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Functional expression and subcellular localization of the Cl^- cotransporters KCC2 and NKCC1 in rodent hippocampal and neocortical neurons

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ACADEMIC DISSERTATION

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Abbreviations

AAC	axo-axonic cell
AE3	chloride bicarbonate exchanger
BDNF	brain-derived neurotrophic factor
CA	*Cornu Ammonis* area of hippocampus
CCC	cation chloride cotransporter
CGP	a potent antagonist of $GABA_B$
CNS	central nervous system
ΔE_{GABA}	somatodendritic E_{GABA} gradient
DGC	dentate gyrus cell
DIC	days in culture
DIP	dynamin inhibitory peptide
E_{Cl}	reversal potential of Cl^-
E_{GABA}	$GABA_AR$- mediated currents
Egr4	early growth response 4
GABA	γ-aminobutyric acid
GFP	green fluorescent protein
KA	kainic acid
KCC	potassium chloride cotransporter
LSO	lateral superior olive
Na-K ATPase	sodium-potassium adenosine triphosphatase
NCC	sodium chloride cotransporter
NKCC	sodium potassium chloride cotransporter
NMDA	N-methyl-D-aspartic acid
P	postnatal day
PKC	protein kinase C
RMP	resting membrane potential
SPAK	Ste20-related proline-alanine-rich-kinase
SPQ	synthetic fluorescent indicator
T4	monoclonal NKCC1 antibody
Thy1	murine thymus cell antigen 1
Trk	tropomyosin receptor kinase
TTX	tetrodotoxin
Vm	membrane potential
WT	wild type

List of original publications

This thesis is based on the following publications herein referred to by their Roman numerals (I-IV)

I. **Khirug S**, Huttu K, Ludwig A, Smirnov S, Voipio J, Rivera C, Kaila K, Khiroug L. Distinct properties of functional KCC2 expression in immature mouse hippocampal neurons in culture and in acute slices. Eur J Neurosci. 2005 Feb;21(4):899-904.

II. Li H, **Khirug S**, Cai C, Ludwig A, Blaesse P, Kolikova J, Afzalov R, Coleman SK, Lauri S, Airaksinen MS, Keinänen K, Khiroug L, Saarma M, Kaila K, Rivera C. KCC2 interacts with the dendritic cytoskeleton to promote spine development. Neuron. 2007 Dec 20;56(6):1019-33.

III. **Khirug S**, Yamada J, Afzalov R, Voipio J, Khiroug L, Kaila K. GABAergic depolarization of the axon initial segment in cortical principal neurons is caused by the Na-K-2Cl cotransporter NKCC1. J Neurosci. 2008 Apr 30;28 (18):4635-9

IV. **Khirug S**, Ahmad F, Puskarjov M, Afzalov R, Kaila K, Blaesse P. A single seizure episode leads to rapid functional activation of KCC2 in the neonatal rat hippocampus. J Neurosci. 2010 Sep 8;30 (36): 12028-35

The doctoral candidate's contribution:

In Study I the candidate participated in the design of the electrophysiological experimental work, performed electrophysiological experiments and participated in analysis of results and manuscript writing.

In Study II the candidate performed electrophysiological experiments and participated in results analysis.

In Study III the candidate had an independent and responsible role in the most difficult experimental electrophysiological work on which the publication is largely based. The doctoral candidate has gathered extensive electrophysiological data using a number of methods, analysed the results, and participated in writing of the manuscript.

In Study IV the candidate had an independent and responsible role in the most difficult experimental electrophysiological work. The doctoral candidate has gathered extensive electrophysiological data using a number of methods, analysed the results, and actively participated in writing the manuscript together with the supervisor.

Publications that were used in other dissertations:

Study II has been used in the thesis of Hong Li "Structural and Functional Roles of KCC2 in the Developing Cortex." 2008 (Faculty of Biosciences, University of Helsinki).

Abstract

The work presented here has focused on the role of cation-chloride cotransporters (CCCs) in (1) the regulation of intracellular chloride concentration within postsynaptic neurons and (2) on the consequent effects on the actions of the neurotransmitter gamma-aminobutyric acid (GABA) mediated by $GABA_A$ receptors ($GABA_ARs$) during development and in pathophysiological conditions such as epilepsy. In addition, (3) we found that a member of the CCC family, the K-Cl cotransporter isoform 2 (KCC2), has a structural role in the development of dendritic spines during the differentiation of pyramidal neurons.

Despite the large number of publications dedicated to regulation of intracellular Cl^-, our understanding of the underlying mechanisms is not complete. Experiments on GABA actions under ”resting” steady-state have shown that the effect of GABA “shifts” from depolarizing to hyperpolarizing during maturation of cortical neurons. However, it remains unclear, whether conclusions from these steady-state measurements can be extrapolated to the highly dynamic situation within an intact and active neuronal network. Indeed, GABAergic signaling in active neuronal networks results in a continuous Cl^- load, which must be constantly removed by efficient Cl^- extrusion mechanisms. Therefore, it seems plausible to suggest that key parameters are the efficacy and subcellular distribution of Cl^- transporters rather than the polarity of steady-state GABA actions. A further related question is: what are the mechanisms of Cl^- regulation and homeostasis during pathophysiological conditions such as epilepsy in adults and neonates?

Here I present results that were obtained by means of a newly developed method of measurements of the efficacy of a K-Cl cotransport. In

Study I, the developmental profile of KCC2 functionality during development was analyzed both in dissociated neuronal cultures and in acute hippocampal slices. A novel method of photolysis of caged GABA in combination with Cl^- loading to the somata was used in this study to assess the extrusion efficacy of KCC2. We demonstrated that these two preparations exhibit a different temporal profile of functional KCC2 upregulation.

In Study II, we reported an observation of highly distorted dendritic spines in neurons cultured from $KCC2^{-/-}$ embryos. During their development in the culture dish, KCC2-lacking neurons failed to develop mature, mushroom-shaped dendritic spines but instead maintained an immature phenotype of long, branching and extremely motile protrusions. It was shown that the role of KCC2 in spine maturation is not based on its transport activity, but is mediated by interactions with cytoskeletal proteins.

Another important player in Cl^- regulation, NKCC1 and its role in the induction and maintenance of native Cl^- gradients between the axon initial segment (AIS) and soma was the subject of Study III. There we demonstrated that this transporter mediates accumulation of Cl^- in the axon initial segment of neocortical and hippocampal principal neurons. The results suggest that the reversal potential of the $GABA_A$ response triggered by distinct populations of interneurons show large subcellular variations.

Finally, a novel mechanism of fast post-translational upregulation of the membrane-inserted, functionally active KCC2 pool during *in-vivo* neonatal seizures and epileptiform-like activity *in vitro* was identified and characterized in Study IV. The seizure-induced KCC2 upregulation may act as an intrinsic antiepileptogenic mechanism.

1. Review of the literature

1.1. Studying the functional activity of ion transporters

The last decade has witnessed a continuous growth of attention on the role that ion transporters play in numerous mechanisms responsible for information processing in the brain. The reason for this is quite obvious: ion transporters seem to be involved not only in static maintenance of transmembrane gradients, but also in network plasticity, neuronal proliferation, differentiation, trauma, disease and recovery (Ben-Ari et al., 2007; Ben-Ari 2008; Blaesse et al., 2009). For a long time, almost all of these phenomena were studied exclusively in the context of membrane receptors and channels. While transporters and channels counteract in terms of their effects on transmembrane ion gradients, they actually cooperate to make possible all electrophysiological phenomena such as the action potential, resting membrane potential (RMP), synaptic transmission and neuronal signaling in general.

1.1.1. Comparison of ion channels and ion transporters

In order for a battery-driven flash-lamp to produce light, the battery must be charged. Similarly, for an ion current to flow through open ion channels, the transmembrane gradient for this particular ion must be generated in the first place. The functional role of transporters is to create the transmembrane ion gradients, while the function of channels is to dissipate them at an appropriate time and in the right place. These two kinds of ion fluxes (gradient generation and dissipation) can be associated with “uphill” and

“downhill” ion movements.

Two distinct types of transporters are known: i) primary active transporters like Na-K ATPase and Ca^{2+} pump , which use directly the energy from ATP hydrolysis and ii) secondary active transporters that exploit gradients already created by primarily active transporters, to co- or counter- transport another ion (the latter transporter types are also called symporters and antiporters, respectively). An example of symporter class of secondary active transporters is the CCC (cation-chloride co-transporter) family (Fig. 1). CCCs are expressed in all organ systems where their ion-transport activity is obviously of key importance in e.g. renal functions, production of endolymph in the inner ear and production of cerebrospinal fluid. The CCCs are also involved in cellular volume regulation in neurons and non-neuronal cells (Payne et al., 2003; Pedersen et al., 2006; Russell, 2000; Flatman, 2008).

1.1.2. Electroneutrality of cation-chloride co-transporters and its impact on their functional analysis

Ion transporters can be classified not only based on their mode of energy consumption (primary *vs.* secondary) but also on whether or not they transfer charge movements across the membrane (electrogenic *vs.* electroneutral). Both primary and secondary active transporters can be either electroneutral or electrogenic. Thus, electroneutral transporters operate with a stoichiometry that results in zero net transfer of charge across the membrane. For example, Cl^- transport mediated by CCCs does not lead to any net charge movement across the membrane, in other words they are electrically neutral (Mercado et al., 2004). All CCCs are electroneutral, which makes electrophysiological studies in this field somewhat challenging. When studying CCCs, researchers rely on the

recording of the reversal potential of GABAergic or glycinergic currents under steady state conditions. However, for accurate estimation of transport efficacy, the system should be exposed to a continuous Cl^- load (Study I, Blaesse et al., 2009).

