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KCC2 AS A MULTIFUNCTIONAL PROTEIN IN BRAIN DEVELOPMENT AND DISEASE

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ACADEMIC DISSERTATION

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To Filip and Lena

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This Thesis is based on the following publications which are referred to in Roman numerals in the text:

I. Khirug S*, Ahmad F*, **Puskarjov M**[1], Afzalov R, Kaila K, Blaesse P (2010) A single seizure episode leads to rapid functional activation of KCC2 in the neonatal rat hippocampus. J Neurosci 30:12028-12035.

II. **Puskarjov M**[2], Ahmad F, Khirug S, Sivakumaran S, Blaesse P, Kaila K (2013) BDNF is required for activity-dependent but not constitutive up-regulation of KCC2 during hippocampal development. Submitted manuscript.

III. **Puskarjov M***[3], Ahmad F*, Kaila K, Blaesse P (2012) Activity-dependent cleavage of the K-Cl cotransporter KCC2 mediated by calcium-activated protease calpain. J Neurosci 32:11356-11364.

IV. Fiumelli H*, Briner A*, **Puskarjov M**[4], Blaesse P, Belem BJ, Dayer AG, Kaila K, Martin JL, Vutskits L (2013) An ion transport-independent role for the cation-chloride cotransporter KCC2 in dendritic spinogenesis *in vivo*. Cereb Cortex 23:378-88.

*Equal contribution

[1]The candidate performed part of the electrophysiological experiments and participated in the analysis of the results.
[2]The candidate performed most of the electrophysiological experiments, contributed to the experimental design, participated in the analysis of the results and wrote the manuscript together with KK.
[3]The candidate performed the electrophysiological experiments, substantially contributed to the experimental design, participated in the analysis of the results and wrote the manuscript together with PB and KK.
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Publications that have been used in other dissertations:

Studies I and III have been included in the Thesis of Dr. Faraz Ahmad, titled “Post-translational regulation of KCC2 in the rat hippocampus” in 2012 (Faculty of Biological and Environmental Sciences, University of Helsinki). Study I has been included in the Thesis of Dr. Stanislav Khirug, titled “Functional expression and subcellular localization of the Cl^- cotransporters KCC2 and NKCC1 in rodent hippocampal and neocortical neurons” in 2011 (Faculty of Biological and Environmental Sciences, University of Helsinki).

Other publications related to the Thesis:

Löscher W, **Puskarjov M**, Kaila K (2013) Cation-chloride cotransporters NKCC1 and KCC2 as potential targets for novel antiepileptic and antiepileptogenic treatments. Neuropharmacology 69:62-74.

Kahle KT*, Deeb TZ*, **Puskarjov M***, Silayeva L, Liang B, Kaila K, Moss SJ (2013) Modulation of neuronal activity by phosphorylation of the K-Cl cotransporter KCC2. Trends Neurosci (in press; http://dx.doi.org/10.1016/j.tins.2013.08.006).

*Equal contribution

LIST OF ABBREVIATIONS

aa	Amino acid
AED	Antiepileptic drug
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
CCC	Cation-chloride cotransporter
CNS	Central nervous system
CTD	C-terminal domain
ΔNTD	N-terminal deletion
DF	Driving force
DIV	Day *in vitro*
E	Embryonic day
E_{GABA-A}	Reversal potential of $GABA_AR$-mediated currents
EEG	Electroencephalogram
E_{Gly}	Reversal potential of GlyR-mediated currents
EGR	Early growth response
EPSP	Excitatory postsynaptic potential
FL	Full-length
$GABA_AR$	Ionotropic γ-aminobutyric acid receptor
GAD	Glutamic acid decarboxylase
GFP	Green fluorescent protein
GlyR	Glycine receptor
IPSP	Inhibitory postsynaptic potential
IUE	*In utero* electroporation
KCC	K-Cl cotransporter
KO	Knockout
LSO	Lateral superior olive
LTCC	L-type Ca^{2+} channel
mEPSC	Miniature excitatory postsynaptic current
mIPSC	Miniature inhibitory postsynaptic current
NCC	Na-Cl cotransporter
NKCC	Na-K-2Cl cotransporter
NL	Neuroligin
nAChR	Nicotinic acetylcholine receptor
NMDAR	N-methyl-D-aspartate receptor
NRSE	Neuron-restrictive silencer element
NRSF	Neuron-restrictive silencing factor
NTD	N-terminal domain
P	Postnatal day
PKC	Protein kinase C
PLCγ	Phospholipase Cγ
Shc	Src homology 2 domain containing transforming protein
TF	Transcription factor
TrkB	Tropomyosin-related kinase B
TTX	Tetrodotoxin
VIAAT	Vesicular inhibitory amino acid transporter
WT	Wildtype

ABSTRACT

Active extrusion of Cl^- from the neuronal cytoplasm by the neuron-specific K-Cl co-transporter isoform KCC2 is necessary for the hyperpolarizing inhibitory Cl^- currents mediated by the GABA receptors ($GABA_A$Rs). Early in development and following cellular trauma or seizures, $GABA_A$R-mediated signaling is often depolarizing and may even, in contrast to its classical inhibitory action, promote action potential firing. Developmental up-regulation of KCC2 is largely responsible for the shift from depolarizing to hyperpolarizing $GABA_A$R-mediated signaling, and conditions associated with brain pathology often lead to loss of KCC2 and re-emergence of depolarizing $GABA_A$R responses. The molecular mechanisms responsible for the up-regulation of KCC2 during development and those mediating its down-regulation, however, remain elusive.

The present Thesis demonstrates that the low level of KCC2 protein in immature neurons is not a limiting factor for its functional activation. A single seizure episode induced with kainate triggers a fast transient enhancement of neuronal Cl^- extrusion capacity paralleled by a large increase in surface-expressed but not total KCC2 protein in the hippocampus of neonatal rodents. This post-translational activation of KCC2 appears to be mediated by BDNF-TrkB signaling, as evidenced by its sensitivity to Trk inhibition and its absence in BDNF knockout mice. In contrast to these fast changes in functional expression of KCC2, no requirement for endogenous BDNF was observed for the developmental up-regulation of KCC2 protein. Another key finding of this work is that down-regulation and inactivation of KCC2 following intense NMDA receptor (NMDAR) activation is mediated via cleavage and truncation of KCC2 by the calcium-activated protease calpain. Importantly, the data obtained using inhibitors of protein degradation and protein synthesis indicate that the basal turn-over of KCC2 protein is slow and, consequently, down-regulation under pathological conditions is likely to result from enhanced degradation rather than from reduced *de novo* KCC2 synthesis. Together, the present findings highlight post-translational regulation as an important mediator of changes in the functional expression of KCC2 in response to conditions of enhanced neuronal activity, such as epileptic seizures.

KCC2 has been traditionally regarded to have the most clearly defined physiological role of all the K-Cl cotransporters, as it is uniquely expressed in central neurons, and determines the neuronal response to activation of $GABA_A$ and glycine receptors. However, such a view has changed drastically following the unexpected observation that KCC2 has also a *structural* role in the morphological maintenance of dendritic spines, one that is independent of its ability to transport ions. The intimate temporal coincidence between the developmental onset of KCC2 expression and the most intense phase of synaptogenesis during the brain growth spurt points to a possible role for this protein in synapse formation. Importantly, whether KCC2 plays a role in spinogenesis i.e. in induction of spines during the brain growth spurt has not been investigated so far. The results of the present work demonstrate that expression of KCC2 is not only a necessary but also a *sufficient* condition for the induction of functional glutamatergic spines during the brain growth spurt.

The results of this work support the idea of KCC2 as an important synchronizing factor in the functional development of glutamatergic and GABAergic signaling.

1 INTRODUCTION

Recently, there has been an increasing interest in the indirect modulation of GABAergic responses in seizure disorders with a therapeutic aim based on a strategy of targeting the plasmalemmal *ion transporters* responsible for the generation and maintenance of the ion gradients that drive $GABA_AR$-mediated currents (Löscher et al., 2013). This is motivated by the fact that short- and long-term changes in the functional properties of ion transporters have a major influence on GABAergic signaling during neuronal maturation and also following trauma and seizures (Fig. 1; Payne et al., 2003; Blaesse et al., 2009; Deeb et al., 2012; Löscher et al., 2013). Notably, standard antiepileptic drugs (AEDs), such as phenobarbital and benzodiazepines, which enhance GABAergic transmission by directly targeting $GABA_ARs$ are less effective in suppressing seizures in neonates than in adults (Painter et al., 1999; Booth and Evans, 2004), and may lose their efficacy in parallel with seizure progression in adults as well (Wasterlain et al., 2009). The reasons for either of these are not fully understood.

Immature neurons show low functional expression of the main neuron-specific Cl^- extruder KCC2 which is associated with depolarizing and sometimes even excitatory actions of GABA on postsynaptic neurons. During neuronal maturation, the expression and functionality of KCC2 are progressively increased, which is the basis for the generation of classical hyperpolarizing inhibitory postsynaptic potentials (IPSPs; Rivera et al., 1999). In rodent hippocampal and neocortical neurons, the 'developmental shift' from depolarizing to hyperpolarizing $GABA_AR$-mediated responses takes place postnatally (Rivera et al., 1999; Ben-Ari et al., 2007; Blaesse et al., 2009; Fig. 1), and the lack of efficacy of drugs such as phenobarbital in human neonates has often been attributed to the lack of KCC2 (cf. e.g. Dzhala et al., 2005; Staley, 2006; Glykys et al., 2009).

In addition to its critical role as a prerequisite for the activity-suppressing action of the main inhibitory neurotransmitters GABA and glycine, KCC2 was recently demonstrated to play a vital, ion transport-independent, role in the morphological maintenance of the dendritic spine, the main target of the excitatory glutamatergic afferent in mature neurons. While genetic deficiencies of KCC2 in animals result in increased excitability (Blaesse et al., 2009), it is unclear to what extent this is attributable to the structural versus transport roles of KCC2, suggesting that, unlike specifically enhancing the transport activity of KCC2, elevating KCC2 protein levels by gene therapy might not be a useful therapeutic strategy.

