ion accumulation (Dietzel et al., 1980). However, simultaneous measurements of the extracellular volume fraction and [K⁺]_o revealed only minimal HFS-induced changes in the volume of the extracellular space in comparison to the evoked [K⁺]_o transient. Therefore, it can be concluded that the activity-dependent increase in [K⁺]_o underlying the tonic excitation results to large extent (~ 93 %) from cellular net release of K⁺.

Remembering that a large intraneuronal Cl⁻ load can be created upon GABA_A receptor activation (see chapter 2.4.1.2), it is interesting to point out that extrusion of Cl⁻ must be coupled to other ion fluxes in order to maintain electroneutrality. After the second postnatal week, hippocampal neurons show a strong expression of KCC2 (Study I of this Thesis) and hence, it is possible that the K⁺ transients accounting for the tonic GABAergic excitation arise, at least to some extent, from a net extrusion of KCl from pyramidal cells via KCC2.

Developmental expression of HFS-induced afterdischarges

While intracellular sharp microelectrode recordings provided information on the developmental profile of HFS-induced tonic GABAergic excitation, and ion sensitive microelectrodes revealed a similar ontogeny for the K⁺_o transients, we used extracellular field recordings to study the synchronization of CA1 pyramidal cell firing. Notably, the HFS paradigm used in this study is similar to what has been classically used in the induction of long-term potentiation in the CA1 area (Bliss and Collingridge, 1993). This provides an obvious link to studies aimed at elucidating mechanisms underlying network activity and synaptic plasticity (Traub et al., 1998; Traub et al., 1999). In the adult rat hippocampal CA1 area, synchronous population oscillations at gamma frequency range can be

measured close (~ 400 μm) to single-site HFS (Colling et al., 1998). With a two-site HFS the firing of principal neurons is synchronized over longer distances and this method can be used to study oscillatory synchronization between different sites (Traub et al., 1996b; Traub et al., 1999). In the present experiments, the single-site HFS-induced afterdischarges in the gamma-frequency range were quantified from the field potential recordings by integrating off-line the apparent power of the population spikes (Figure 1 in the original publication). Experiments were done using both submerged slices and slices at saline-gas interface in order to exclude the possible increase in field effects caused by hypo-osmotic conditions that has been reported to take place in poorly perfused interface chambers (see Whittington et al., 2001).

Field potential recordings from rat hippocampal CA1 stratum pyramidale, both in the presence and absence of ionotropic glutamate receptors, revealed a striking temporal correlation between the expression of pyramidal carbonic anhydrase activity and the appearance of HFS-induced network activity at around P12. The likely causal relation between these two events was further validated in experiments examining the effects of EZA. Once the extracellularly measured afterdischarges could be evoked, their apparent power was always significantly suppressed by the membrane permeable CA inhibitor EZA whereas BA did not decrease the apparent power. The strict dependency of the HFS-induced afterdischarges on the availability of HCO₃⁻ was confirmed in experiments where the standard CO₂/HCO₃⁻-buffered solution was replaced with a nominally CO₂/HCO₃⁻ free, HEPES-buffered solution. Washout of CO₂/HCO₃⁻ rapidly and reversibly abolished the HFS-induced afterdischarges.

A developmental increase in excitability at around P13 has been reported by Kohling et al. (2000) for 0-Mg²⁺ induced gamma oscillations. Our results readily explain their findings and point to a crucial role of

intrapyrimalidal CAVII in the generation of developmental upregulation of network excitability. Even though one should be cautious in comparing data obtained *in vitro* and *in vivo*, the dependency of HFS-induced after discharges (as well as 0-Mg²⁺ induced activity; Kohling et al., 2000) on CA_i activity is intriguing. In humans, CA inhibitors have been used to control epileptic seizures both as monotherapy and in combination with other antiepileptic medication (Wyllie, 1997; Supuran et al., 2003; see also Katayama et al., 2002). Our finding thus places CAVII as a possible novel drug target in the therapy of epilepsy.