In contrast, the activity of electrogenic transporters generates a non-zero charge transfer across the membrane, due to which they have a direct effect on the membrane potential (Vm). The Na-K ATPase is the best studied example of this group of transporters. Three α isoforms have been identified: α1, α2 and α3 (Blanco, 2006; Zhang et al., 2009). The Na-K ATPase plays a crucial role in the maintenance of transmembrane electrochemical gradients and thus provides a major source of driving force for a variety of ion currents, at least in certain neuronal types (Ikeda et al., 2004; Hilgenberg et al., 2006). Maintenance of the Na^+ and K^+ transmembrane gradients consumes tremendous amounts of energy in animal cells – up to 70% of total metabolic energy in neural tissue (Siesjö, 1978; Mellergård and Siesjö, 1998; Attwell and Laughlin, 2001, Raichle and Mintun, 2006).

1.1.3. Classical voltage (hyperpolarizing) inhibition

Distribution of chloride ions across the membrane plays a crucial role in a number of physiological processes that are fundamental in GABAergic neurotransmission (Misgeld et al., 1986; Ben-Ari et al., 2007; Farrant et al., 2007). Both inhibitory neurotransmitters GABA and glycine can hyperpolarize the membrane by opening Cl^- permeable channels and allowing Cl^- influx from the extracellular space. This is the basis for hyperpolarizing inhibition, a phenomenon which reduces the likelihood of action potential generation. Along

with ionotropic $GABA_A$ receptor channels ($GABA_AR$), the key players that contribute to the GABAergic hyperpolarizing inhibition are plasmalemmal transporters that extrude Cl^- from the mature neurons. Their activity results in the reversal potential of Cl^- (E_{Cl}) becoming more negative than the resting membrane potential (Vm) (Deisz and Lux, 1982). This difference creates a driving force that gives rise to Cl^- influx, which corresponds to the outward (hyperpolarizing) current.

In addition to ionotropic $GABA_A$ receptors, there are also metabotropic $GABA_B$ receptors. Although $GABA_B$ receptors are also inhibitory, they differ from $GABA_A$ receptors both in structure and in the mechanism of action. The inhibitory mode of action of $GABA_B$ receptors is realized through G-protein mediated activation of K^+ channels in the target cell and inactivation of Ca^{2+} channels at the presynaptic terminal (Misgeld et al., 1995; Uusisaari et al., 2002; Ben-Ari et al., 2007).

There is a common misunderstanding in the assumption that the reversal potential of $GABA_AR$- mediated currents (E_{GABA}) is the same as (E_{Cl}). This would be true if $GABA_A$ R channels were permeable only to Cl^- which is clearly not the case because they are also permeable for HCO_3^- (Kaila 1994). Importantly, the lower the intracellular Cl^- concentration gets, the greater becomes the influence of HCO_3^- on the GABA reversal potential. The permeability ratio between HCO_3^- and Cl^- (HCO_3^- : Cl^-) for $GABA_AR$ channels is in the range of 0.2-0.4 (Kaila and Voipio, 1987; Bormann et al., 1987; Kaila et al., 1993; Farrant and Kaila., 2007). Under some circumstances, HCO_3^- current keeps E_{GABA} more positive than E_{Cl} and may even be able to cause net depolarization of the membrane (Kaila et al., 1989b; Kaila et al., 1993; Gulledge and Stuart, 2003).

Because of the $GABA_A$ permeability to Cl^-, the type of action

(hyperpolarizing or depolarizing) will depend mostly on the transmembrane gradient of Cl^- concentrations. Moreover, shifting between these two modes may occur quite rapidly, on a scale of tens of seconds to minutes (Kaila et al., 1997; Gaiarsa et al., 2002; Woodin et al., 2003; Fiumelli et al., 2005; Dan and Poo, 2006; Study IV).

1.1.4. Shunting inhibition

In addition to the "classical" hyperpolarizing inhibition, GABA also mediates inhibition via the so called "shunting" mechanism. During a rapid increase of the post-synaptic $GABA_A$Rs membrane conductance, the probability of post-synaptic action potential firing may decrease regardless of the direction of membrane potential shift. Indeed, a depolarizing action of GABA may still cause inhibition rather than excitation, because GABA opens a large conductance which makes the membrane leaky, thus creating a "shunt" that renders glutamatergic inputs incapable of driving membrane potential to supra-threshold levels required to generate action potentials. Shunting inhibition lasts as long as the ion channels remain open, in other words its duration is in the range of several milliseconds up to tens of milliseconds. In the case of voltage inhibition the time constant of the membrane sets the duration of the inhibitory voltage effect which therefore is typically longer than shunting inhibition. (Bartos et al., 2001)

1.1.5. Basic properties of chloride transport

The pioneer studies that characterized CCCs were performed on vertebrate red blood cells. Starting in 1960s, those studies described movements

of K^+ and Cl^- across the membrane, mediated by transporter molecules identified later as NKCC and KCC (Hoffman and Kregenow, 1966; Funder and Wieth, 1967). At the time, the idea of passive distribution of Cl^- was a widely accepted part of the “cationocentric point of view” (Russell, 2000). It was an ironic coincidence that CCC mechanisms were studied on models such as blood cells and skeletal muscle cells. In fact, one peculiarity of these cells is their extremely high passive Cl^- permeability, which leads to an equilibrium distribution of Cl^- across their membranes. In contrast to these early studies, subsequent research performed on several different tissues including neurons revealed that the majority of cell types does not have high Cl^- permeability, and they show very active regulation of intracellular Cl^- to keep its concentration far from the electrochemical equilibrium potential. (Ben-Ari, 2002; De Koninck, 2007; Blaesse et al., 2009)

There are three groups in the CCC gene family. First, NCCs: Na-Cl cotransporters, expression of which has only been reported outside the CNS. Second, NKCCs: Na-K-2Cl co-transporters responsible for the active Cl^- accumulation. The third group, KCCs: K-Cl co-transporters - reduces intracellular Cl^- concentration. It is worth mentioning that all CCCs are bi-directional electroneutral carriers, which means that, depending on the transmembrane distribution of transported ions, they can act either as inward or as outward transporters of Cl^-. Indeed, K-Cl co-transport is readily reversed by an increase in extracellular K^+. The predicted secondary structure of CCCs, confirmed only for NKCC1 so far (Gerelsaikhan and Turner, 2000), consists of 12 membrane spanning segments that are flanked by intracellular termini that constitute about half of the protein (Fig. 1). However, NKCCs are far from equilibrium and thus do not reverse their transport direction under physiological or even pathophysiological conditions.

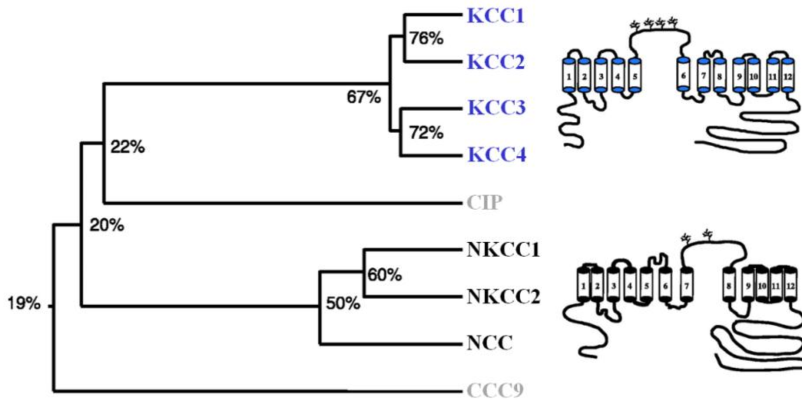

Figure 1. Phylogenic tree of CCC family with indicated degree of identity and proposed topologies for KCC's and NKCC's. They are large integral membrane proteins, possessing 12 transmembrane domains flanked by hydrophilic amino and carboxy terminal domains. Modified from Gamba (2005)

The CCC family in the central nervous system is represented by four members of potassium-chloride cotransporters, namely KCC1, KCC2, KCC3 and KCC4. In addition to them, one sodium-chloride cotransporter, NKCC1, is widely expressed in the CNS. Out of this group of cotransporters KCC2 shows an exclusively CNS specific neuronal expression pattern (Payne et al., 1996; Rivera et al., 1999; Williams et al., 1999), while all others are broadly

expressed in peripheral neurons and in many non-neuronal tissues (Delpire et al., 1994; Mount et al., 1999). Not much is known about the functionality of KCC1 and KCC4 in the CNS. KCC3 was reported to be involved in the chloride regulation in neurons as well as in the mechanisms responsible for the resistance to seizures (Boettger et al., 2003; Byun and Delpire, 2007). KCC2 gene generates two neuron-specific isoforms, isoform a and isoform b (Uvarov et al., 2007). Interestingly, KCC2b rather than KCC2a seems to be responsible for the developmental shift in the GABA polarity (Blaesse et al., 2009). Thus, the transgenic mouse by Woo et al., 2002, which was originaly thought to be a KCC2 KO or hypomorph, was turned out to be a KCC2b KO (Uvarov et al., 2007; Blaesse et al., 2009).

1.1.6. Methods of studying the efficacy of cation-chloride co-transporters

Electroneutrality of CCCs makes their study very challenging. Since researchers can't measure activity of these transporters directly, they have to rely on anion-permeable channels, like $GABA_A$ or glycine receptors, using them as "read-out devices". The most commonly used way is to measure reversal potentials of Cl^- currents by means of the gramicidin-perforated patch technique. In this technique, the polypeptide antibiotic gramicidin is added in the electrode filling solution. Within 30 minutes after the formation of a tight contact between the membrane and electrode tip, gramicidin creates tiny pores that are not permeable for Cl^- but are capable of conducting Na^+ and K^+ currents. The cation selectivity sequence of gramicidin in cell membranes is similar to that measured in artificial lipid bilayers:

H+>NH4+>Cs+>Rb+>K+>Na+>Li+ (Myers and Haydon, 1972; Tajima et al., 1996). The advantage of this method is that it prevents exchange of Cl^- between the pipette and intracellular compartment but allows the electrical coupling to the inside of the cell (Abe et al., 1994; Ebihara et al., 1995; Kyrozis and Reichling, 1995; Akaike, 1996; Kakazu et al., 1999, 2000).