2 REVIEW OF THE LITERATURE

2.1 IONIC BASIS OF $GABA_AR$- AND GLYR-MEDIATED SIGNALING

Electrical signals in the brain are essentially mediated by means of dissipation of ion transporter-generated transmembrane ion gradients following voltage- and neurotransmitter-gated opening of ion channels. The main inhibitory neurotransmitters of the CNS, GABA at supraspinal pathways and glycine in the brainstem and spinal cord, act on anion channel receptors primarily permeable to Cl^- but also to HCO_3^- (Bormann et al., 1987; Kaila and Voipio, 1987; Kaila et al., 1993; Kaila, 1994; Ikeda et al., 2004). The electrochemical gradient of a given ion species is set by the activities of plasmalemmal transport mechanisms and the gradient-dissipating conductive channels. The ion gradient is the potential energy for ion movement stored in the chemical potential of the transmembrane concentration gradient and the transmembrane voltage difference. When the chemical and electrical forces governing the net movement of ions across the cell membrane are equal and opposite, no transmembrane charge transfer by the ion species takes place. The membrane potential difference at this energy equilibrium is defined as the *equilibrium potential* E_i for the current I_i carried by the given ion (*i*) species. Increasing the membrane conductance by opening channels selectively permeable to ion species *i* drives the membrane potential V_m towards E_i. While the amount of the ionic current, $I_i = G_i(V_m - E_i)$ depends on the ionic conductance G_i and the driving force ($DF_i = V_m - E_i$), where G_i is determined by the number and properties of ion channels, and DF_i is generated by the action of plasmalemmal ion transport mechanisms. Importantly, DF_i is responsible for setting the *polarity* i.e. direction of I_i. Thus, ion transporters of excitable cells are in a position to determine both the quantitative as well as the *qualitative* consequences of ion-channel mediated signaling.

$GABA_AR$ and GlyR-mediated hyperpolarization of the membrane potential to a level more negative than the resting V_m requires the presence of an active Cl^- extrusion mechanism (Kaila, 1994). The transmembrane electrochemical Cl^- gradient i.e. the DF for $GABA_AR$- and GlyR-mediated inhibitory postsynaptic potentials (IPSPs), is generated by secondary active K-Cl cotransport (Thompson et al., 1988), which was later shown to be largely mediated by the neuron-specific K-Cl cotransporter KCC2 (Rivera et al., 1999; Hübner et al., 2001b; Zhu et al., 2005; Blaesse et al., 2009; Seja et al., 2012). KCC2 is initially expressed at very low levels in the developing central nervous system (Blaesse et al., 2009), which fully accounts for the *lack of hyperpolarizing responses* to GABA observed in immature neurons which do not express KCC2 or other Cl^- extruding mechanisms (Fig. 1; Rivera et al., 1999; Blaesse et

al., 2009). It is obvious, however, that the presence of additional mechanisms, such as Na-K-2Cl cotransport mediated by NKCC1 (see section 2.2), which actively accumulate Cl^-, are required for a Cl^--dependent *depolarization* of the membrane potential following $GABA_AR$ or GlyR activation (Fig. 1).

$GABA_AR$-mediated depolarization is, however, not always an indication of active Cl^- accumulation because $GABA_ARs$ are not solely permeable to Cl^- (Kaila, 1994). The term *reversal potential* E_r, often inappropriately used synonymously with the *equilibrium potential* E_i, is equal to E_i only if a channel is ideally permeable to one ion species only. Thus, the reversal potential for currents mediated by $GABA_ARs$ (E_{GABA-A}), receptor channels permeable to both Cl^- and HCO_3^-, is determined by the electrochemical gradients and by the relative permeabilities of these ions:

$$E_{GABA-A} = \frac{RT}{F} ln \frac{P_{Cl^-}[Cl^-]_{in} + P_{HCO_3^-}[HCO_3^-]_{in}}{P_{Cl^-}[Cl^-]_{out} + P_{HCO_3^-}[HCO_3^-]_{out}}$$

While the relative ionic permeability of $GABA_ARs$ is larger for Cl^- than for HCO_3^- ($P_{HCO3}^-/P_{Cl}^- \approx 0.2$-$0.3$; Kaila, 1994), it is evident from the above equation that contribution of HCO_3^- to the value of E_{GABA-A} can be substantial in neurons with low $[Cl^-]_i$, typically mature neurons, and E_{GABA-A} can therefore significantly deviate from E_{Cl}^- towards the more depolarizing E_{HCO3}^- (see Farrant and Kaila, 2007), especially during intense $GABA_AR$ activation leads to a HCO_3^--dependent intracellular accumulation of Cl^- (Kaila, 1994; Viitanen et al., 2010). In contrast, the contribution of HCO_3^- to E_{GABA-A} is minimal in neurons with high $[Cl^-]_i$, such as immature neurons which often lack the capacity to extrude Cl^-.

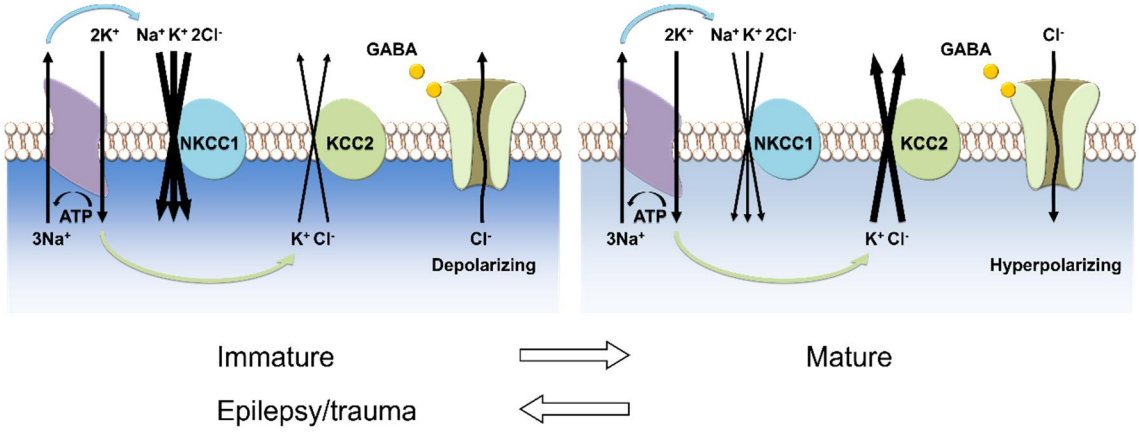

Figure 1. A shift from depolarizing to hyperpolarizing $GABA_A$ receptor ($GABA_AR$)-mediated Cl^- currents takes place during neuronal development, and an opposite effect is often seen following epilepsy and trauma. In cortical and hippocampal neurons, the Na-K-2Cl cotransporter isoform 1 (NKCC1) mediates Cl^- uptake, while the K-Cl cotransporter isoform 2 (KCC2) extrudes Cl^-. The energy for both of these electrically neutral ion-transport processes is derived from the ion gradients generated by the Na-K ATPase. NKCC1 is driven by the Na^+ concentration gradient and KCC2 by the K^+ gradient. In immature neurons (left) $GABA_AR$-mediated Cl^- currents are depolarizing. During neuronal development, up-regulation of KCC2 (rightward arrow) renders $GABA_AR$-mediated Cl^- currents hyperpolarizing (right). Exposure of neurons to recurrent seizures and other traumatic insults can lead to down-regulation of KCC2 and to a re-establishment of NKCC1-dependent depolarizing GABAergic signaling.

2.2 CATION-CHLORIDE COTRANSPORTERS

KCC2 is a member of the SLC12A electroneutral cation-chloride cotransporter (CCC) family, which in turn is part of the solute carrier (SLC) superfamily comprising ~300 transporter proteins (Hediger et al., 2004). SLC transporters are expressed in the plasma membrane and in the membranes of intracellular compartments of virtually all cells and organelles, where they control uptake or efflux of sugars, amino acids (aa), nucleotides, ions, and drugs. Active translocation of solutes across cell membranes is mediated by primary active adenosine triphosphate (ATP)-driven transporters, such as the Na-K ATPase, and secondary active transporters that couple the energetically favorable 'downhill' solute electrochemical gradients, generated by primary active transporters, to the transport of other solute species 'uphill' across cell membranes. The mammalian SLC12A family in humans and rats is encoded by the genes *SLC12A1*-9/*Slc12a1-9*. The gene products of *SLC12A1-7* are secondary-active electroneutral cation-Cl symporters that couple the energy stored in the Na^+ and/or K^+ electrochemical transmembrane gradients generated by the Na-K ATPase to actively transport Cl^- across the plasma membrane (Blaesse et al., 2009; Gagnon and Delpire, 2013). CCCs are 1000-1200 aa glycoproteins of 120-200 kDa, and their

predicted structure, so far confirmed only for SLC12A2 (Gerelsaikhan and Turner, 2000; Payne, 2012), comprises a short N-terminal and a long C-terminal domain, 12 transmembrane spanning domains, and an extracellular loop containing putative glycosylation sites (Gamba, 2005; Arroyo et al., 2013). Based on both their transport properties and amino acid sequences the CCCs can be divided in two groups (Gagnon and Delpire, 2013), (i) the Na^+-dependent Cl^- importers and (ii) the K^+-dependent Cl^- extruders. Na-K-2Cl cotransporter isoforms 1 and 2 (NKCC1; *SLC12A2* and NKCC2; *SLC12A1*) and the Na-Cl cotransporter (NCC; *SLC12A3*) exploit the inwardly-directed Na^+ electrochemical gradient to import Cl^- into cells. K-Cl cotransporter isoforms 1-4 (KCC1-4; *SLC12A4-7*) use the outwardly-directed K^+ gradient to extrude Cl^- from the cell interior.