To verify that the nominal absence of CO₂/HCO₃⁻ did not result in a general blockage of hippocampal oscillations we studied the effect of CO₂/HCO₃⁻ withdrawal in another model of network activity, the GDPs of the developing hippocampus (also called the spontaneous population oscillations in Study II in this Thesis). While HFS-induced after discharges could not be evoked in the absence CO₂/HCO₃⁻, GDPs readily occurred after withdrawal of CO₂/HCO₃⁻. In fact, the amplitude of GDPs increased significantly in the HEPES-buffered solution. These results, together with the early onset of GDPs (Ben-Ari et al., 1989; Khazipov et al., 2004) which precedes CAVII expression, showed that there is no universal mechanism of hippocampal neuronal network activity that would depend on the presence of CO₂/HCO₃⁻ or on fast functional intrapyrimalidal CA activity. This conclusion is further supported by the observation that hippocampal network activity induced by cholinergic agonists (Fisahn et al., 1998) starts at around P7 (A. Fisahn personal communication; E. Ruusuvuori, K. Kaila, and J. Voipio, results based on 41 slices from eleven P6-21 rats, unpublished observations).

On the basis of results of Study II it can be concluded that intrapyrimalidal CA activity is a key factor in the ontogeny of tonic GABAergic excitation that drives HFS-induced afterdischarges. It is only after the expression of the intrapyrimalidal CA that a pronounced, HFS-induced

GABAergic excitation becomes functional. Moreover, Study II shows that the up-regulation of the cytoplasmic CA isoform CAVII accounts for the developmental increase in the intracellular CA activity in hippocampal pyramidal neurons. This puts CAVII in a key position in governing the electrophysiological behavior of CA1 pyramidal neurons, the major output pathway of the hippocampus, in situations where large-scale GABA_A receptor activation takes place.

5.3. Mice with targeted *Slc4a10* gene disruption have small brain ventricles and show reduced neuronal excitability (III)

The Na⁺-driven HCO₃⁻ transporter NCBE (encoded by *Slc4a10* gene) belongs to the SLC4 gene family of HCO₃⁻ transporters which have been shown to be closely involved in solute transport and pH regulation. To study the role of NCBE in physiological processes mice with targeted *Slc4a10* gene disruption was generated by deleting exon 12 of the gene.

Two independent correctly targeted embryonic stem cell clones were injected into blastocysts and gave heterozygous offspring that had no obvious phenotype. From heterozygous mating, homozygous KO mice were born in the expected Mendelian ratio. However, in these litters NCBE KO pups had a higher mortality rate than the heterozygous and wild-type (WT) pups. After the second postnatal week NCBE KO mice did not gain weight at the same rate as their littermates and many of them died around weaning. Only if provided moistened food at the age of P14-30, NCBE KO mice grew normally and the increased mortality around weaning was avoided. In litters from KO mating pairs the pups gained weight and developed normally. These results most likely exclude the possibility that NCBE deletion per se would have an effect on the viability and development of the animals. Rather, it seems that juvenile NCBE

KO mice are unable to compete for maternal care with their heterozygous and WT littermates and therefore have a higher mortality. Adult NCBE KO mice were indistinguishable from their heterozygous and WT littermates, they were fertile even as KO mating pairs, and had no major behavioural abnormalities, as tested for circadian rhythm, overall locomotor activity, motor coordination and spatial learning. The slightly delayed habituation to novel surroundings and objects could be taken as an indication of a type of hippocampal-related memory impairment (Malleret et al., 1999).

Multiple tissue Northern blots revealed a prominent expression of NCBE in the mouse brain, retina and spinal cord. In line with the normal phenotype of the NCBE KO animals, the gross morphology of the brain tissue of adult KO mice revealed no major changes except for a dramatic decrease in the total volume of brain ventricles (see below). With quantitative real-time PCR it was further verified that the loss of Slc4a10 was not compensated by an up-regulation of other solute carriers from the BTs (Slc4), the CCCs (Slc12), the NHEs (Slc9), or from the gene family of multifunctional anion exchangers (Slc26).

To detect NCBE at the protein level, a novel polyclonal antibody against an N-terminal epitope of murine NCBE was generated. This antibody should detect both of the NCBE isoforms with distinct carboxyterminal inserts (see Giffard et al., 2003). The specificity of the antibody, both in immunoblots and in immunohistochemistry, was confirmed by using brain tissue from KO animals. In immunoblots the antibody detected an approximately 180 kDa band in protein lysates from whole WT brain. To examine the expression of NCBE in neurons and glia, protein lysates were made from mixed neuronal/glial and from pure glial cell cultures. Transcripts of a NCBE splice variant with a terminal PDZ motif had been detected in cultured rat astrocytes (Giffard et al., 2003). We detected NCBE in protein lysates from co-cultured mouse

neurons and glia but not in lysates from pure glial cultures.