Measurement of the Cl^- reversal potential with the gramicidin perforated patch technique provides an estimate of the steady-state intraneuronal Cl^- concentration which results from two opposing processes: i) passive flow of Cl^- through the Cl^- -permeable channels, and ii) active Cl^- transport by CCCs and other Cl^- transporters including the Cl/HCO_3 exchanger AE3. Indeed, under conditions where the cellular Cl^- load is kept at a low level, even a very weak Cl^- extrusion mechanism will be capable of keeping the E_{GABA} at a hyperpolarized level. A disadvantage of the gramicidin-based method is that if it is used under conditions with no Cl^- load (as is usually the case; but see Kakazu et al., 1999; Achilles et al., 2007) it does not provide information on the efficacy of Cl^- transport.

Another method based on preserving the plasma membrane intact was recently proposed by Tyzio and co-workers (Tyzio et al., 2006). The method involves single channel recording of $GABA_A$ and NMDA receptor reversal potentials. However, this method has also been used in the absence of a Cl^- load and hence the data obtained share the disadvantage of gramicidin-perforated patch clamp technique as it measures steady-state E_{GABA} under "resting conditions" rather than Cl^- transport efficacy.

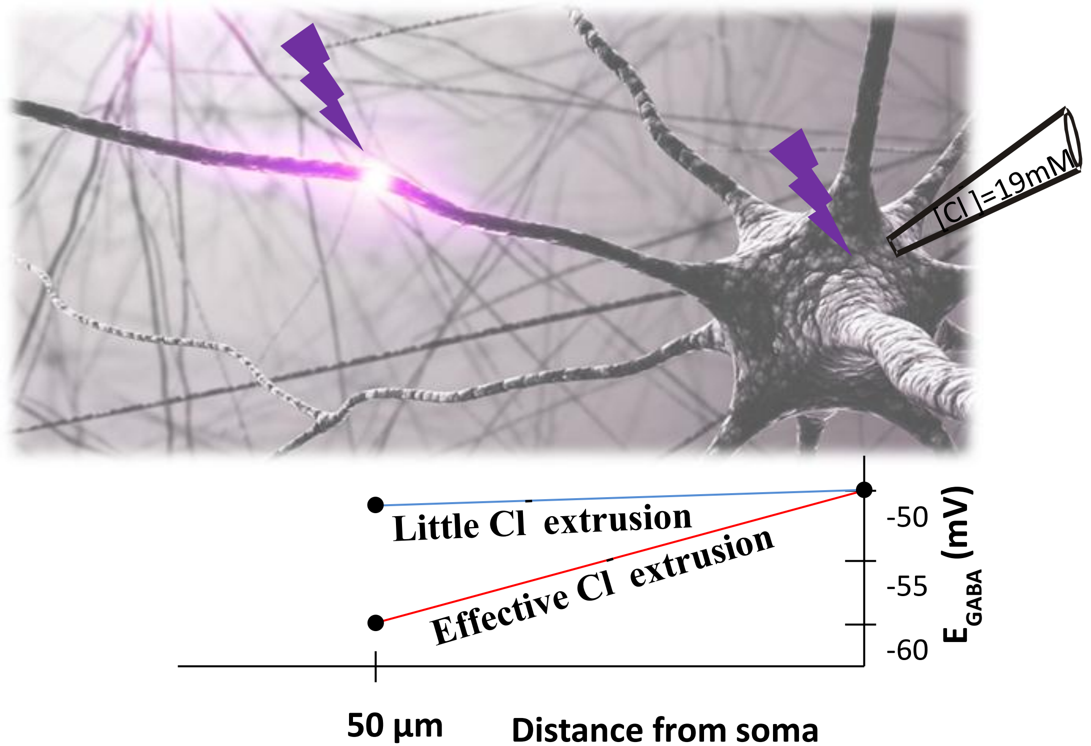

Figure 2. Measuring KCC2 transport efficacy and local $[Cl^-]_i$: GABA uncaging where E_{GABA} values are compared for currents evoked from soma and from dendrites, and under the present conditions the somato-dendritic gradient can be attributed to Cl^- extrusion by KCC2, which leads to more negative dendritic E_{GABA} relative to the clamped somatic one.

To overcome the limitation of the above techniques, one should expose the neuron to a Cl^- load. By doing so, experimenters can assess and quantify the Cl^- extrusion efficacy. A method of Cl^- loading via the recording pipette was introduced by Jarolimek and co-authors who used dissociated cells in culture (Jarolimek et al., 1999). Subsequently, this method was adapted for acute brain slices by us (Study I). An innovative way of inducing $GABA_AR$-mediated currents selectively in certain cellular compartments (soma and dendrites 50 to 100 µm apart) was a critical step in the adaptation of a constant somatic Cl^-

loading protocol in acute slices (Fig. 2). We employed local photolysis of caged GABA (Khiroug et al., 2003) (described in detail in Chapter 4.1. of Results and Discussion), which remains the only reliable method of rapid and selective exogenous agonist delivery to distinct neuronal compartments (Park et al., 2002; Blaesse et al., 2009).

Although patch clamp (with its various modifications) is the golden standard in single-cell studies of intracellular Cl^-, it is not a method of choice if a greater number of cells needs to be analyzed simultaneously. Therefore, a variety of methods based on fluorescence imaging of Cl^- have been proposed as an adequate alternative to electrophysiological recordings. A synthetic fluorescent indicator SPQ (Illsley and Verkman, 1987; Verkman 1990) was the first one introduced. SPQ is excited at about 450 nm, i.e. in the ultraviolet (UV) part of the spectrum. The harmful nature of UV is an obvious disadvantage of SPQ in studies of living cells. Another Cl^- indicator 6-methoxy-N-ethyl-1,2-dihydroquinoline (diH-MEQ) should be mentioned because of its improved sensitivity to Cl^- as compared to SPQ, as well as because of its non-invasive loading into living cells (Biwersi and Verkman, 1991). There are a number of other synthetic Cl^- sensing probes, but we will not discuss them here because they all share a set of common limitations, such as their strong sensitivity to pH and relatively dim fluorescence.

A genetically encoded, brightly fluorescent Cl^- indicator Clomeleon was introduced by Kuner and Augustine in 2000. Clomeleon is a fusion protein consisting of the cyan fluorescent protein (CFP) and topaz variant of yellow fluorescent protein (YFP) (Kuner and Augustine, 2000). The sensor function of Clomeleon is mediated by an intrinsic sensitivity of YFP to halides (Wachter and Remington, 1999). Clomeleon has been used in some studies, such as: measurement of resting Cl^- in mature neurons (Berglund et al., 2006, 2008),

pathological changes in Cl^- (Pond et al., 2006) as well as in studies on compartmentalization of Cl^- in neurons (Duebel et al., 2006). Importantly, Clomeleon can be used for Cl^- imaging *in vivo.* To this end, Clomeleon has been expressed in stable mouse lines using a *Thy1* promoter. Two-photon microscopy has enabled the visualization of pyramidal neurons within living mice as deep as neocortical layer 5 (Krieger et al., 2007; Berglund et al., 2008). Clomeleon's major limitation stems from its strong pH sensitivity and low Cl^- affinity which renders interpretation of the imaging data ambiguous. More recently, two groups have developed new Cl^- sensors based on fluorescent protein pairs (Markova et al., 2008; Arosio et al., 2010). Compared to Clomeleon, the Cl^- sensor exhibits a higher affinity to Cl^- (EC_{50} of 30 mM *vs.* 160 mM for Clomeleon), which enabled compartment-specific detection of intracellular Cl^- microdomains (Waseem et al., 2010). A further improvement was introduced with ClopHensor (Arosio et al., 2010), which allowed simultaneous ratiometric measurement of both pH and $[Cl^-]i$, thus reducing detrimental effects of pH sensitivity which are intrinsic to both Clomeleon and Cl^- Sensor. A major limitation of ClopHensor is the requirement for three precisely distinct excitation wavelength, which prevents *in vivo* use of this sensor in combination with two-photon microscopy. When talking about those methods we should also remember that they provide us data obtained in steady-state measurements at rest. Also the driving force, a crucial parameter, remains unknown which makes this data useless in thermodynamic considerations of Cl^- transport.

It is likely that newly available imaging and electrophysiological data on the GABA actions obtained from experiments performed *in vivo* with or without anesthesia will expand our current knowledge or even will bring some unexpected surprises.

1.2. Developmental changes in CCC expression and activity

GABAergic transmission is a very complex and highly plastic system which undergoes dramatic changes during development of the organism as well as in the course of certain pathophysiological conditions such as brain trauma and epilepsy. Unlike, for example, the glutamatergic system characterized by the invariably depolarizing effect on neurons, the action of GABA may vary from depolarizing (and sometimes even excitatory) in the immature rodent brain to hyperpolarizing (and often inhibitory) in the brain of mature animals. This change of GABA responses, often called "switch" or "shift", is often considered a hallmark of the developing rodent brain (Ben-Ari et al., 1989; Cherubini et al., 1991; Owens et al., 1996). It is generally attributed to the differential temporal expression patterns of two major Cl^- cotransporters: NKCC1 and KCC2 (Rivera et al., 1999; Payne et al., 2003; Stein et al., 2004). Thus, the shift in the polarity of GABA action results from the persistent reduction in intracellular Cl^- concentration that is associated with an increase in the KCC2 expression and perhaps with a simultaneous downregulation of the NKCC1 protein.