Because the CCCs transfer an equal number of cations and anions per transport cycle, their operation does not result in net charge movement across the plasma membrane rendering them electroneutral. In contrast, the $3Na^+/2K^+$ antiport stoichiometry of the Na-K ATPase is *electrogenic* and gives rise to an outward current. The electroneutral mode of CCC is particularly important in the context of excitable cells, such as muscle and nerve, as it enables regulatory control over intracellular anion activity and cell volume without affecting the membrane potential (Payne, 2012). CCCs are expressed in all organ systems and are critical for a wide range of physiological processes, including cell volume regulation, transepithelial transport of solute and water, blood pressure regulation, and regulation of intraneuronal Cl^- concentration (Hebert et al., 2004; Gamba, 2005; Blaesse et al., 2009; Arroyo et al., 2013; Gagnon and Delpire, 2013). Disease-related alterations in CCC-functionality have been implicated as part of several etiologically heterogeneous diseases, including arterial hypertension, osteoporosis, cancer, and epilepsy (Gamba, 2005). Pharmacological antagonists of some of the CCCs are among the most commonly used drugs in medicine (Bartholow, 2012) and they, most notably, include the loop and thiazide diuretics, which are indicated for a variety of fluid-balance disturbance-related conditions, such as hypertension, glaucoma, and edema (Sarafidis et al., 2010; Pacifici, 2012). In addition to their clinical use, the loop diuretics furosemide and bumetanide have proven to be valuable tools for research on KCCs and NKCCs. Bumetanide at low micromolar concentrations can be used to selectively inhibit NKCCs, while bumetanide and furosemide concentrations in the millimolar range non-selectively antagonize both K-Cl and Na-K-2Cl cotransport (Adragna et al., 2004; Löscher et al., 2013). A major limitation in studies on K-Cl cotransport has been the absence of selective inhibitors (Adragna et al., 2004). Recent high throughput screening of potential drug candidates has identified small-molecule inhibitors for the neuron-specific KCC isoform (Delpire et

al., 2009; Lindsley et al., 2010), however, characterization of the off-target actions of such molecules is still in progress (Delpire et al., 2012).

The clinical relevance of CCCs was highlighted following the identification of a number of mutated *SLC12A* genes in several human Mendelian diseases (Gamba, 2005; Arroyo et al., 2013). These include Bartter syndrome type I and Gitelman syndrome, both of which are characterized by low blood pressure due to renal failure and hypokalemic alkalosis, involving mutations in NKCC2 and NCC, respectively (Simon et al., 1996a; Simon et al., 1996b); as well as Andermann syndrome, a severe peripheral neuropathy with agenesis of the corpus callossum that involves mutations in KCC3 (Howard et al., 2002; Rudnik-Schoneborn et al., 2009). Although no human diseases have so far been directly linked to mutations in either NKCC1 or KCCs other than KCC3 (Rudnik-Schoneborn et al., 2009), phenotypes of mice with full or partial genetic disruptions in CCCs (for review, see Blaesse et al., 2009; Gagnon and Delpire, 2013) exhibit inner ear dysfunction (NKCC1, KCC3, KCC4; Delpire et al., 1999; Flagella et al., 1999; Howard et al., 2002; Boettger et al., 2002; Boettger et al., 2003), blood pressure regulation impairments (NKCC1, KCC3; Flagella et al., 1999; Rust et al., 2006), as well as reduced threshold for seizure generation (KCC2; KCC3; Woo et al., 2002 Boettger et al., 2003), and generalized seizures (KCC2 [splice variant KCC2b; see below]; Woo et al., 2002).

Apart from NCC and NKCC2 which are predominantly expressed in the kidney (Gamba et al., 1994; Mastroianni et al., 1996) all other CCCs are expressed at some stage in mammalian CNS development (Fig. 2; Blaesse et al., 2009; Arroyo et al., 2013). The ubiquitously expressed NKCC1 is present neurons of rodents (Delpire et al., 1994; Plotkin et al., 1997a; Kanaka et al., 2001; Li et al., 2002) and humans (Munoz et al., 2007; Hyde et al., 2011). Descriptions of the developmental expression patterns of NKCC1 in the CNS have been controversial. Plotkin et al. (1997b) first reported that a developmental peak in NKCC1 expression is reached in the rat forebrain around the first postnatal week, and down-regulation of NKCC1 mRNA and protein takes place thereafter (see also Wang et al., 2002). Such data is also in line with a reported shift from predominantly neuronal to largely glial expression of NKCC1 mRNA during CNS development in mouse (Hübner et al., 2001a), although, curiously, no NKCC1 *protein* was detected in glial cells by Plotkin et al. (1997a). In contrast, no developmental down-regulation of NKCC1 mRNA was observed by Clayton et al. (1998) in the rat cortex. The authors suggested that the loss of NKCC1 expression observed by Plotkin et al. (1997b) may actually reflect changes in the alternative splicing and not expression of NKCC1, as the region of NKCC1 mRNA and protein detected by the oligonucleotide probes and antibodies used by Plotkin et al. (1997b) was found to participate in alternative splic-

ing (see Randall et al., 1997). In the human CNS, no down-regulation, but rather progressive up-regulation of NKCC1 mRNA across the entire lifespan has been demonstrated (Hyde et al., 2011; see also Szabadics et al., 2006; Munoz et al., 2007; Fig. 2). Such data is not, however, sufficient to yield information regarding the functional roles of NKCC1, as the subcellular expression pattern of NKCC1 seems to determine its physiological actions (cf. O'Donnell et al., 2004; Khirug et al., 2008; Bos et al., 2011). However, the lack of specific NKCC1 antibodies has complicated the interpretation of immunochemical studies on the subcellular distribution of NKCC1 (Blaesse et al., 2009). For example, Marty et al. (2002), reported localization change of NKCC1 from soma to dendrites in hippocampal pyramidal neurons during postnatal life, yet later electrophysiological work on NKCC1 knockout (KO) animals pinpointed the importance of this transporter in the regulation of GABAergic signaling at the axon initial segment of neocortical and hippocampal principal neurons (Khirug et al., 2008; see also Szabadics et al., 2006).

Among the KCCs, only the expression of KCC2 is restricted to central neurons (Payne et al., 1996; Williams et al., 1999; Rivera et al., 1999; Karadsheh and Delpire, 2001; Uvarov et al., 2005) and it is also the major KCC isoform expressed in the mature rodent and human CNS (Fig. 2; Rivera et al., 1999; Boettger et al., 2003; Karadsheh et al., 2004; Blaesse et al., 2009; Seja et al., 2012). Although KCC2 is broadly expressed among neurons of the adult CNS, certain neuronal subpopulations, including the dopaminergic neurons of substantia nigra (Gulacsi et al., 2003), vasopressinergic neurons of the dorsolateral part of the paraventricular nucleus (Kanaka et al., 2001; Haam et al., 2012), of the dorsomedial part of the suprachiasmatic nucleus (Kanaka et al., 2001; Belenky et al., 2008; Belenky et al., 2010), reticular thalamic neurons (Kanaka et al., 2001; Bartho et al., 2004), neurons of the medial habenular nucleus (Kanaka et al., 2001; Wang et al., 2006; Kim and Chung, 2007), as well as the neurons of the mesencephalic trigeminal nucleus (Kanaka et al., 2001; Toyoda et al., 2005) have been reported to lack KCC2.

The ubiquitously expressed KCC1 is often considered as a ‘housekeeping’ isoform involved in cell volume regulation (Hebert et al., 2004), however, in central neurons it appears to be expressed at very low levels (Payne et al., 1996; Rivera et al., 1999; Kanaka et al., 2001; Li et al., 2002; Rust et al., 2007). Similarly, except during the embryonic phase, the CNS expression of KCC4 is limited (Karadsheh et al., 2004). The relatively high mRNA expression of both KCC1 and KCC4 during early embryonic development in the rodent (Li et al., 2002) and human (Fig. 2) CNS is an intriguing observation that prompts future investigation. KCC3 is alternatively spliced producing three variants. Of these, KCC3a and KCC3c are expressed in glia and neurons, respectively,

while KCC3b is found outside the CNS (Le Rouzic et al., 2006; Blaesse et al., 2009).

In the rodent CNS the expression of both KCC2 and KCC3 is up-regulated during CNS development (Rivera et al., 1999; Pearson et al., 2001; Blaesse et al., 2009). Although up-regulation in the expression of both KCC2 and KCC3 temporally coincides with the emergence of hyperpolarizing $GABA_AR$ and GlyR-mediated responses (Ben-Ari et al., 2007; Blaesse et al., 2009), functional up-regulation of KCC2 appears both necessary (Rivera et al., 1999) and sufficient (Lee et al., 2005) to account for the observed qualitative change in GABAergic signaling in principal neurons. Indeed, based on a recent knockdown study, performed in cerebellar Purkinje and granule cells, the contribution of KCC3 to the total neuronal Cl^- extrusion capacity is small compared to KCC2 (Seja et al., 2012; see also Boettger et al., 2003). The relatively higher expression of KCC2 compared to KCC3 is also likely to be explained by the striking finding that KCC2, not KCC3, is required for the morphological maturation of cortical dendritic spines, in a manner that is independent of its ability to transport ions (Li et al., 2007; see section 2.3.1).