Localization and function of NCBE in choroid plexus

Double staining immunofluorescence confirmed the basolateral localization of NCBE in choroid plexus epithelial cells, previously described by Praetorius et al., (2004) and Bouzinova et al., (2005). The high expression of NCBE in the choroid plexus together with the obvious change in the ventricular volume seen in the histological sections, pointed to an important role of NCBE mediated Na^+ -uptake in CSF production. The reduction in the ventricular volume was analyzed in more detail by *in vivo* MRI scans. These measurements showed that the total ventricular volume was diminished from $10.49 \pm 1.8 \text{ mm}^3$ (mean \pm SEM) in WT mice to $2.31 \pm 0.39 \text{ mm}^3$ in KO mice.

An elevated intracranial pressure due to brain edema could perhaps also result in collapsing of ventricles. However, this would be accompanied with cellular edema or increased water content of the brain, and result in other anatomical changes such as in protrusion of the cerebellum towards the *foramen magnum*. No anatomical changes were observed and the possibility of edema was excluded by further analysis of T_2 relaxation times and apparent diffusion coefficient maps calculated from the MRI scans. The results thus strongly support the conclusion that the collapsing of ventricles in NCBE KO mice results from abnormally low CSF production. Closer examination of the NCBE KO choroid plexus epithelia with electronmicroscopy revealed an intact epithelial lining but with strongly reduced apical microvilli and increased lateral intercellular spaces.

The role of NCBE in Na^+ -dependent pH_i regulation in choroid plexus epithelial cells was studied in clusters of isolated epithelial cells (see Fig. 2 in the original publication). pH_i was measured with the pH sensitive fluorescent indicator BCECF and the recovery from an exogenous acid load was studied using the ammonium pre-

pulse technique (Boron and De Weer, 1976). pH_i recovery was fully dependent on extracellular Na^+ and it was significantly slower in epithelial cells from KO than from the WT mice. This shows that NCBE markedly contributes to the basolateral Na^+ -dependent pH_i regulation in choroid plexus epithelial cells. While the critical roles of $Na^+-K^+-ATPase$ and AQP1 water channels for CSF production have been recognized and their importance in apical Na^+ secretion and osmotic water flow emphasized, the basolateral Na^+ uptake mechanism has so far remained unidentified (for a recent review see Praetorius, 2007). Combining the results from pH_i measurements with those from the enhanced MRI scans, it can be speculated that NCBE is a likely candidate to mediate the basolateral uptake of Na^+ , and thereby NCBE would make a significant contribution to the production of CSF.

Localization of NCBE in the hippocampus

The regional distribution of NCBE in the brain parenchyma was studied in diaminobenzidine stained, free-floating brain sections. The protein was broadly expressed in various brain regions but was absent from fiber tracts such as the corpus callosum or the white matter of the spinal cord. In the hippocampus there was a striking regional difference in NCBE expression. The barely detectable labeling of CA1 pyramids and the dentate granule cells was in sharp contrast to the strong signal obtained from CA3 *stratum oriens*, *stratum pyramidale*, and *stratum radiatum*.

To examine the cell-type specific expression of NCBE in more detail, double-staining with glial cell, interneuronal, axonal, and dendritic markers were performed. Immunohistochemical double-staining revealed no overlap of NCBE with markers of oligodendrocytes (CNPase) or astrocytes (glial fibrillary acidic protein, GFAP). Interneurons, identified by their expression of glutamic acid decarboxylase, were strongly immunopositive for NCBE in

all regions of the *cornu ammonis*. On the subcellular level NCBE was abundantly detected in somata and proximal and apical dendrites of CA3 pyramidal cells. With the results obtained from immunoblots, our data point to a neuronal expression of NCBE.

Electron micrographs suggested that in the CA3 area NCBE localized to the somatic region and to dendritic spines within the *stratum oriens* and the *stratum radiatum*. As most somatic synapses are inhibitory, whereas dendritic spines receive excitatory input, NCBE appears to be expressed in the postsynaptic membranes of both inhibitory and excitatory synapses. These findings on ultrastructural NCBE localization wait to be verified in studies using immunogold labeling.

NCBE-mediated pH_i regulation controls neuronal network excitability in CA3

The high expression of NCBE in CA3 pyramidal neurons led us to address the role of NCBE in neuronal pH regulation and in the control of network excitability in the CA3 area.