It was proposed that NKCC1 during development undergoes a reduction in the protein expression in a number of different regions. This has been proposed for the neocortex, hippocampus, cerebellum, thalamus, brainstem and olfactory bulb (Yamada et al., 2004; Clayton et al., 1998; Kanaka et al., 2001; Wang et al., 2002; Dzhala et al., 2005). It was shown also that the NKCC1 expression switched from a neuronal to a more glial pattern in the adult (Hubner et al., 2001a). In contrast to the protein levels, the NKCC1-encoding mRNA was reported to be upregulated during development (Yan et al., 2001a; Wang et al., 2002; Mikawa et al., 2002). This can be also appreciated from *in situ*

hybridization showing the broad distribution of NKCC1 mRNA in the adult as compared to the low intensities found in the early postnatal brain (Fig. 3). At least partly, this inconsistency might be explained by rather low specificity of the antibodies used to estimate expression levels of NKCC1 protein. Taken together, a more detailed analysis of developmental profile of NKCC1 is vitally needed, and new molecular tools, such as more specific antibodies, will be required to reach this goal. However, NKCC1 seems to be expressed even in mature mouse brain.

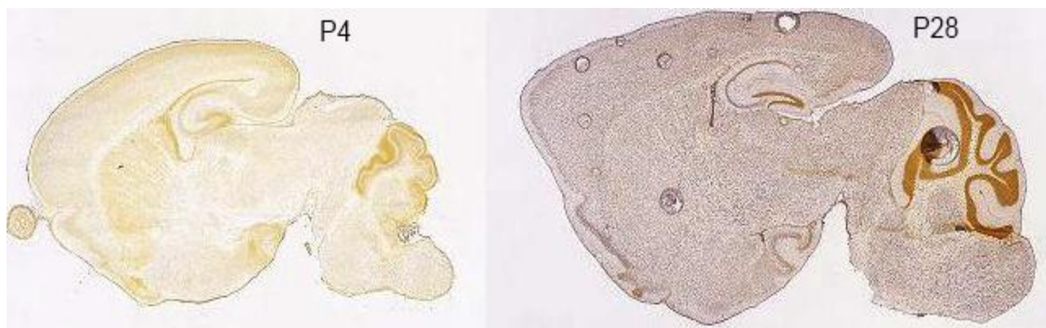

Figure 3. Sagittal section of P4 and P28 mouse brain, NKCC1 mRNA expression is not reduced during development, rather the opposite takes place. (From the Allen Institute for Brain Science, www.brain-map.org)

An important functional role of NKCC1 as an inward Cl^- "pump" was demonstrated in the NKCC1 knockout mice (Delpire and Mount, 2002). Although animals with disrupted NKCC1 gene did not demonstrate any obvious neuronal phenotype, a number of abnormalities in the development were

revealed including salivation impairment, hearing loss caused by lack of endolymph in the inner ear, growth retardation and deficient spermatogenesis (Delpire et al., 1999; Flagella et al., 1999; Evans et al., 2000; Pace et al., 2000).

NKCC1 seems not to be the only mechanism mediating intracellular Cl^- accumulation in the CNS. There are several brain regions where high intracellular chloride concentration was detected despite lack of NKCC1 expression and absence of effect of bumetanide. This was reported for amacrine cells of immature retinal ganglion (Zhang et al., 2007) and for lateral superior olive (LSO) cells of the brainstem (Balakrishnan et al., 2003; Blaesse et al., 2006). It should be noted, however, that a mere increase or decrease of the total cotransporter protein itself is not a limiting factor that determines its functionality. Balakrishnan and co-workers demonstrated that, although in LSO neurons KCC2 is expressed already at very early developmental stages, it exists there in an inactive form. A similar observation was made by our group on dissociated hippocampal cultured neurons (Study I) and *in vivo* (Study IV). In more details this topic will be addressed in Chapter 1.4.

Although KCC2 upregulation during development is a common feature of the majority of brain regions, there are exceptions from this mainstream rule. In the auditory brainstem, KCC2 is highly expressed already at P2, but it is expressed in an inactive form incapable of active extrusion of Cl^- from brainstem neurons. In response to the application of GABA or glycine, these neurons generate depolarizing actions, meaning that they maintain a relatively high intracellular Cl^- concentration in the absence of NKCC1, which failed to be detected in these neurons (Balakrishnan et al., 2003; Blaesse et al., 2006). Thus, a still unknown mechanism must be in place to mediate the active inward transport of Cl^- in these neurons. A possible candidate for somatic Cl^- accumulation was proposed in our paper (Study III). Preliminary experiments

suggest that this could be a bicarbonate-dependent mechanism of chloride uptake (our unpublished data). The elucidation of the active chloride uptake mechanism will be an important subject for future studies.

In contrast to NKCC1 which is broadly expressed in almost all tissue types, KCC2 is a neuron-specific isoform. The CNS-specific expression of KCC2 is controlled by transcription factors, such as Egr4, and by neuron-restrictive silencing elements (Uvarov et al., 2005 and 2006). Expression of KCC2 is quite broad within the CNS regions, as it has been detected in e.g. cortical neurons (Gulyas et al., 2001; Szabadics et al., 2006), in cerebellum (Williams et al., 1999), in thalamus (Bartho et al., 2004), and in brainstem (Blaesse et al., 2006).

What is the physiological role of the depolarizing (and often excitatory) GABA in the immature brain? It has been proposed (Ge et al., 2006; Wang and Krigstein, 2008) that, due to the increased network activity during the first postnatal week, immature cortical neurons exhibit large Ca^{2+} transients and activation of NMDA receptors, i.e. the processes that are strongly implicated in the neuronal proliferation and migration (Owens and Krigstein, 2002). The significance of depolarizing GABA during normal neuronal development was demonstrated recently by several groups. In those studies, early overexpression of KCC2 has resulted in significant drop in the intracellular Cl^- level with subsequent impairment of neuronal development (Chudotvorova et al., 2005; Akerman and Cline, 2006). In the first study by Chudotvorova and collaborators, KCC2 overexpression *in vitro* was achieved by transfecting cultured hippocampal neurons. This procedure resulted in a decreased Cl^- concentration as well as in an increased density of GABAergic receptors and formation of additional GABAergic synapses. An *in vivo* study was performed on the electroporated immature tectal cells of the Xenopus tadpole. In this

study, normal development of the glutamatergic system was altered and GABAergic inputs were enhanced as a result of early overexpression of KCC2 (Akerman and Cline, 2006). However, the interpretation of these results should be reconsidered in view of the recently reported structural role of KCC2 in neuronal morphogenesis (Study II; Horn et al., 2010).

1.3. Intracellular Cl^- gradients and E_{GABA} compartmentalization. Cl^- microdomains

1.3.1. Concept revision: neuron as more than a unit with a singular E_{GABA}

It was a very common and well accepted point of view that intracellular Cl^- is evenly distributed across the whole neuron under steady-state conditions. Today, however, the even distribution of intracellular Cl^- is not taken for granted any longer. Accumulating evidence coming from different brain regions and obtained with a variety of techniques suggests that Cl^- is not evenly distributed. Instead, steady intracellular Cl^- gradients, or in other terms, intracellular Cl^- microdomains, seem to be present in various parts of neurons. In such crucial subcellular locations as axon initial segment (AIS), local Cl^- gradients may revert the polarity of GABA action and strongly affect neuronal excitability, which probably explains the particular interest of researchers to this region and the fierce debate around it (Freund and Buzsaki, 1996; Howard et al., 2005; Szabadics et al.,2006; Woodruff and Yuste, 2008; Glickfeld et al., 2009). By means of patch clamp, Cl^- imaging and other methods it has been made clear that within the same neuron Cl^- concentrations might differ by

several mM between different subcellular compartments. As a result, the steady-state gradients in E_{GABA} between distinct neuronal compartments may reach up to 15-20 mV (Szabadics et al., 2006; Study III; Waseem et al., 2010; Báldi et al., 2010; but see Glickfeld et al., 2009). It is tempting to speculate that Cl^- compartmentalization forms subcellular microdomains, which may share some similarities with Ca^{2+} or pH microdomains (Augustine et al., 2003; Schwiening and Willoughby, 2002). However, the rate of diffusion of Ca^{2+} in the cytosol is very low, and therefore Ca^{2+} and Cl^- microdomains cannot be compared in quantitative terms. Intraneuronal pH microdomains in some neurons can influence E_{GABA} through the unevenly distributed bicarbonate (Schwiening and Willoughby, 2002).

1.3.2. Spatially distinct expression patterns of NKCC1 and KCC2 resulting in steady-state E_{GABA} gradients

The first and most straightforward explanation of Cl^- compartmentalization is a difference in spatial patterns of expression of Cl^- transporters, primarily KCC2 and NKCC1 (Fig. 4). Available literature on the spatio-temporal expression of NKCC1 is extremely confusing and contradictory (see above). The majority of the data published up to date are based on the use of a monoclonal NKCC1 antibody T4 (Lytle et al., 1995). Although T4 was shown to be a very reliable tool for immunoblotting, estimation of NKCC1 protein level in neurons is a challenging task because of non-exclusive neuronal expression of NKCC1, which is also widely expressed in glia. In addition to T4, other NKCC1 antibodies are available, such as a phospho-specific antibody (anti-phospho-NKCC1 antibody R5) raised against a diphosphopeptide containing Thr212 and Thr217 of human NKCC. The advantage of this

antibody is that it is capable to detect the functional state of the protein, because it detects the phosphorylated NKCC1. Actually NKCC1 can be phosphorylated at least at five different residues (Fig. 1), and R5 antibody recognizes only one of them (Flemmer et al., 2002).

All limitations in the analysis of NKCC1 expression listed above make the subneuronal immunohistochemical detection of this Cl^- cotransporter challenging if not unfeasible at the moment. In such a situation, functional analysis with a specific pharmacological block by means of low doses of the diuretic bumetanide combined with NKCC1 knockout mice seems to be the only reliable alternative (Study III).