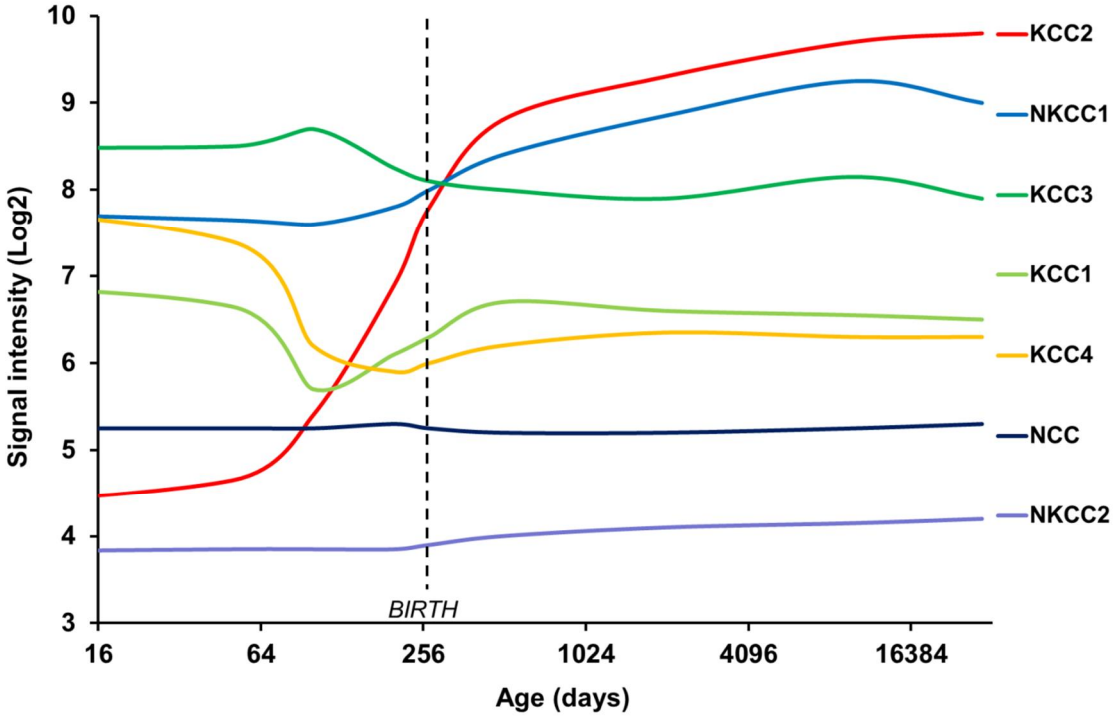

Figure 2. Expression profiles of the SLC12A1-7 gene products in the human neocortex. Average fits of log2-transformed exon array signal intensity data from the Human Brain Transcriptome data bank (www.hbatlas.org; cf. Kang et al., 2011). Broken vertical line denotes approximate time of birth.

2.3 KCC2

KCC2 is a glycoprotein with a predicted topology of 12 transmembrane domains, an N-glycosylated (where N is amino acid asparagine) extracellular domain between the 5th and the 6th transmembrane domains, and two intracellular domains, a smaller (~100 aa) N-terminal domain (NTD) and a larger (~480 aa) C-terminal domain (CTD), which flank the transmembrane domains and together account approximately for half of the size of the KCC2 molecule (1116 aa; Fig. 3). Deletion of the NTD (aa 1-100; KCC2 ΔNTD) renders KCC2 transport-inactive in neurons and HEK-293 cells, while at least the latter are able to express the ΔNTD variant at the cell membrane (Li et al., 2007). Similarly, the CTD is necessary for the K-Cl cotransport function (Mercado et al., 2006; Acton et al., 2012). Especially the latter of the two terminal domains has been identified as critical cytoplasmic target for post-translational regulation of KCC2. The majority of the phosphorylation sites predicted for KCC2 appear in its CTD (Song et al., 2002; Chamma et al., 2012) and several of these residues have been implicated in regulatory phosphorylation of KCC2 during development and in response to neuronal activity (Chamma et al., 2012; Kahle et al., 2013).

The mammalian KCC2 gene is N-terminally spliced to produce two neuron-specific isoforms, KCC2a and KCC2b, with comparable co-transport properties (Uvarov et al., 2007). KCC2b has been established as the major isoform contributing to almost 90% of total KCC2 protein in the adult murine cortex (Uvarov et al., 2007; Uvarov et al., 2009). While the expression of KCC2a remains relatively low throughout development, the expression of KCC2b is strongly up-regulated during postnatal life (Stein et al., 2004; Uvarov et al., 2007; Uvarov et al., 2009). A particularly high increase in KCC2b expression has been observed in mouse hippocampal and cortical regions, where KCC2b mRNA is up-regulated 10- and 35-fold, respectively, between embryonic day (E) 17 and postnatal day (P) 14 (Uvarov et al., 2007). The specific disruption of KCC2b leads to a seizure phenotype, however such mice are viable until the third postnatal week (Woo et al., 2002; Uvarov et al., 2007; cf. Blaesse et al., 2009). Thus, the genetic disruption of KCC2-mediated neuronal Cl^- extrusion is not lethal at birth as was originally thought (Hübner et al., 2001b).

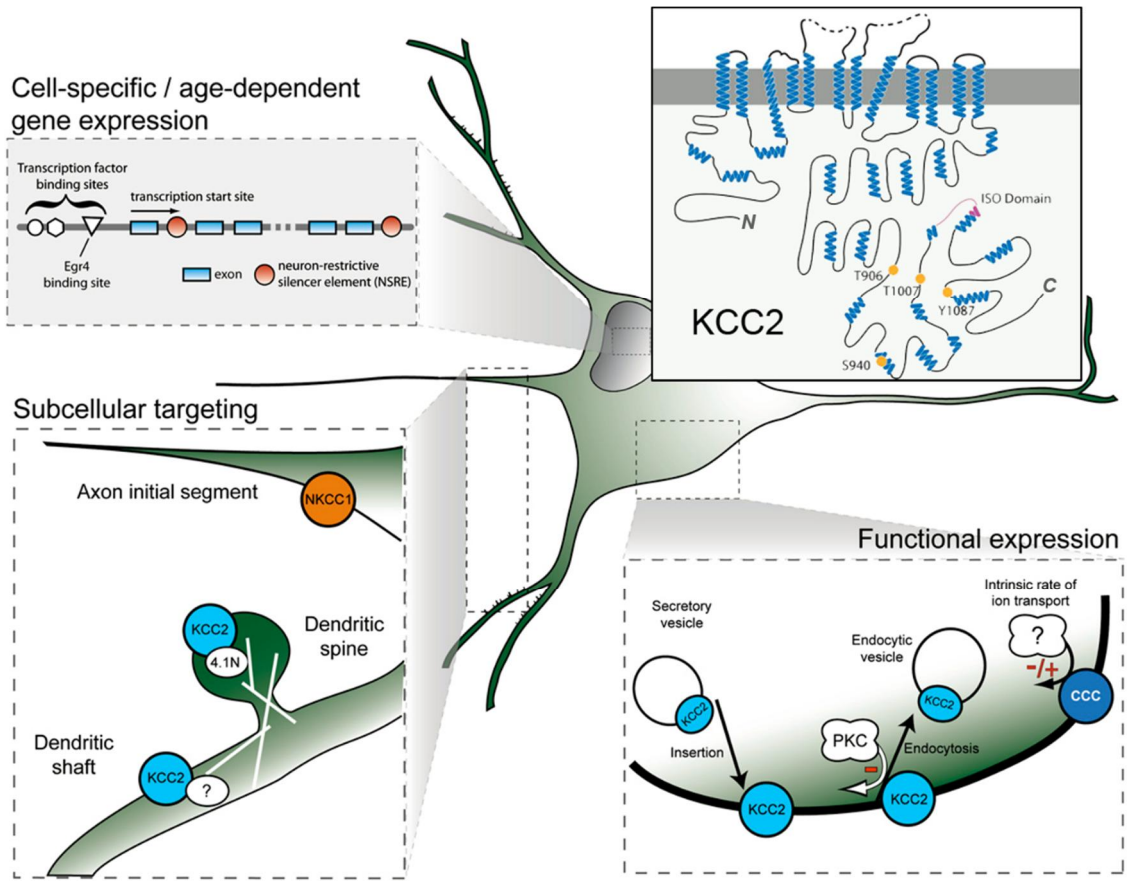

Figure 3. KCC2 functions are regulated by transcriptional control, subcellular targeting, and post-translational modifications such as protein phosphorylation. ***Upper left*** Transcriptional control of KCC2 expression in central neurons is mediated by several, most likely redundant, regulatory mechanisms. ***Lower left*** While protein 4.1N is involved in the anchoring of spine KCC2 to the cytoskeleton, it is not clear whether this is true for KCC2 located in dendritic shafts and neuronal somata (indicated by a question mark). The mechanisms whereby KCC2 is excluded from the axon and NKCC1 targeted to the axon initial segment are unclear. ***Lower right*** Kinases and phosphatases acting on the phosphorylatable residues of KCC2 (see inset upper right; e.g. PKC acting on S940) can modify KCC2 function by influencing the relative rates of endo- and exocytosis. The lack of evidence (indicated by a question mark) for direct modulation of the intrinsic rate of ion transport of neuronal CCCs is highlighted. Modified with permission from Blaesse et al. (2009). Inset with KCC2 diagram (and notable putative phosphorylation sites and the "ISO" domain) adapted, with permission, from Kahle et al. (2013).

2.3.1 FUNCTIONS OF KCC2

During neuronal development, depolarizing GABAergic signaling promotes the opening of voltage-gated Ca^{2+} channels, activation of NMDARs and sometimes the firing of action potentials. The resulting transient elevations in $[Ca^{2+}]_i$ and activation of downstream signaling cascades underlie the trophic effects of depolarizing GABAergic signaling during development (Ben-Ari et al., 2007). These effects have been observed *in vitro* at numerous levels of neuronal and network development, ranging from synthesis of DNA to neuronal proliferation, migration, and morphological maturation of neurons and synapses (Represa and Ben Ari, 2005; Blaesse et al., 2009). The loss of depolarizing and acquisition of hyperpolarizing $GABA_AR$-mediated signaling through functional up-regulation of KCC2 is believed to bring to an end the trophic effects of depolarizing GABA (cf. Akerman and Cline, 2006; Cancedda et al., 2007; Bortone and Polleux, 2009).