The contribution of NCBE in intrapyramidal pH regulation was studied in adult hippocampal slices. The fluorescence pH imaging experiments showed that the steady-state pH of the CA3 pyramidal neurons was similar in slices from NCBE KO and WT mice. However, the recovery from an acid load, induced by 20 mM propionate, was significantly slower in NCBE KO slices than in WT controls. Consequently, reflecting the faster pH_i recovery, the alkaline over-shoot upon propionate washout was more prominent in the WT slices. The impaired ability to recover from an imposed acid load could modify the excitability of CA3 neurons. This aspect was examined in experiments where intense, periodic bursting of CA3 neurons was induced by bath application of the non-selective K^+ channel blocker 4-aminopyridine (4-AP). The activity of the CA3 network was measured with extracellular field potential microelectrodes.

There was no difference in the baseline frequency of the 4-AP induced interictal-like activity in WT and KO slices, and intracellular acidification with propionate initially suppressed the frequency of the bursts in a similar manner in both genotypes. In WT the activity recovered close to the baseline value, or slightly above, within 15 minutes in the continuous presence of propionate. In contrast, the frequency of neuronal bursts in KO slices showed only little recovery during the propionate application. The longer-lasting suppression of neuronal activity in KO slices reflects the inability of the NCBE-deficient neurons to maintain pH homeostasis when faced with an exogenous acid load. If the effect of the endogenous acid-load generated by neuronal activity is taken into account, the time scale of the propionate-induced suppression of network activity is strikingly similar with the pH_i responses described earlier. Together, these results provide further evidence for the pH_i -dependent modulation of neuronal excitability and suggest a significant role for NCBE in mediating neuronal recovery from acid load.

The *in vitro* experiments provided the background for studying the role of NCBE-mediated pH_i regulation on neuronal excitability *in vivo*. Also, studies from another KO strain with impaired pH_i -regulation, the AE3 KO, have shown that epileptic-like seizures are sensitive indicators of imbalances in cellular pH (Hentschke et al., 2006). To test the susceptibility of the NCBE KO mice to seizures we used proconvulsant substances (pentylenetetrazole and pilocarpine) as well as a non-pharmacological approach to provoke epileptic activity, the recently improved rat pup model of febrile seizures (Schuchmann et al., 2006). In this model ictal activity is caused by hyperthermia-induced hyperventilation with a concomitant rise of cortical pH.

After an intraperitoneal injection of pentylenetetrazole (40 mg/kg) three successive seizure phases could be distinguished in WT mice: myoclonic jerks which could progress to clonic seizures and then to generalized

tonic/clonic seizures. The latency to the onset of myoclonic jerks was almost twice as long in NCBE KO compared to WT mice, and the epileptic activity never progressed to generalized seizures in the KO animals. There was a clear influence of the *Slc4a10* disruption on the mortality of the mice. NCBE KOs were protected from lethal seizures both after a higher dose of pentylenetetrazole (60 mg/kg) or after administration of pilocarpine (350 mg/kg). Also the results from the hyperthermia model suggested that disruption of the *Slc4a10* gene suppressed seizure susceptibility.

Thus, both *in vitro* and *in vivo* findings point to the conclusion that NCBE has a crucial role in modulation of network excitability. Neuronal activity can induce an intracellular acid load, which, in turn, suppresses seizures. In light of the results presented, it is plausible that the effects of NCBE on modulation of network excitability and seizure susceptibility are directly attributable to its role in the regulation of intraneuronal pH.

There are several pathways by which the impaired HCO_3^- uptake may affect network excitability. The excitatory effects of GABA in mature neurons and neuronal networks (Voipio and Kaila, 2000) are dependent on intraneuronal HCO_3^- and, consequently, on mechanisms that regulate neuronal pH_i (Bevensee and Boron, 1998): a rise in pH_i enhances GABA_A-receptor mediated excitation by increasing the depolarizing HCO_3^- current component while an acid shift has the opposite effect (Kaila et al., 1993). Also, upon intense GABA_A receptor activation the depolarizing HCO_3^- -efflux drives the fast anionic redistribution (Kaila, 1994; Voipio and Kaila, 2000), and the rapid replenishment of HCO_3^- has been shown to be essential for the prolonged GABAergic depolarization mediated by increased $[K^+]_o$ (Study II of this Thesis). Finally, in addition to the GABAergic transmission, the glutamatergic synaptic responses are modified by intracellular pH. Lee et al. (1996) have shown that monosynaptic

AMPA receptor-mediated transmission is suppressed by intracellular acidification. On the network level, acidification is expected to reduce gap-junctional coupling (Spray et al., 1981). Non-selective pharmacological blockage of gap junctions or genetic deletion of a single, interneuronal gap-junction connexin (connexin 36) has been shown to affect neuronal population oscillations in the gamma frequency range (Hormuzdi et al., 2001; Traub et al., 2003).