In terms of subcellular plasmalemal distribution, KCC2 expression is restricted to the plasma membrane of somata and dendrites but almost absent from the axon initial segment (AIS) (Gulyas et al., 2001; Hubner et al., 2001b; Szabadics et al., 2006; Study III; Báldi et al., 2010). On the other hand, KCC2 expression in the terminals of retinal ON bipolar neurons was reported by Vardi and co-workers (Vardi et al., 2000). But this may be explained based on the peculiarity of these cells, as their neurites do not generate action potentials and their definition as axons is arguable. In addition, functional dependence of cotransporters on membrane rafts was recently reported (Hartmann et al., 2009 but see Watanabe et al., 2009). These authors have shown that, in the mature rat brain, NKCC1 was mainly insoluble in Brij 58 and co-distributed with the membrane raft marker flotillin-1 in sucrose density flotation experiments. In contrast, KCC2 was found in the insoluble fraction as well as in the soluble fraction, where it co-distributed with the non-raft marker transferrin receptor. Authors conclude that, membrane raft association appears to represent a mechanism for co-ordinated regulation of chloride transporter function meaning that membrane rafts render KCC2 inactive and NKCC1 active.

High expression of KCC2 in dendritic spines reported by the group of Freund was a somewhat enigmatic observation in light of preferential localization of glutamatergic synapses at dendritic spines (Gulyas et al., 2001), and GABAergic synapses on the somata and dendritic shafts. This surprising situation was hard to explain until recently when an unexpected role of the Cl^- cotransporter KCC2 was found in formation and maintenance of dendritic spines (Fig. 4), a novel function which is independent of the transport of Cl^- by KCC2 (Study II).

In conclusion, the high specificity of subcellular and regional expressional patterns of NKCC1 and KCC2 pointed out the importance of intracellular Cl^- fine tuning, which is reflected in intraneuronal gradients and even Cl^- microdomains (Study III; Waseem et al., 2010).

1.4. Fast post-translational regulation of KCC2 function

1.4.1. Complementary roles of gene expression-mediated regulation and post-translational regulation

Regulation of transporters, similarly to regulation of all membrane-bound proteins, is a very complex mechanism which takes place at different levels. First, slow regulation is at the transcriptional level that determines cell-type or tissue specificity as well as temporal expression by a promoter of a specific transporter gene. Post-translational regulation, which takes place after the transcriptional one (there is also one step after transcriptional regulation- namely- translational regulation), is much faster and consists of a trafficking of the protein to the membrane and kinetic modulation where it should be first

inserted and later on removed. There is a constant addition and replacement of transport proteins by newly synthesized and recycled ones respectively. This implies that the functional expression of these proteins can be easily and rapidly affected by any physiological and/or pathophysiological conditions challenging the tissue. Moreover, transporters that are already in the membrane may further undergo conformational changes that affect their functional activity.

Transcriptional regulation of cotransporters plays an important role during development of the CNS. Postnatal upregulation of KCC2 is a good example of this (Lu et al., 1999; Rivera et al., 1999). There is evidence that brain-derived neurotrophic factor (BDNF) mediates, at least partially, this increase (Aguado et al., 2003; Rivera at al., 2002).

Post-translational processing, which follows the translational phase, is characterized by several modifications that allow proper assembly and trafficking of protein to the plasma membrane. Glycosylation plays a role in the trafficking of many membrane-bound transport proteins. During this process, glycan groups are enzymatically linked to proteins like CCCs. Its importance was demonstrated for NKCC cotransporters trafficking (Delpire et al., 1994; Payne et al., 1995). For KCCs not much is known about the role of glycosylation, except for the identification of four conserved sites in the loop (Fig. 1) between transmembrane segments 5 and 6 (Gillen et al., 1996; Payne et al., 1996; Hiki et al., 1999; Mount et al., 1999). Another type of post-translational regulatory modification is oligomerization. Casula with collaborators were the first ones to show that KCC proteins are able to form oligomers (Casula et al., 2001). A similar observation was recently published also for NKCC1 (Simard et al., 2007). To show that KCC2 oligomerization occurs also under *in vivo* conditions and has functional effects, Blaesse and colleagues performed analysis of immature and mature rat brainstem neurons.

This analysis revealed the absence of oligomerized KCC2 in immature neurons. Interestingly, KCC2 is expressed at the plasma membrane of brainstem neurons already at that early developmental stage but is not functionally active. In contrast, the mature neurons were found to contain more multimers than monomers. This change during maturation was paralleled by progressive functional activation of KCC2, indicating that multimers are the functional units (Blaesse et al., 2006 but see Uvarov et al., 2007).

A very important factor in transporter regulation is the protein phosphorylation state. Regulation of transporter trafficking in the membrane (i.e., its surface expression level, or surface stability) was shown to depend on phosphorylation by protein kinase C (PKC) (Cheung et al., 1999; Galibert et al., 2001; Xiao et al., 2002). Interestingly, PKC phosphorylation has opposite effects on KCC2 and NKCC1 cotransporters. For the former, it was shown that the rate of internalization from the membrane was decreased (leading to an increase in cell surface stability) as a result of PKC activation (Lee et al., 2007 but see Lee et al., 2010). In contrast, phosphorylation of NKCC1 by PKC induces internalization, which leads to the decreased surface expression of this cotransporter (Del Castillo et al., 2005). Thus, PKC activity leads to net decrease in $[Cl-]_i$ by enhancing KCC2-mediated Cl^- clearance and simultaneously decreasing NKCC1-mediated Cl^- accumulation.

The efficacy of the transport function of a given transporter depends on the number of the functional protein molecules that are physically located in the plasma membrane during a certain period of time. This implies that a number of variables might affect the transporter function. Phosphorylation was reported to be involved in the activation of NKCC1 (Lytle and Forbush, 1992) as well as in inactivation of KCCs (Payne et al., 2003). NKCC1 regulation is also very sensitive to the intracellular Cl^- concentration, where reduction of the Cl^- below

a certain homeostatic “set point” activates the transporter by means of the direct protein phosphorylation (Vitari et al., 2006) Nevertheless, the role of phosphorylation in KCC2 function is not straightforward. A number of reports indicated that tyrosine phosphorylation actually activates KCC2 (Vale et al., 2005; Wake et al., 2007).

During the first 10 days *in-vitro* in hippocampal neurons, KCC2 cotransporter is present (Ludwig et al., 2003) but it is in a non-functional form incapable of efficient Cl^- transport (Study I). Interestingly, it was possible to induce a rapid activation of Cl^- transport by application of broad spectrum kinase blocker staurosporine (Study I).

1.4.2. Functional regulation of KCC2 during neonatal seizures

In mature cortical neurons, as it was mentioned earlier, intracellular concentration of Cl^- stays at relatively low levels due to the abundant functional expression of KCC2 and low expression of NKCC1. Thus, GABA release induces a hyperpolarizing influx of Cl^- which results in neuronal inhibition. In contrast, in cortical neurons of developing CNS, NKCC1 plays a key role in creation and maintenance of high intracellular Cl^- concentration, while KCC2 is either poorly expressed or expressed in an inactive form. This leads to the intracellular Cl^- being above the electrochemical equilibrium, to efflux of Cl^- during opening of Cl^- permeable channels, and to depolarization of the plasma membrane, which often has an excitatory effect. Interestingly, a very similar situation can be also observed in the mature CNS suffering from different forms of epilepsy or brain trauma (Pathak et al., 2007; Katchman at al., 1994; Pond et al., 2006; Pitkänen and Lukasiuk, 2011). In such clinical cases, benzodiazepines

and barbiturates are often not efficient in reducing seizure activity because they act by prolonging opening of GABAR channels and increasing the frequency of GABAergic events (Huberfeld et al., 2007). These drugs are also in clinical use in attempts to prevent seizures in neonates and a major problem here is that usually they are not effective in controlling ictogenesis, and may actually make patients' conditions even worse (Rennie and Boylan, 2007; Blaesse et al., 2009).

Ionotropic GABA receptors are pentameric assemblies of subunits that form a central ion channel which is gated by the binding of GABA. Each subunit has four transmembrane domains (Lester et al., 2004; Peters et al., 2005). Two GABA molecules bind at the extracellular interfaces between α and β subunits. Nineteen $GABA_A$ receptor subunit genes in mammals are grouped in eight families based on their sequence similarity. They are $\alpha_{1\text{-}6}$, $\beta_{1\text{-}3}$, $\gamma_{1\text{-}3}$, δ, ε, θ, π and $\rho_{1\text{-}3}$ with additional variation due to alternative splicing (Barnard et al., 1998). It is obvious that such subunit diversity predicts enormous heterogeneity of receptor types, with theoretically as many as more than two million unique pentameric permutations. However, due to the "basic roles of assembly" and differential distribution of subunit types in different brain regions, a much smaller number of receptor subtypes exists in the CNS (Wisden et al., 1992; Pirker et al., 2000; Kittler et al., 2002; Luscher and Keller, 2004). Combinations of α and β subunits are sufficient to form functional $GABA_A$ receptor, however, the majority of endogenous receptors contain a third subunit type. The most abundant $GABA_A$ receptor subtype is formed from α_1, β_2 and γ_2 subunits (McKernan and Whiting, 1996; Sieghart and Sperk, 2002).

Benzodiazepines bind at the interface of the α and γ subunits on the $GABA_A$ receptor. Binding requires that alpha subunits contain a histidine acid residue at position 101 in the α_1 subunit, (*i.e.*, α_1, α_2, α_3, and α_5 containing

$GABA_A$ receptors). For this reason, benzodiazepines show no affinity for $GABA_A$ receptors containing α_4 and α_6 subunits with an arginine instead of a histidine residue in that position (Wafford et al., 2004).