The functions of KCC2 in neurons have been studied using *in vitro* and *in vivo* models where KCC2 has been either disrupted or overexpressed. Such work has linked KCC2 to formation and function of GABAergic and glutamatergic synapses (for review, see Blaesse et al., 2009; Chamma et al., 2012). Original work using antisense oligonucleotide knockdown of KCC2, first defined the causal role for KCC2 in the generation of the driving force for hyperpolarizing actions of GABA (Rivera et al., 1999). Later, a second *structural* function, one that is independent of ion transport, was demonstrated for KCC2 using transport-inactive variants of KCC2 in the morphogenesis of glutamatergic synapses (Li et al., 2007; see also Horn et al., 2010). Consequently, in light of this recent finding, many effects concluded in an *a priori* manner to arise from changes in KCC2-mediated Cl^- extrusion may in fact partly or fully be accounted for by the structural roles of KCC2. For instance, the KCC2-C568A mutant used by Cancedda et al. (2007) and Reynolds et al. (2008) to support the idea that premature expression of KCC2-mediated Cl^- extrusion disrupts neuronal development, was later found to be unable not only of ion transport (Reynolds et al., 2008; Puskarjov et al., unpublished) but *also* of interactions with the actin cytoskeleton (Horn et al., 2010). Thus, in attempts to infer the roles of this multifunctional protein in CNS physiology and disease states, it is imperative to consider both the contribution of ion transport as wells as other functions of KCC2 that may be unrelated to K-Cl cotransport.

Neuronal K-Cl cotransport

K-Cl cotransport was initially identified in red blood cells (Kregenow, 1971; Dunham et al., 1980; Lauf and Theg, 1980), where (and as later discovered in most other cells of the body) it is activated by hypotonic cell swelling and mediates regulatory volume decrease through an efflux of K^+, Cl^- and osmoti-

cally obliged water. Compared to the other KCC isoforms, which are exclusively swelling activated, the neuron-specific isoform KCC2 is unique as it is capable of constitutive K-Cl cotransport under isotonic conditions (Payne, 1997; Song et al., 2002; Gamba, 2005; Mercado et al., 2006). This important feature of KCC2 has been pinpointed to a unique stretch of amino acids termed the "ISO domain" and located in the distal C-terminus of the transporter (Fig. 3; Mercado et al., 2006). Deletion of the KCC2 ISO domain in neurons leads to loss of Cl^- extrusion under isotonic conditions whilst apparently sparing that induced by experimentally-induced hypotonic shock (Acton et al., 2012). However, physiologically-induced swelling of neurons results from activity-dependent ionic loads, not from hypotonic stress (Gulyas et al., 2001; Payne et al., 2003). Thus, massive synaptic activity and excitotoxic conditions are thought to lead to neuronal swelling caused by an enhanced cellular ionic influx which is accompanied by the net movement of water (Choi, 1987; Allen et al., 2004). In contrast, under hypotonic conditions, the intracellular solute level is reduced (Basavappa and Ellory, 1996), and under these conditions the volume of glial but not of neuronal cells is likely to be affected, due to the apparent lack of aquaporins in neurons (Amiry-Moghaddam and Ottersen, 2003). The abundant expression of KCC2 near excitatory synapses in hippocampal neurons has been proposed to limit dendritic swelling in response to intense glutamatergic signaling (Gulyas et al., 2001). However, experimental tests of this hypothesis are lacking. Nonetheless, the exclusive ability of KCC2 among the KCCs, to extrude Cl^- under isotonic conditions (Gamba, 2005), supports the conclusion that it is the major functional KCC isoform in neurons. The salient developmental expression profile of KCC2b, together with the fact that GABAergic responses in cultured cortical neurons from KCC2b-specific KO mice remain depolarizing (Zhu et al., 2005), indicates that the KCC2b isoform is responsible for the shift from depolarizing to hyperpolarizing $GABA_AR$-mediated responses during development (Blaesse et al., 2009).

Regarding the role of ion transport in controlling the efficacy of inhibition, the determining factor is the capability of KCC2 to maintain the $GABA_A$ driving force ($DF_{GABA_A} = V_m - E_{GABA\text{-}A}$) at a sufficiently negative level to prevent the neuron from firing action potentials (Farrant and Kaila, 2007). The above does not necessarily stipulate hyperpolarizing levels, as an $E_{GABA\text{-}A}$ level that is close to resting V_m or even slightly depolarizing does not imply an absence of an inhibitory GABAergic action. This is because the opening of $GABA_ARs$ leads not only to a change in V_m towards $E_{GABA\text{-}A}$, but also to *shunting* of excitatory postsynaptic potentials (EPSPs), i.e. to a decrease in membrane resistance and a consequent decrease in the efficacy of EPSPs to sum up in space and in time and to reach the action potential threshold (Farrant and Kaila, 2007;

Bartos et al., 2007). An important distinction between voltage inhibition and shunting inhibition lies in the fact that the latter is local and lasts only for the duration of the change in synaptic conductance i.e. as long as the $GABA_A$Rs reside in an open and conductive state. In contrast, hyperpolarizing or depolarizing synaptic potentials outlast the conductance change that generates them, and their spread in space and time is determined by passive membrane properties and voltage-gated channels (Farrant and Kaila, 2007).

The electroneutral transport mode of CCCs obviously precludes direct electrophysiological monitoring of their transport function in a manner used to assess electrogenic transporters. A notable example of such is the Na-K ATPase, the transport cycle of which generates an outward transmembrane current. Nevertheless, because the reversal potential of $GABA_A$Rs and GlyRs is strongly influenced by the mechanisms regulating $[Cl^-]_i$, these receptor channels can be used as indirect read-out devices for the assessment KCC2-mediated Cl^- extrusion. Measurements of the steady state E_{GABA-A} or E_{Gly} values can, however, at best provide information about the presence or absence of Cl^- extrusion as, in the absence of a cellular Cl^- load, even an inefficient extrusion mechanism is able to maintain a hyperpolarizing E_{GABA-A} or E_{Gly} (Jarolimek et al., 1999; Blaesse et al., 2009). Techniques involving an imposed Cl^- load are thus warranted to assess the *capacity* of a neuron to extrude Cl^- (Jarolimek et al., 1999; Khirug et al., 2005; Jin et al., 2005; Zhu et al., 2005; Blaesse et al., 2009; Nardou et al., 2011b; Seja et al., 2012). The validity of this approach to assess changes in KCC2 function was well demonstrated by Prince and colleagues in cortical pyramidal neurons, where, in the absence of a Cl^- load, even a marked damage-induced down-regulation of KCC2 function could not be detected by recordings of the steady-state E_{GABA-A} (Jin et al., 2005).

Because neurons in active networks *in vivo* are under constant barrage of excitatory and inhibitory inputs that promotes Cl^- loading (Buzsaki et al., 2007), it is obvious that an efficient Cl^- extruding mechanism such as KCC2 is a requirement for maintaining E_{GABA-A} below action potential firing threshold in such cells. However, under conditions of strong $GABA_A$R activation, high elevations in $[Cl^-]_i$, generated by a large electrogenic uptake of Cl^- driven by efflux of HCO_3^- through $GABA_A$Rs, have been demonstrated to promote generation of depolarizing extracellular $[K^+]$ transients via KCC2 (Viitanen et al., 2010). Thus, under conditions of intense $GABA_A$R activation, such as during bursts of ictal activity, KCC2 may paradoxically act as a mediator of *excitatory* $GABA_A$R signaling (Kaila et al., 1997; Viitanen et al., 2010; see also Miles et al., 2012; Pavlov et al., 2013).

Ion transport-independent roles of KCC2

Work by Gulyas et al. (2001) first showed that a considerable part of KCC2 is associated with dendritic spine heads and bases of all hippocampal principal cells and parvalbumin-positive interneurons, with a particularly high level in the thorny excrescences of CA3 neurons. In light of the notion that the vast majority of excitatory synapses are formed on dendritic spines and most inhibitory inputs are made onto dendritic shafts rather than spines (Somogyi et al., 1998; Hering and Sheng, 2001; Yuste, 2010), the high level of a key molecule for GABAergic transmission in the vicinity of glutamatergic synapses in spines (Gulyas et al., 2001) raised the question that perhaps 'spine KCC2' may have a role that is not directly related to inhibitory signaling.

Li et al. (2007) were first to provide evidence for a role of KCC2 in the formation of excitatory synapses. Neurons from organotypic or dissociated embryonic cortical cultures from KCC2 KO ($KCC2^{-/-}$) mice exhibited elongated filopodia-like dendritic protrusions, instead of dendritic spines, and a reduced number of functional excitatory synapses. The latter was seen as a reduction in synaptic clusters, in the number of active presynaptic elements as well as in the frequency of miniature excitatory postsynaptic currents (mEPSCs; Li et al., 2007). Importantly, the authors also demonstrated that this effect was specifically attributable to the loss of KCC2 function unrelated to ion transport. Transfection of day *in vitro* (DIV) 9 $KCC2^{-/-}$ neurons with full-length KCC2 (KCC2-FL) or with an N-terminally-deleted transport-deficient KCC2 construct (KCC2-ΔNTD; deletion of aa 1-100) prevented the above effects of constitutive KCC2 disruption, observed on DIV14, while transfection with GFP or KCC3 had no effect (Li et al., 2007). Accordingly, the impaired spine maturation in $KCC2^{-/-}$ neurons was apparently not attributable to the lack of functional inhibition and consequent hyperexcitability, as culturing these neurons in the continuous presence of the voltage-gated Na^+ channel inhibitor tetrodotoxin (TTX) had no effect on the length of their dendritic protrusions (Li et al., 2007; but see also Richards et al., 2005). For corroboration of the *in vitro* data, Li et al. (2007) utilized hypomorphic heterozygous KCC2 mice ($KCC2^{hy/-}$), which express ~20% of wildtype (WT) KCC2 protein but, unlike the complete KO, they are viable (Tornberg et al., 2005). In cortical slices from $KCC2^{hy/-}$ mice the constitutively decreased KCC2 expression was associated with elongation of dendritic protrusions, albeit to a much lesser extent than that seen in $KCC2^{-/-}$ cultures (Li et al., 2007). Alterations in the *density* of dendritic protrusions were observed neither *in vitro* in cultured $KCC2^{-/-}$ cortical neurons at DIV14, nor in *ex vivo* cortical neurons from P16 $KCC2^{hy/-}$ mice (Li et al., 2007). Knockdown of KCC2 starting from the second postnatal week in cerebellar Purkinje neurons *in vivo* using Cre-mediated exon

excision (Seja et al., 2012) or from DIV14 in cultured hippocampal neurons using siRNA (Gauvain et al., 2011) had no effect on the length or density of dendritic protrusions, as seen in adult neurons from these preparations. Curiously though, in the study by Gauvain et al., an increase in the proportion of mushroom-type spines was observed (see also Khalilov et al., 2011). When KCC2 was knocked down starting from DIV4, i.e. before spine formation, a larger proportion of filopodia-type protrusions but no change in the overall density of dendritic protrusions was observed at DIV14 (Gauvain et al., 2011). Moreover, recent data by Sun et al. (2013) demonstrated that the dramatic reduction of spine density of DIV15 primary mouse cortical neurons following shRNA-mediated knockdown of the cell adhesion molecule neuroligin-2 (NL-2), could be completely prevented by co-transfecting the neurons with NL2shRNA and FL-KCC2 at DIV2. Together these studies (Li et al., 2007; Gauvain et al., 2011; Seja et al., 2012; Sun et al., 2013), might suggest that expression of KCC2 may be required for the *induction* rather than for the *maintenance* of dendritic spines. However, further studies are required to investigate whether knocking KCC2 down after spine formation in hippocampal and cortical pyramidal neurons has an effect on spine maintenance under *in vivo* conditions. While no effect of such a manipulation on dendritic spines was observed in cerebellar Purkinje neurons (Seja et al, 2012), given the likelihood of fundamental differences in the mechanisms of spine formation between pyramidal and Purkinje neurons (see Yuste and Bonhoeffer, 2004; Ethell and Pasquale, 2005), generalization of this observation to pyramidal neurons warrants caution.