As a whole, mice with targeted Slc4a10 disruption have so far revealed two physiologically important mechanisms of function for the transporter. The impaired HCO_3^- uptake plays a key role in pH-dependent modulation of neuronal network excitability. In choroid plexus epithelial cells the NCBE mediated basolateral Na^+ and HCO_3^- uptake is likely to make a significant contribution to the transepithelial solute transport, and hence, to the secretion of CSF.

6 Conclusions

This Thesis has assessed the physiological function and development of ion regulatory proteins that make a major contribution to the maintenance of neuronal Cl^- and pH homeostasis.

Study I of this Thesis was the first report to identify the molecular mechanism that renders GABA_A -receptor mediated transmission from depolarizing to hyperpolarizing during the development of central neurons. In the rat, the steep upregulation of KCC2 in hippocampal pyramidal neurons ends the neonatal period when depolarizing GABA_A ergic transmission contributes in different aspects of neuronal development. Although the KCC2 mediated Cl^- extrusion renders GABA_A -receptor mediated hyperpolarizing, the strictly inhibitory action of GABA is not its only mode of action. Depolarizing and excitatory actions mediated by GABA_A -receptors are now well-recognized in adult neurons. In mature neurons strong GABA_A -receptor activation can result in transient ionic redistribution, and under these conditions the depolarizing phase of GABA depends on the availability of HCO_3^- (Voipio and Kaila, 2000). The extent of the activity-dependent shift in $E_{\text{GABA-A}}$ to more depolarizing values and hence, the power of the 'ionic modulation' on GABA_A ergic transmission may differ between neuronal subpopulations, probably arising from differences in Cl^- - HCO_3^- homeostasis in distinct cell types (Marty and Llano, 2005; Blaesse et al., 2008).

In hippocampal pyramidal neurons the appearance of intrapyramidal CA activity parallels the developmental onset of HFS-induced tonic GABA_A ergic depolarization and the underlying $[\text{K}^+]_o$ transients that show a strict dependency on the fast replenishment of HCO_3^- . The results of Study II of this Thesis provide a mechanistic explanation for the depolarizing GABA_A ergic transmission in hippocampal neuronal networks and urge future studies to assess the role of intrapyramidal CA activity under conditions where its contribution to physiological processes could be further evaluated. Interestingly, recent findings have revealed that depolarizing GABA_A ergic responses in mature neocortical neurons are generated not only as a consequence of activity induced ionic shifts or due to the HCO_3^- permeability of GABA_A receptor channels. The existence of subcellular Cl^- pools within a neuron, created by spatially compartmentalized Cl^- transporters, can also generate local, cell region-specific differences in $E_{\text{GABA-A}}$ with depolarizing IPSPs in the AIS. Therefore, both the temporal patterning of activity in inhibitory interneurons and the subcellular localization of their synapses on target neurons are essential determinants that control and modulate GABA_A ergic responses. Evidently, the traditional division of neurotransmitter responses to excitatory and inhibitory overlooks many aspects of GABA_A ergic neurotransmission.

The physiological significance of the Na^+ -driven HCO_3^- transporter NCBE (Slc4a10) was the focus of the Study III. This work combined several methods at different organizational levels in order to elucidate the (sub)cellular localization of the molecule and to study the importance of NCBE in pH-regulation and in epithelial solute transport. Combined together, the basic *in vitro* findings and the *in vivo* studies suggest that NCBE has a crucial role in the pH_i -dependent modulation of neuronal excitability. They also point to a NCBE-mediated epithelial Na^+ and HCO_3^- uptake in choroid plexus that is likely to make a significant contribution to CSF production.

All of the Studies in this Thesis emphasize the importance of gaining mechanistic explanations from *in vitro* experiments that can further be exploited to understand the role of a single molecule in a larger context, and finally to assess its function in physiological and/or pathophysiological functions *in vivo*.

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Eva

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