Recently, pyramidal neurons in slices from P5-P7 rats were found to show a step-like increase in the KCC2 cotransporter activity after a single seizure episode *in vivo,* or after a brief episode of enhanced epileptiform-like network activity *in vitro* (Study IV). This leads to a hyperpolarizing shift in E_{GABA} which might act as an intrinsic antiepileptogenic mechanism. An interesting idea is that such activity-dependent modulation of E_{GABA} plays also a role in mechanisms that promote homeostatic plasticity under more physiological conditions.

2. Aims of this study

The mechanisms underlying intracellular Cl^- regulation in the developing and epileptic cortex were the aim of this study. In particular:

1. Design of a photolysis-based assay for quantitative analysis of Cl^--transporting function of KCC2 in brain slices
2. Application of the assay in studies of developmental regulation of the dual role of KCC2 in Cl^- transport and dendritic spine stabilization
3. Determination of intracellular Cl^- distribution patterns and Cl^- microdomains in neocortical neurons
4. Investigation of fast regulation of KCC2 during neonatal seizures

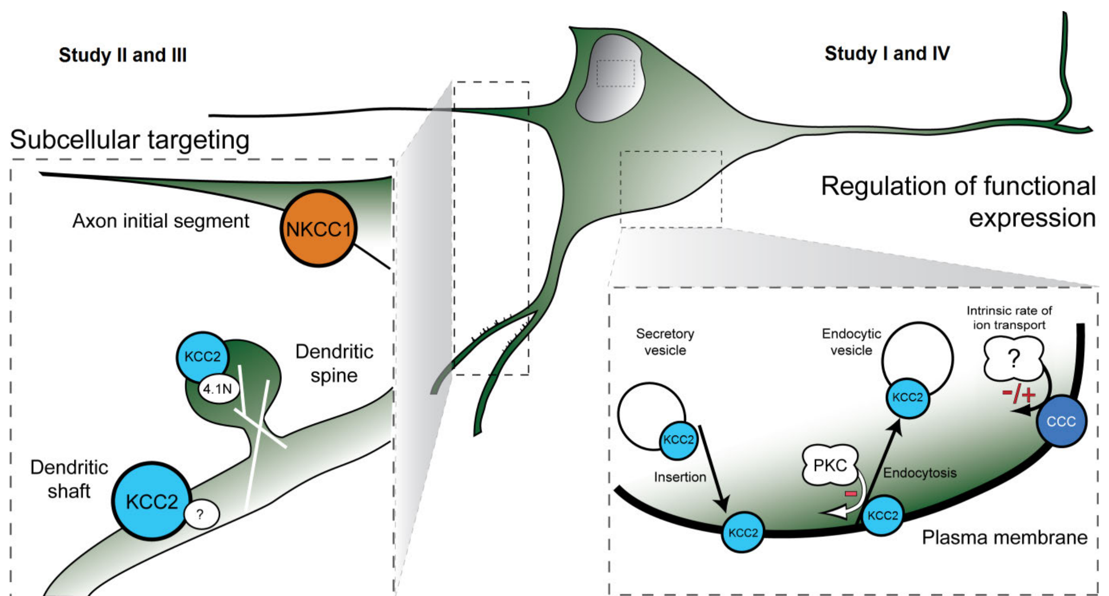

Figure 4. Regulation of CCC functionality (modified from Blaesse et al., 2009)

3. Experimental procedures

All materials and methods that were used in this study are described in detail in the “Materials and Methods” sections of the original papers included in this thesis. All experiments were approved by Ethics Committee for Animal Research at the University of Helsinki. Personal contribution of the author is described in detail in the list of original publications included in this thesis. Only those procedures are listed in this chapter where the author was personally involved. All drug and equipment suppliers are indicated in the original publications and are not mentioned here.

3.1. Acute hippocampal slice preparation (I, III, IV)

Rats or mice 3 to 32 postnatal days of age were used in the experiments described here. The more mature animals (>P10) were anesthetized with pentobarbital or halothane prior to decapitation. Transverse or horizontal hippocampal slices (350-600 μm thick) were obtained from P3-P32 mice or rats using VT 1000S or Vibratome 3000 vibrating blade microtome. After cutting, slices were allowed to recover at 36°C for one hour before the recordings were started.

3.2. Induction of neonatal seizures (IV)

Intraperitoneal injections of kainic acid (2 mg/kg of body weight) were performed to induce neonatal seizures in male P5-P7 Wistar rat pups. Saline injections were performed as controls. To determine the onset of seizures, continuous behavioral monitoring was performed by video recording of

animals. The onset of seizures in kainate-injected animals occurred within half an hour after the injection and was characterized by the initial stage of immobility. Subsequent development of *status epilepticus* was characterized by bouts of scratching behavior, hyperactivity, ataxia, isolated myoclonic jerks ending in tonic or tonic-clonic seizures without recovery during one hour before decapitation.

3.3. Induction of seizure-like activity *in vitro* (IV)

In order to induce a seizure-like activity, horizontal hippocampal slices (600 µm) were incubated for 10 min in 300 nM kainate which was dissolved in physiological solution at 32°C. After that, slices were transferred to the physiological solution containing 1 µM TTX and 10 µM bumetanide to block neuronal activity and NKCC1 respectively.

3.4. Local photolysis of caged GABA and visualization of the targeted neurons (I, II, III, IV)

Caged GABA was diluted in the standard physiological solution and delivered to the vicinity of the patch-clamped cell using a syringe micropump. Use of the micropump allowed us to avoid recycling of small volumes and at the same time ensured application of maximal concentrations of fresh caged GABA, thus addressing the major concerns associated to other known protocols of caged compound delivery. For photolysis, UV light from a laser source was focused to the uncaging spot of approximately 10 µm in diameter.

To visualize fine processes (dendrites and axons) in the whole-cell patch-clamp experiments, AlexaFluor 488 was added directly to the patch

pipette solution. In gramicidin perforated patch clamp recordings, visualization of neurons and their processes was achieved by single-cell electroporation with AlexaFluor 488 or by using slices from *Thy1-mGFP* L21 mice, a mouse line engineered to express a fluorescent protein in a subset of hippocampal neurons. A confocal microscope (Radiance 2100) was used for real time imaging and three-dimensional reconstruction of neuronal morphology. All those techniques were used to study NKCC1-mediated accumulation of Cl^- in the axon initial segment (Study III).

3.5. Electrophysiology: patch clamp and field-potential recordings (I, II, III, IV)

Somatic recordings of neurons from dissociated cultures or from acute hippocampal slices were performed in the whole cell voltage-clamp or gramicidin perforated patch-clamp configuration using an HEKA EPC 10 patch-clamp amplifier. To assess the efficacy of KCC2-mediated Cl^- extrusion, the Cl^- concentration in the patch pipette was set to 19 mM in order to clamp the somatic E_{Cl} at a -50 mV as calculated based on the Nernst equation. KCC2 function was pharmacologically isolated from that of NKCC1 by blocking the latter with 10 µM bumetanide. The $GABA_B$ receptors were blocked with 1 µM CGP 55845 and action potentials with 1 µM TTX. The E_{GABA} values recorded by UV flash stimulation in different neuronal compartments (soma or dendrite) were compared. Under these conditions, the somato-dendritic gradient can be attributed to Cl^- extrusion mediated by KCC2 cotransport. In Study III, a modified protocol was used: Instead of the whole-cell patch-clamp with Cl^- loading of somata, measurements of native Cl^- concentrations and native axo-somato-dendritic gradients were performed by means of gramicidin perforated

patch-clamp recordings. NKCC1-mediated Cl^- transport was not blocked, thus it was possible to measure Cl^- accumulation in the axon initial segment (AIS) mediated by this cotransporter.

To characterize epileptiform like network activity, in Study IV we performed field-potential recordings. Thick (600 µm) horizontal hippocampal slices were recorded in the submerged chamber.

4. Results and discussion

4.1. A novel optical-electrophysiological assay for quantitative analysis of KCC2 functional activity in brain slices

The assessment of efficacy of the cation-chloride cotransporter KCC2 in the cultured hippocampal neurons as well as in acute hippocampal slices was our goal during the development of a novel optical-electrophysiological assay. We decided to use a technique where caged GABA is photolyzed with brief pulses from a UV laser (Pettit & Augustine, 2000; Khiroug et al., 2003) along the dendrite at varying distances from soma of pyramidal neurons. To expose KCC2 to the Cl^- load at soma, we patch-clamped the neurons in the whole cell configuration with 19 mM Cl^- in the patch pipette. Values of E_{GABA} were obtained from I-V curves (Study I, Fig. 1B). Under these conditions, the experimentally recorded somatic E_{GABA} was very close to the value calculated on the basis of Nernst equation. NKCC1 was pharmacologically blocked throughout the experiments by bumetanide, a selective blocker of this inward cotransporter at low micromolar concentrations. In addition, action potentials were blocked with TTX, and $GABA_B$ receptors with CGP 55845.

To determine the diameter of the effective photolysis spot we applied 25 ms pulses of the UV light and moved the spot in a direction perpendicular to the dendrite of a patch-clamped cell. The resultant current amplitude was plotted against the linear distance between the dendrite and the center of the spot. Thus, the effective uncaging spot was found to be approximately 10 μm in diameter (Study I, Fig. 1D).

To validate the method we used it first on hippocampal cultured neurons. An obvious advantage of the GABA uncaging stimulation is that it is

applicable also for the acute slices. Thus we performed somato-dendritic E_{GABA} measurements with photolysis of caged GABA in acute hippocampal slices obtained from mice up to one month old. Combining the uncaging technique with whole-cell patch-clamp recordings and somatic Cl^- load in acute mouse hippocampal slices makes this novel method a unique tool to assess the efficacy of Cl^- transport mechanisms. We have successfully used this combination to study KCC2 efficacy under pathological conditions (Study IV). A further modification of this technique, namely GABA uncaging on gramicidin-perforated patch-clamped neurons, was instrumental in revealing the native steady-state Cl^- intracellular gradients (Studies III and IV).