Using cultured hippocampal neurons Gauvain et al. (2011) also found that chronic suppression of KCC2 expression after spine formation *in vitro* was associated with a reduction in mEPSC amplitude paralleled by increased lateral diffusion of the AMPA receptor (AMPAR) GluR1 subunits in spines. The authors suggested that this effect was attributable to an ion transport-independent function of KCC2, as increased lateral diffusion of AMPARs was observed also after dominant-negative expression of the C-terminal domain of KCC2 (KCC2-CTD; aa 637-1116), but not following incubation of neuronal cultures with a recently synthesized KCC2 cotransport inhibitor VU0244051 for longer than 72 hours (Gauvain et al., 2011). This conclusion warrants confirmation because it is largely based on the data obtained using a relatively long incubation with VU0240551 (Delpire et al., 2009), which has been reported to have significant off-targets (Lindsley et al., 2010; Delpire et al., 2012), including inhibition of L-type Ca^{2+} channels (LTCCs). It is by no means excluded that such off-target effects may play a role in the present context, as LTCCs are expressed hippocampal pyramidal neurons and actively partake in plasticity of dendritic spines

(cf. Matus, 2000; Obermair et al., 2004; Oertner and Matus, 2005; Nakata and Nakamura, 2007; Di Biase et al., 2011). One potential approach to assess the non-dependence of the effect of lateral diffusion of AMPARs on KCC2 transport function would be to knock down endogenous KCC2 using shRNA against an N-terminal sequence of KCC2 mRNA and attempt a rescue with an N-terminally deleted KCC2 construct, such as the KCC2-ΔNTD, which is transport-deficient but has been shown to interact with the cytoskeleton (see Li et al., 2007).

Also Li et al. (2007) reported a dominant-negative effect of KCC2-CTD overexpression in cultured cortical neurons, but this was on the length of dendritic protrusions. Transfection of WT neurons on DIV9 with KCC2-CTD led to an increase in protrusion length as observed on DIV14 that was comparable to what was observed in $KCC2^{-/-}$ neurons transfected with GFP alone (Li et al., 2007). However, no dominant-negative effect of KCC2-CTD on protrusion length was observed at DIV24 in hippocampal neurons that where transfected on DIV14 (Gauvain et al., 2011), suggesting either dependence on the cell type or a restricted time window for the effect. The dominant effect of KCC2-CTD in particular, and the morphogenic effect of KCC2 in general, have been attributed to stem from the interaction of the C-terminal domain of KCC2 with the 4.1N protein (Li et al., 2007; Horn et al., 2010), a structural protein found in neurons that binds actin and crosslinks the spectrin/actin skeleton with transmembrane proteins (Bennett and Baines, 2001; Baines et al., 2001; Denker and Barber, 2002). Interestingly, a point mutation (KCC2-C568A) located outside the C-terminal domain of KCC2 also renders KCC2 unable to bind 4.1N, to interact with the cytoskeleton (Horn et al., 2010), and to transport ions (Reynolds et al., 2008; Puskarjov et al., unpublished). It is likely that this mutation results in misfolding of KCC2 protein precluding its membrane expression and/or cytoskeletal interactions.

While the landmark study of Li et al. (2007) established a novel primary function for KCC2, the ion transport-independent role of KCC2 in synaptogenesis is not clear cut. For instance, while early overexpression of KCC2, but not of a transport-deficient mutant (KCC2-Y1087D; Strange et al., 2000), resulted in reduced amplitude and frequency of mEPSCs, in *Xenopus* tectal neurons (Akerman and Cline, 2006), overexpression of KCC2 in cultured hippocampal neurons had no effect on mEPSC frequency or amplitude or the density of glutamatergic terminals (Chudotvorova et al., 2005). In the latter study, overexpression of KCC2 was, however, reported to increase the frequency of miniature inhibitory postsynaptic currents (mIPSCs) and the density of $GABA_AR$ clusters (Chudotvorova et al., 2005). Surprisingly, a reduction in the frequency of mIPSCs but not of mEPSCs was reported in CA1 pyramidal neurons of KCC2 hypomorphic mice (Riekki et al., 2008). However, it is not

possible to assert, whether such effects of KCC2 overexpression or disruption on the properties of GABAergic synapses are a result of KCC2 functions dependent on K-Cl cotransport, on those independent of ion transport, or both. Intriguingly, an increased frequency of both spontaneous IPSCs and EPSCs in CA3 pyramidal neurons was observed in $KCC2^{-/-}$ mice already at E18.5, well before KCC2-mediated Cl^- extrusion is up-regulated in these cells (Khalilov et al., 2011). The existence of an early embryonic ion-transport independent function of KCC2 in morphological maturation of neurons is also supported by the study of Horn et al. (2010). The authors of this work employed pronuclear DNA injection of fertilized mouse oocytes with KCC2 constructs under the nestin promoter, limiting their expression to neuronal progenitors. They demonstrated that constitutive neuron-specific overexpression of KCC2-FL or of the transport-deficient KCC2-ΔNTD, but not of KCC2-C568A (a mutant incapable of both ion transport and actin-binding) severely impaired the development of neural tube- and neural crest-related structures, resulting in death of the implanted embryo at E13.5-15.5. Horn et al. (2010) also observed something that may shed light on the early ion transport-independent function of KCC2 in the early embryonic CNS. The authors demonstrated not only aberrant cytoplasmic distribution of 4.1N and actin but also impairments in neuronal differentiation and migration in the neural tube of embryos overexpressing FL-KCC2, KCC2-ΔNTD but, again, not KCC2-C568A (Horn et al., 2010; see also Wei et al., 2011). This is an intriguing finding as premature expression of the transport active KCC2-FL but not of KCC2-ΔNTD has been shown to terminate the postnatal migration of cortical interneurons (Bortone and Polleux, 2009). However, no effects on migration but rather impairments in dendritic arborization of cortical pyramidal neurons were reported following *in utero* electroporation at E17-18 of KCC2-FL, but not KCC2-C568A (Cancedda et al., 2007).

2.3.2 EXPRESSION OF KCC2

Most of the data published on the spatiotemporal expression patterns of KCC2 reflects that of both KCC2a and KCC2b, as the mRNA probes and antibodies used do not differentiate between the two spice variants. Therefore in the present Thesis "KCC2" represents both splice variants (unless otherwise stated), except when referring to the study by Stein et al. (2004) where the antibody used was KCC2b-specific, although the authors were not aware of this at the time of publication of the original study (cf. Uvarov et al., 2009).

Ontogeny of KCC2 in rodents and humans

In all of the many species studied, including humans, an almost invariant feature observed during development of the CNS, is an up-regulation of KCC2 expression. However, in keeping with the general differences in the milestones of CNS development (cf. Clancy et al., 2001; Erecinska et al., 2004b; Semple et al., 2013), also the timing of KCC2 induction appears to be species- and CNS region-specific. In the CNS of the mouse and the rat, two species most studied with this regard and the model species used in the present Thesis, KCC2 is up-regulated strictly in parallel with neuronal differentiation, with a gradual increase in the caudal-to-rostral direction of the CNS (Li et al., 2002; Wang et al., 2002; Stein et al., 2004).

In the caudal parts of the rodent CNS, such as the spinal cord and parts of the brainstem, perinatal KCC2 expression patterns are comparable to those observed in older animals (Balakrishnan et al., 2003; Stein et al., 2004; Blaesse et al., 2006; Uvarov et al., 2009). In the more rostral parts, such as the hippocampus and the neocortex, a steep up-regulation of KCC2 mRNA commences by the time of birth (Rivera et al., 1999; Wang et al., 2002; Li et al., 2002; Balakrishnan et al., 2003; Stein et al., 2004) and reaches a plateau around the third postnatal week (Rivera et al., 1999; Wang et al., 2002).

In the mouse hippocampus, no detectable KCC2 protein was observed prenatally using Western blotting (Stein et al., 2004). However, the low resolution achieved with this technique may not detect the expression of KCC2 at this stage in subpopulations of neurons (cf. e.g. Khalilov et al., 2011). Postnatally, a very low level of expression of KCC2 protein was seen in the ~P1-P4 mouse hippocampus (Stein et al., 2004; Blaesse et al., 2006; Zhu et al., 2008), and has been estimated to increase ~4-fold between P6-9 (Liu et al., 2006) and ~3-fold between ~P6-20 (Sipilä et al., 2009). Interestingly earlier expression has been reported in the CA1 than in the neighboring CA3 region (Zhu et al., 2008).