A very interesting and promising application of this optical assay is its possible use under *in vivo* conditions, i.e. in the intact brain of a living anaesthetized rodent. The ultimate physiological relevance of such model makes such studies extremely valuable, but renders conventional drug application protocols (e.g., iontophoresis, pressure puff or fast perfusion) totally unfeasible. To photolyse caged GABA in cortical layers or deeper brain regions such as hippocampus, two-photon microscopy will have to be used. This *in vivo* assay can be further refined by combining patch-clamp and photolysis with a Cl^- sensor imaging (Kuner and Augustine, 2000; Waseem et al., 2010).

4.2. Developmental regulation of structural and Cl^- transport activity of KCC2 in mouse hippocampal neurons

4.2.1. Developmental time course of KCC2 Cl^--extruding activity in cultured neurons and brain slices

During first two postnatal weeks of rodent's life, $GABA_A$- mediated responses in cortical neurons undergo a shift from depolarizing to hyperpolarizing. The question is whether a mere increase in the amount of KCC2 protein is the rate-limiting factor for the functional Cl^- outward transport. To directly assess the efficacy of neuronal Cl^- extrusion we have established a protocol where the load of a defined high Cl^- concentration was imposed on the neuron in order to test the neurons capability to maintain its $[Cl^-]i$. The measured difference in E_{GABA} recorded during photorelease of caged GABA at soma and at a certain distance along the dendrite provided us with quantitative and physiologically relevant information about the functional activity of KCC2.

Immature cultured neurons grown for 5 to 10 days in culture (DIC 5 - 10) demonstrated only a slightly more hyperpolarized dendritic E_{GABA} as compared to the somatic one (Study I, Fig. 2B and 3A). In contrast, neurons cultured for more than 15 days were able to keep dendritic E_{GABA} much more negative compared to soma, resulting in a very steep somato-dendritic E_{GABA} gradient (Study I, Fig. 2B and 3A). This gradient was almost completely abolished by bath application of 0.5 mM furosemide, indicating that it was KCC2-mediated. We then studied E_{GABA} gradients in acute slices, a more physiological preparation in comparison to dissociated cultured neurons. In slices obtained during the first postnatal week, E_{GABA} gradients were not different from these observed in cultures at DIC 5 – 10 (Study I, Fig.3A and B).

In contrast, the slices from more mature animals (second postnatal week) demonstrated much larger E_{GABA} gradients than slices from newborn mice (Study I, Fig. 3B). Interestingly, E_{GABA} values observed in slices from older mice (>P14) were similar to those recorded from older cultured neurons (>DIC 14), suggesting that maximal levels of Cl^--pumping activity are similar in both preparations. However, E_{GABA} levels increased gradually in slices from P3 to P13, whereas in cultured neurons the gradient increased more abruptly from DIC 10 to DIC 14 (Study I, Fig. 3A *versus* 3B). It is important to note here that KCC2 protein expression has similar developmental profile in slices and cultures (Ludwig et al 2003). Thus, the difference in E_{GABA} gradients is likely due to the presence in acute hippocampal slices of a yet unidentified factor that is required for KCC2 functional activation, whereas in cultures this factor is missing.

The action of this putative factor is mimicked by exposure of cultured neurons to a broad-spectrum protein kinase inhibitor staurosporine (100 nM). After 5 minutes in staurosporine, the E_{GABA} gradients in immature cultural neurons were three-fold larger than control values. Interestingly, intracellular applications of staurosporine produced the same result, indicating that the effect is at the level of a single neuron and does not depend on changes in network activity. In contrast to cultures, the P3 – P6 acute slices showed no change in E_{GABA} gradients in response to staurosporine application, even when very high concentrations of staurosporine (up to 2 mM) were applied for much longer (0.5 -2 hours). This observation reinforces the argument that cultured neurons lack a factor that is required for the gradual up-regulation of functional KCC2 during normal physiological development. Because staurosporine is a broad spectrum kinase inhibitor, it is not possible to exactly identify this “missing factor”.

However, our results do suggest that the phosphorylation state of the protein is important and involved in functional KCC2 activation.

4.2.2. A novel structural role of KCC2 in dendritic spine development

It was known for quite a while that KCC2 in dendrites of cortical neurons has a clear tendency to localize around dendritic spines (Gulyas et al., 2001). This was difficult to interpret due to the established fact that dendritic spines primarily act as the postsynaptic sites of excitatory synapses. Therefore, it was extremely intriguing that neurons cultured from the $KCC2^{-/-}$ embryos had highly distorted dendritic spines (Study II). During their maturation in the culture dish, KCC2-lacking neurons failed to develop mature, mushroom-shaped dendritic spines but instead maintained an immature phenotype with long, branching and extremely motile protrusions (Study II, Fig 1D).

To test whether abnormal development of neocortical spines in the KCC2 knockout mice affected the maturation of excitatory synapses, we used immunostaining and a number of functional measurements (Study II, Fig. 2). All these methods pointed to a significant reduction in the number of functional excitatory synapses. Thus the structural data was supported by the observation of reduced number of functional excitatory synapses in the KCC2-lacking neurons.

In order to test the specificity of the changes in KCC2 knock-out on spine morphology and glutamatergic synaptic transmission, we performed rescue experiments by overexpressing KCC2 in the knockout neurons. Full-length KCC2 completely restored both structure and function of glutamatergic synapses (Study II, Fig. 3).

We then asked whether the Cl^--extruding activity of KCC2 is required for its newly discovered spine-stabilizing function. Our hypothesis was that, similarly to KCC1, the N-terminal domain of KCC2 is required for its Cl^- transporting activity (Casula et al., 2001) but may not be involved in its spine-stabilizing function. To test this hypothesis, we first applied the uncaging-based assay to measure E_{GABA} gradients and estimate Cl^--pumping activity of KO neurons overexpressing either full-length (KCC2-FL) or the N-terminal domain deletion construct (KCC2-ΔNTD). We found that N-terminal-truncated KCC2 was transport inactive (Study II, Fig. 3 A and B). Importantly, KCC2-ΔNTD overexpression resulted in a restoration of the normal spine structure in $KCC2^{-/-}$ neurons as well as in enhanced expression of functional excitatory synapses (Study II, Fig. 4).

These results led us to conclude that the novel spine-stabilizing role of KCC2 is independent of its established role in Cl^- transporter activity. Developmental implications of this discovery are prominent. Indeed, it suggests that KCC2 may be a synchronizing factor in the development of both excitatory (via spine stabilization) and inhibitory (via Cl^- extrusion) transmission.

4.3. Intracellular Cl^- distribution patterns in neocortical neurons

The idea of uneven intraneuronal Cl^- distribution is not new to the field, however, its experimental demonstration has not been a trivial task. Interestingly, GABAergic terminals of axo-axonic cells (AACs), that are exclusively located on the axon initial segment (AIS) of cortical principal neurons, can exert depolarizing and even excitatory actions (Szabadics et al., 2006). This observation is of particular importance because those terminals are considered to be a site of powerful inhibition. The authors' explanation of these

phenomena was an absence of KCC2 expression in the AIS. However, a mere absence of active Cl^- extrusion is not sufficient to lead to accumulation of Cl^- in the AIS. Rather, this suggests the presence of an active accumulation mechanism, such as NKCC1, in this particular subcellular region. Therefore, in Study III we used local photolysis of caged GABA to estimate the local Cl^- concentration in the AIS compared with other cellular compartments by measuring reversal potentials of $GABA_A$ receptor-mediated currents at different localizations.

A very crucial question in this content was whether E_{GABA} gradients can be observed under nonperturbed conditions in dentate gyrus cells (DGCs). We used gramicidin-perforated patch-clamp technique to record currents evoked by uncaging of GABA. To overcome the obvious problem of visualization of individual neuron with its fine processes in the absence of dye loading through the pipette, we used hippocampal slices from mice that expressed EGFP in a subset of DGCs under *Thy1* promoter (De Paola at al., 2003; Galimberti at al., 2006). Local uncaging of GABA was performed on AIS (50 µm from the soma), somata and at dendrite (50 µm from the soma) (Study III, Fig. 1). A marked depolarizing driving force of about 15 mV for the GABAergic AIS responses from the (RMP) was revealed. In addition, a negative axo-dendritic E_{GABA} gradient was present, indicating a 5 mM native steady-state gradient of intracellular Cl^- between AIS with 11 mM and dendrite with 6 mM. The distance between those two uncaging locations was only 100 µm, and a remarkable difference between subcellular compartments is present.

To identify the mechanism of Cl^- accumulation in the AIS we have used three approaches: i) gramicidin perforated patch recordings from DGCs of NKCC1 knockout mice and their WT littermates (Study III, Fig. 2), ii) whole-cell patch-clamp recordings of E_{GABA} gradients in rat DGCs under

pharmacological block of NKCC1 with 10 μM bumetanide in the perfusion (Study III, Fig. 3) and iii) similar recordings from neocortical neurons in layer 2/3 (Study III, Fig. 4).

In the first approach i) WT neurons showed axo-somatic E_{GABA} gradients similar to the gradients observed in the *Thy1*-mGFP neurons, while in striking contrast in NKCC1$^{-/-}$ neurons no axo-somatic gradients were observed. Somato-dendritic gradients were present and, interestingly, they were similar in both WT and knockout groups. The mechanism that generates those gradients is still unknown, but our preliminary experiments suggest a bicarbonate-dependent chloride uptake in the soma. These data pointed to the NKCC1 co-transporter as the mechanism responsible for the AIS-specific accumulation of the Cl^-. Furthermore, it is obvious from these data that NKCC1 has no significant influence on dendritic E_{GABA}.

The experimental approaches ii) and iii) were used to study possible species- and cell-specific differences in the generation of depolarizing GABA responses in the AIS. In the Study I we demonstrated that plasmalemmal ion transporter KCC2 is able to maintain Cl^- gradients along the dendrites during a somatic Cl^- load in the whole-cell mode of patch-clamp (Study I, Fig. 1B). Whole-cell recordings performed in the DGCs of rats revealed a robust axo-somatic E_{GABA} gradient of about 7 mV. In agreement with our results from NKCC1$^{-/-}$ neurons, application of the specific blocker of NKCC1-mediated Cl^- uptake, bumetanide, resulted in the collapse of axo-somatic E_{GABA} gradient without having any effect on the somato-dendritic E_{GABA} gradient. Similar results were obtained from the whole-cell recordings in rat neocortical layer 2/3 pyramidal neurons (Study III, Fig. 4). The observed similarity between different cortical regions and between mice and rats supports the presence of a prominent steady-state axo-somato-dendritic E_{GABA} gradient as a common feature of most

if not all types of cortical principal neurons. E_{GABA} gradients have also been demonstrated in cortical interneurons (Martina et al., 2001; Banke and McBain, 2006).

To conclude, our data presented in the study III, demonstrate that NKCC1-mediated Cl^- uptake results in a depolarizing action of AACs at the AIS of cortical principal neurons. Axo-somatic Cl^- gradient can be explained on the basis of the distinct spatial expression of NKCC1 and KCC2 along axo-somato-dendritic axis (Gulyas et al., 2001; Szabadics et al., 2006). This microdomain-like compartmentalized intraneuronal Cl^- suggests that a widely used way of assigning a "typical" E_{GABA} to a given principal neuron is misleading. Indeed, it would be more appropriate to specify the E_{GABA} value of a given interneuronal input on the postsynaptic neuron. The second important conclusion from our study is the need to re-examine the "developmental shift" from depolarizing to hyperpolarizing GABA transmission not only at cellular, but also at the subcellular level. Obviously, this input specificity can have broad implications in network plasticity at different levels.

4.4. Post-translational regulation of KCC2 activity during neonatal epileptogenesis

It is a widely accepted view that maturation of Cl^--dependent $GABA_A$ receptor-mediated inhibition correlates strongly with the functional expression of KCC2 in developing central neurons. In Study IV, we show that a single neonatal seizure episode evoked during the first postnatal week results in a very rapid (minutes range) increase in the Cl^- extrusion capacity of rat hippocampal CA1 neurons.

First we estimated the effect of a single kainate-induced seizure episode on KCC2 Cl^- transport activity. To this end, we performed a single intraperitoneal injection of kainate in P5-7 rats for the induction of seizures. Gramicidin-perforated patch-clamp recordings revealed a step-like hyperpolarizing shift (about 10 mV) in somatic E_{GABA} in post-seizure CA1 hippocampal neurons. In fact, the E_{GABA} in the kainate-treated neonatal rats was very close to the level of E_{GABA} in animals older than two weeks (Study IV, Fig. 1 A and B).

It was reported in Study III that cortical neurons from mice older than two weeks demonstrated a native somatodendritic E_{GABA} gradient (ΔE_{GABA}) in gramicidin perforated patch recordings. Thus we tested whether the single seizure episode leads to a similar native E_{GABA} gradient in rats during the first week of life. Interestingly, under control conditions almost no native gradient of E_{GABA} was observed. But, post-seizure, ΔE_{GABA} was similar to that in P16 rats, with hyperpolarized responses in the dendrite (Study IV, Fig. 1D-F). We also used bumetanide, a selective blocker of NKCC1 in micromolar concentrations, to test the possible role of this cotransporter in the creation and maintenance of those somatodendritic gradients. In the present study, pharmacological block of NKCC1 did not induce any change in the native ΔE_{GABA} both in post-seizure and control neurons. This result points to KCC2 as a mechanism responsible for the establishment of the native ΔE_{GABA} in post-seizure slices.

In this study, to obtain a quantitative estimate of the effect of seizures on the Cl^- extrusion, we used an assay with a constant somatic Cl^- load on the neuron as was described in details in Study I. Similar to results published in Study I, uncaging of GABA at soma and dendrite of control CA1 hippocampal neurons revealed the absence of an efficient mechanism of Cl^- extrusion. In contrast, the same photolysis of GABA in post-seizure slices demonstrated

almost a threefold increase in somatodendritic ΔE_{GABA} (Study IV, Fig. 1 E, F). Thus we conclude that functional activation of KCC2 in CA1 neurons in post-seizure slices is the mechanism responsible for the hyperpolarization of E_{GABA}. This conclusion was supported by the effect of furosemide (nonspecific KCC2 blocker) which abolished the increase in ΔE_{GABA} (Study IV, Fig. 1 F).

Next, we tested if an increase in total KCC2 protein expression in CA1 region is responsible for the increased Cl^- extrusion efficacy in animals that experienced seizures. Western blot analysis did not detect any difference in the total KCC2 level in saline- *vs.* kainate- injected rats (Study IV, Fig. 2 A and B), indicating that an increase of KCC2 does not play a role in these phenomena. Based on the previously published data on the role of KCC2 trafficking to the plasma membrane, we asked if the change in the number of functionally active KCC2 cotransporter molecules in the membrane can lead to its upregulated pumping activity (Lee et al., 2007; Wake et al., 2007). To this end, two different biochemical approaches were used: i) protease treatment of hippocampal slices with trypsin to detect both the cleaved KCC2 (which corresponds to a surface/membrane pool) and the uncleaved KCC2 (intracellular pool) (Ahmad et al., 2011); and ii) a well-established biotinylation protocol (Thomas-Crusells et al., 2003). Both methods showed an almost twofold increase in the surface KCC2 after seizure (Study IV, Fig. 2 C-G). Interestingly, both recently identified KCC2 isoforms a and b (Uvarov et al., 2007) showed a similar increase in the surface KCC2 expression. In addition, surface expression of α2 subunit of the Na-K-ATPase (which generates the K^+ transmembrane gradient that is actively exploited by KCC2) was also increased after seizures. The α2 subunit which has been shown to interact physically with KCC2 (Ikeda et al., 2004) exhibited an almost twofold higher surface expression in post-seizure rats than in control saline injected littermates.

Then, we addressed the questions of the time course of this increase of the KCC2 activity as well as its mechanism. To do so, we took advantage of the *in vitro* model of kainate-induced elipeptiform-like activity in acute hippocampal slices. Incubation of slices in 300 nM kainate resulted in enhanced network activity (Study IV, Fig. 3 A). A brief period of seizure-like activity *in vitro* resulted in activation of Cl^- extrusion was reflected in a threefold increase in the ΔE_{GABA} with a somatic Cl^- load (Study IV, Fig. 3 B-E). This effect was fast and progressive over the 30 minute period after kainate-induced network activity, and it was blocked by addition of TTX during the pre-incubation period with KA. These data indicate that KCC2 activation was activity dependent, and not induced by kainate itself (Study IV, Fig. 3D). To reveal possible mechanisms which could underlie this fast activation of KCC2 function we used K252a, a tyrosine receptor kinase TrkB blocker. Interestingly, both the network activity-induced increase in the surface expression and the increase in transport efficacy of KCC2 were abolished by K252a (Study IV, Fig. 3E-G). To test whether a decrease in the endocytosis of KCC2 could be involved in functional KCC2 activation, we used dynamin inhibitory peptide (DIP) intracellularly to block endocytosis. Neurons patched with DIP demonstrated after 10-30 minutes a much larger ΔE_{GABA} compared to control cells (Study IV, Fig. 3H).

To summarize, Study IV revealed that immature hippocampal neurons with relatively low expression of KCC2 are able to activate a more efficient use of the available total cellular pool of transporter molecules. Fast and progressive time course of this activation pointed to the posttranslational nature of this mechanism. These data do not exclude the existence of parallel long-term effects of enhanced KCC2 protein de-novo synthesis. This study describes a brief initial phase of changes that take place during neonatal epileptogenesis.

5. Conclusions

The overall aim of this Thesis was to elucidate molecular mechanisms of Cl^- transporter regulation. To achieve this, we first had to develop a relevant experimental assay that would allow us to quantify the efficacy of transmembrane Cl^- transport. The assays that had been available at the time this thesis work was initiated were designed to estimate the steady-state transmembrane distribution of Cl^- rather than the actual efficacy of Cl^- transport. Study I documents the development of such an assay based on imposing a somatic Cl^- load and local photolysis of caged GABA in subcellular compartments of interest.

We successfully applied the newly developed assay to reveal developmental changes in KCC2 Cl^- transporting efficiency under both *in vitro* and *in situ* conditions (cultured neurons and hippocampal slices, respectively). Study I demonstrates existence of functionally inactive KCC2 in immature cultured neurons that was rapidly activated by staurosporine.

Study II presents evidence of a novel structural function of KCC2 in stabilizing dendritic spines and formation of functional excitatory synapses. Importantly, the spine-stabilizing function of KCC2 was independent of its ability to transport Cl^-. These findings imply a pivotal role for KCC2 in developmental regulation of both excitatory and inhibitory synapses.

Applying our original photolysis-based assays to mapping putative subcellular microdomains of distinct Cl^- concentrations allowed us to demonstrate for the first time the existence of standing Cl^- gradients along the axo-somato-dendritic axis (Study III). Furthermore, we were able to document a central role of NKCC1 in the formation of high Cl^- microdomains in the (AIS).

Finally, we extended our developmental findings to pathophysiological domain of neonatal epilepsy investigations. Study IV presents compiling evidence that a seizure-induced acute increase in Cl^- transport activity and resulting shifts in E_{GABA}.

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