A similar picture prevails also in the rodent cerebral cortex, with very little KCC2 mRNA before birth, low but increasing levels during the first postnatal week, followed by robust up-

regulation reaching adult levels during or soon after the third postnatal week (Clayton et al., 1998; Wang et al., 2002; Shimizu-Okabe et al., 2002; Ikeda et al., 2003; Stein et al., 2004). KCC2 protein levels in the mouse neocortex appear very low perinatally up to P3-4, and increase robustly around the second postnatal week, as seen from Western blot data from the whole cortex (Stein et al., 2004; Sun et al., 2013) or immunohistochemical analysis of the somatosensory cortex (Takayama and Inoue, 2010; Kovacs et al., 2013). For example, a ~3-fold increase in KCC2 protein level was seen in Western blots of the whole mouse neocortex between P4 and P16, reaching ~65% of the level observed at P20 (Sun et al., 2013). Immunohistochemical analysis of KCC2 expression in the rat cortex during the first two postnatal weeks demonstrated presence of KCC2 already at birth in the piriform and entorhinal cortices, with gradually increasing expression in the superficial layers of the neocortex during the first postnatal week. By the end of the first week an adult-like pattern of KCC2 expression, including discrete dendritic expression, was observed in cortical cells of all neocortical and paleocortical areas (Kovacs et al., 2013). All these observations are at striking odds with a highly cited study (Dzhala et al., 2005) containing Western blot data obtained from an unspecified cortical region of the rat brain, which failed to detect any KCC2 protein prior to P11.

A default assumption in the vast majority of studies seems to be that in the rodent hippocampus, adult levels of KCC2 protein expression are reached during the third postnatal week, most commonly around P15 (Stein et al., 2004). However, this appears to be largely based on an extrapolation from a plateau in KCC2 mRNA levels beginning around ~P15 (cf. Rivera et al., 1999; Wang et al., 2002). To the best of my knowledge, the only report so far providing a direct quantitative comparison of total KCC2 protein levels at later time points in the mouse hippocampus demonstrated a ~2-fold increase in KCC2 protein levels between P15 and P30 (Uvarov et al., 2006; see also Zhu et al., 2008). Nevertheless, after P15, no further increase in KCC2-mediated Cl^- extrusion capacity of mouse CA1 pyramidal neurons (Khirug et al., 2005), or additional shifts in E_{GABA-A} or DF_{GABA-A} of rat CA3 pyramidal neurons (Tyzio et al., 2008), were observed. Such observations suggest either a higher safety factor (cf. Diamond, 2002) for total cellular KCC2 protein expression in older animals, and that part of the total KCC2 pool in these cells is not immediately contributing to Cl^- extrusion at all. Obviously, these options are not mutually exclusive.

Most of the studies on the developmental shift in E_{GABA-A} have been performed using rodents (Ben-Ari et al., 2007). The timing of the E_{GABA-A} shift in human CNS structures has not been identified. In altricious rodents such as rats and mice, the most intense phase of the developmental increase in KCC2 protein, paralleled by a hyperpolarizing

shift in E_{GABA-A}, takes place after birth during the first weeks of life (for review, see Ben-Ari et al., 2007). In contrast, in developmentally precocious species, such as the guinea pig, KCC2 mRNA up-regulation takes place already *in utero* and $GABA_AR$-mediated responses are hyperpolarizing at birth (Rivera et al., 1999). Humans are conventionally regarded as an altricious species but, in terms of CNS development, human neonates are born at a much more advanced stage compared to rats and mice, which are born at a stage of cortical development which roughly corresponds to that of the second half of human gestation (Clancy et al., 2001; Avishai-Eliner et al., 2002; Erecinska et al., 2004b; Khazipov and Luhmann, 2006; Semple et al., 2013). Analysis of several GABAergic parameters in standardized regions of the human cerebral cortex has demonstrated that the period from the second half of gestation to early infancy is a critical period for rapid development of the cortical GABAergic system (Xu et al., 2011). Notably, in human preterm babies changes in the properties of the electroencephalogram (EEG) parallel up-regulation of KCC2 mRNA, and in the healthy human newborn the salient EEG properties correspond to a developmental stage in rodents where GABAergic signaling is no longer depolarizing (Vanhatalo et al., 2005). Furthermore, a study on the macaque hippocampus *ex utero* has demonstrated that epileptiform discharges can be evoked by $GABA_AR$ antagonism by the last third of gestation (Khazipov et al., 2001), which roughly corresponds to the middle of the second trimester in humans (Clancy et al., 2001). Little KCC2 immunostaining is observed in the human neocortex before midgestation (Bayatti et al., 2008; Wang et al., 2010), and the above functional inferences as well as analyses of KCC2 expression patterns (Fig. 2; Vanhatalo et al., 2005; Bayatti et al., 2008; Hyde et al., 2011) suggest that a robust increase in KCC2 protein takes place during the second half of gestation and continues postnatally. This is in striking contrast to a study reporting strictly postnatal expression of KCC2 protein in human parietal cortex (Dzhala et al., 2005). The late postnatal expression of another key GABAergic protein, the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD65 and GAD67), has been suggested to contribute to the susceptibility of the neonatal brain to perinatal hypoxia-ischemia (Xu et al., 2011). Further translational work on this topic is obviously needed.

Influence of activity on KCC2 up-regulation

Studies on the effects of chronic blockade of glutamatergic signaling and action potentials in cultured hippocampal neurons suggest that endogenous ionotropic glutamatergic signaling and even the firing of spikes are not needed for the developmental induction of KCC2 mRNA or protein (Ganguly et al., 2001; Ludwig et al., 2003). The requirement of signals mediated by $GABA_ARs$ for up-regulation of KCC2 has been subject to

dispute. While Ganguly et al. (2001) reported a significant reduction of KCC2 mRNA by ~25% at DIV9 and by ~70% at DIV12-15 following $GABA_AR$ blockade starting at DIV3 (see also Leitch et al., 2005), a study by Ludwig et al. (2003) did not observe any effect on KCC2 protein levels when $GABA_ARs$ were blocked from DIV2 to DIV15. In support of the latter observation, chronic blockade of $GABA_ARs$ failed to prevent the developmental hyperpolarizing E_{GABA-A} shift of cultured midbrain neurons (Titz et al., 2003). Furthermore, KCC2 mRNA and protein levels remain unperturbed despite the lack of depolarizing GABAergic signaling (Pfeffer et al., 2009; Sipilä et al., 2009) and even in complete absence of GABAergic synaptic transmission (Wojcik et al., 2006), as seen in mice lacking NKCC1 or the vesicular inhibitory amino acid transporter (VIAAT), respectively. In contrast to blockade of endogenous glutamatergic and GABAergic signaling, the effects of the glutamatergic proconvulsant kainate suggest that increased neuronal activity associated with pathophysiological insults may have pronounced effects on the developmental expression of KCC2 (Galanopoulou, 2008; Briggs and Galanopoulou, 2011).

Chronic treatment of neonatal rats with nicotine has been reported to increase hippocampal expression of KCC2 and BDNF mRNA (Damborsky and Winzer-Serhan, 2012). This is an intriguing observation, as impaired up-regulation of KCC2 protein was reported in the hippocampi of KO mice deficient for the α7 subunit of the nicotinic acetylcholine receptor (nAChR) (Liu et al., 2006). However, whether there is a potential role for BDNF in this case is unclear as BDNF has been demonstrated to *inhibit* functional activation of nAChRs containing the α7 subunit (Fernandes et al., 2008). Moreover, in view of the neurotrophin hypothesis of BDNF as an instructive signal for the developmental up-regulation of KCC2 (Aguado et al., 2003; Carmona et al., 2006; Ludwig et al., 2011b), it is intriguing that general anesthetics, which are powerful modulators of not only GABAergic and glutamatergic activity (Rudolph and Antkowiak, 2004), but also of the BDNF-TrkB signaling pathway (Lu et al., 2006; Ponten et al., 2011; Popic et al., 2012), appear not to have any effect on the progression of up-regulation of the total KCC2 protein level during the brain growth spurt (Lacoh et al., 2013). However, it is of interest to note here that striking modulatory effects of these agents have been demonstrated on the development of spine density (De et al., 2009; Briner et al., 2010; 2011).

Subcellular distribution of KCC2 in pyramidal neurons

In hippocampal and neocortical pyramidal neurons, KCC2 is associated with the plasma membrane and transport vesicle membranes of somato-dendritic compartments (Gulyas et al., 2001; Baldi et al., 2010; Kovacs et al., 2013), including dendritic spines (Gulyas et al., 2001;

Baldi et al., 2010; Gauvain et al., 2011; Kovacs et al., 2013). It is absent from the axonal compartments, including the axon terminals and the initial segment (Szabadics et al., 2006; Baldi et al., 2010; see also Williams et al., 1999; Hübner et al., 2001; Bartho et al., 2004).
A somato-dendritic KCC2 concentration gradient, with relatively higher expression of immunogold-labeled KCC2 in the apical dendritic membrane and cytoplasm, was observed in dentate granule cells (DGCs) and CA1 pyramidal neurons of the rat hippocampus (Baldi et al., 2010; see also Bartho et al., 2004). A similar, dendritically declining, native somato-dendritic gradient of E_{GABA-A}, reflecting an uneven distribution of functionally active KCC2 along the cell membrane, has been reported using gramicidin-patch recordings in adult mouse DGCs and rat CA1 pyramidal neurons (Khirug et al., 2008). Along the apical dendrite itself KCC2 appears evenly distributed in DGCs (Baldi et al., 2010), but in CA1 pyramidal neurons the highest KCC2 membrane densities have been observed in the proximal part of stratum radiatum and in the stratum lacunosum moleculare, i.e. the dendritic parts which are closest and the furthest away from the soma (Gulyas et al., 2001; Baldi et al., 2010). Although no detailed comparative somato-dendritic distribution analysis has been published for cortical pyramidal neurons, the strong dendritic versus somatic KCC2 labeling in all cortical layers (Kovacs et al., 2013) and the robust somato-dendritic E_{GABA-A} gradient recorded in cortical layer 2/3 pyramidal neurons (Khirug et al., 2008) suggest that a somato-dendritic KCC2-density gradient is a feature of both hippocampal and neocortical principal neurons.

Considerable developmental and regional variations in the distribution of KCC2 between the membrane and cytosolic compartments have been observed. A gradual decrease in cytoplasmic transport vesicle-associated KCC2 paralleled by an increase in plasma membrane-bound KCC2 was reported using immunogold-labeling during early postnatal development in rat hippocampal principal cells (Gulyas et al., 2001; see also Zhang et al., 2006). However, no such effect was seen in either the entorhinal or the somatosensory cortex (Kovacs et al., 2013; see also Vale et al., 2005; Blaesse et al., 2006). On the contrary, the authors reported an increase in association of KCC2 with transport vesicles from ~20% of total immunogold-labeled KCC2, at P2, to ~40-50% by P12 in superficial cortical layers (Kovacs et al., 2013). Moreover, in deep layers of these cortical regions, more than half of all KCC2 was associated with transport vesicles at all postnatal ages studied. A recent study, implementing trypsin-mediated enzymatic 'shaving' of the cell surface for quantitative analysis of surface-expressed proteins (Tjalsma et al., 2008), reported that only ~20% of the total KCC2 protein in the P19-22 rat hippocampus is expressed in the plasma membrane (Ahmad et al., 2011). As this approach is, by definition, able to detect only proteins that are

integrated in the plasma membrane, it avoids overestimation of membrane expression inherent to immunocytochemical approaches, where proteins located near the membrane may be erroneously interpreted as surface-expressed. This notion is especially relevant in the case of KCC2, as the commonly used antibodies are directed against either epitopes in the C- or N-termini (Blaesse et al., 2006; Chamma et al., 2012), both of which are cytosolic (see above).

Studies on the ultrastructural localization of KCC2 in principal neurons suggest also differential expression profiles of KCC2 in spines of cortical and hippocampal neurons. Quantification of the spine plasma membrane-associated KCC2 in adult rat CA1 pyramidal cells from adult rats has been estimated as ~40% of that labeled by immunogold in the shaft membrane (Baldi et al., 2010). In mature cultured hippocampal neurons, the intensity of KCC2 immunostaining was reported as ~76% higher in spines compared to dendritic shafts, and as three-fold higher than that in the cytoplasm (Gauvain et al., 2011; see also Chamma et al., 2012). In contrast, according to a recent study, by P12 only ~10% and ~15% of the total KCC2 immunogold particles in a given neuron appear to be associated with spines in the rat superficial somatosensory and entorhinal cortices, respectively (Kovacs et al., 2013). Moreover, in the rat hippocampus, robust KCC2 expression within both the necks and heads of spines was demonstrated (Gulyas et al., 2001; Gauvain et al., 2011), and in cortical neurons KCC2 was observed to preferentially localize near the spine neck or even to *the spine apparatus*, but not to spine heads (Kovacs et al., 2013). The spine apparatus, which is found in a fraction of spines, consists of specialized smooth endoplasmic reticulum (ER), and because spines are not typically associated with rough ER (Yuste, 2010), a requirement for the production of membrane proteins, the reported localization of KCC2 to this structure is intriguing but warrants confirmation.

2.3.3 REGULATION OF KCC2

Transcriptional regulation of KCC2 expression

Unlike other mammalian KCCs, which are expressed widely or even ubiquitously (Becker et al., 2003; Arroyo et al., 2013) the expression of both KCC2 splice variants is largely restricted to central neurons with negligible expression in peripheral neurons and non-neuronal cells (Payne et al., 1996; Rivera et al., 1999; Williams et al., 1999; Song et al., 2002; Stein et al., 2004; Uvarov et al., 2007; 2009; Uvarov, 2010). However, the mechanisms responsible for driving expression of KCC2 in central neurons but not in other cells of the body are not clear-cut. Since the fairly recent discovery of the KCC2a isoform (Uvarov et al., 2007), no studies have yet addressed the mechanisms behind the confinement of its expression to neurons. Interestingly, KCC2a expression was recently reported also in avian cardiomyocytes (Antrobus et al., 2012), suggesting profound differences in transcriptional regulation between the two KCC2 splice variants.

For KCC2b, a potential neuron-restricting mechanism emerged after a consensus sequence for the neuron-restrictive silencer element (NRSE aka RE1) was identified in intron 1b of the genes coding for KCC2 in mouse and human (Fig. 3; Karadsheh and Delpire, 2001; Song et al., 2002). Binding of the neuron-restrictive silencing factor (NRSF aka REST) to NRSE has been shown to repress the expression of multiple neuron-specific genes in non-neuronal cells (Chong et al., 1995; Schoenherr and Anderson, 1995). Karadsheh and Delpire (2001) were the first to propose that NRSE-NRSF-mediated repression of transcription of the gene encoding for KCC2 in non-neuronal cells might underlie the neuron-restricted expression profile of KCC2. However, this view was challenged by Uvarov et al. (2005) using transgenic mice with a deletion of the NRSE sequence in a KCC2 reporter gene. While exogenous NRSF was able to down-regulate reporter KCC2 activity, it failed to derepress non-neuronal KCC2 expression. Interestingly, another NRSE site, located upstream from the transcription start site on the KCC2 gene was reported more recently (Yeo et al., 2009). It is thus possible that redundancy in the transcriptional machinery regulating KCC2 gene repression in non-neuronal cells accounts for the absence of derepression after deletion of only one NRSE site in the gene coding for KCC2. To substantiate this, studies on the effects of a dual NRSE deletion are needed.

A 1.4 kb promoter fragment upstream from the transcription start site of the KCC2 gene has been demonstrated sufficient to mediate neuron-specific KCC2 expression (Uvarov et al., 2005). This evolutionarily conserved promoter area was found to contain multiple transcription factor (TF) binding sites, including that for the TF early growth response 4 (EGR4 aka NGFI-C) of the EGR family of zinc finger TFs (Fig. 3;

Uvarov et al., 2006), whose expression can be induced by neurotrophins and neuronal activity (O'Donovan et al., 1999).

Of the neurotrophins, signaling mediated in particular by the brain-derived neurotrophic factor (BDNF) and its receptor tropomyosin-related kinase B (TrkB) (Park and Poo, 2013) has been suggested to play a role in the developmental up-regulation of KCC2 mRNA (Aguado et al., 2003; Carmona et al., 2006; Ludwig et al., 2011a; 2011b). Embryonic over-expression of BDNF (Aguado et al., 2003) and genetic deletion of TrkB (Carmona et al., 2006) were reported to increase and decrease, respectively, KCC2 mRNA levels. However, absence of TrkB signaling did not result in apparent impairment of the developmental shift in GABAergic action (Carmona et al., 2006). The similarity between KCC2 and EGR4 with respect to the developmental and neuron-specific expression patterns in the rat brain (Crosby et al., 1992; Uvarov et al., 2006), prompted the hypothesis that neuron-specific developmental up-regulation of KCC2 is driven via neurotrophin-induced expression of the transcription factor EGR4 (Uvarov et al., 2006; Ludwig et al., 2011a; 2011b). Application of BDNF or of neurturin, a member of the glial cell-derived neurotrophic factor family (Airaksinen and Saarma, 2002), to immature cultured hippocampal neurons was shown to induce extracellular signal-regulated kinase 1/2 (ERK1/2)-dependent expression of EGR4 and activation of the KCC2b promoter and to significantly increase the expression of KCC2 mRNA and protein (Ludwig et al., 2011a; 2011b). Also, injections of neurturin into hippocampi of rats at P5 led to an increase, albeit modest, in KCC2 immunostaining at P8 (Ludwig et al., 2011a). The effect of exogenous BDNF application was prevented by mutating the EGR4 site in the KCC2 promoter, rendering it unable to bind endogenous EGR4 (Ludwig et al., 2011b). Mutating the EGR4 binding site, knockdown of EGR4, or expression of a dominant negative EGR4 isoform resulted in ~25-50% decrease in KCC2 expression in neuroblastoma and dissociated cortical cultures (Uvarov et al., 2006). However, as only maximum of ~50% of total KCC2 expression was down-regulated under various conditions of diminished EGR4 signaling (Uvarov et al., 2006), transcriptional regulation of KCC2 expression is likely to be under control of additional transcription factors, e.g. upstream stimulating factors USF1-2 (cf. Markkanen et al., 2008). Up-regulation of KCC2 through developmental down-regulation microRNA-92 has been also suggested (Barbato et al., 2010). Of the EGR family members (EGR1-4) expressed during human brain development, the transcript expression especially of EGR1 robustly follows that of KCC2 (Kang et al., 2011; http://hbatlas.org/hbtd/images/wholeBrain/EGR1.pdf), while expression of EGR4 is up-regulated much less (Kang et al., 2011; http://hbatlas.org/hbtd/images/wholeBrain/EGR4.pdf). This suggests that,

unlike in the rat (Crosby et al., 1992; Uvarov et al., 2006), in humans, EGRs other than EGR4 may be important for up-regulation of KCC2 expression during brain development.

Taken together, neuron-specific expression of KCC2b during development is unlikely to rely on a single transcriptional mechanism. A redundancy in transcriptional regulation of KCC2 might serve the purpose of minimizing perturbations in KCC2 expression, which, as exemplified by constitutive KO (KCC2a and KCC2b; Hübner et al., 2001b; KCC2b; Woo et al., 2002; see also Khalilov et al., 2011) or overexpression (Reynolds et al., 2008; Horn et al., 2010) of KCC2, can have devastating effects on CNS development and perinatal survival.

Post-translational regulation of KCC2

Post-translational modifications of the KCC2 protein have been suggested to regulate its functional expression in developing and mature neurons. For example, work on the developing brainstem has demonstrated that despite similarly high expression levels of KCC2 protein in the early postnatal period and by the second month of life in the lateral superior olive (LSO; Balakrishnan et al., 2003; Blaesse et al., 2006) and the cochlear nucleus (Vale et al., 2005), the Cl^- extrusion capacity of LSO neurons emerges gradually during the first weeks of life (Balakrishnan et al., 2003; Blaesse et al., 2006). Likewise, in the retina KCC2 is expressed but largely localized in the cytosol of ganglion cells during the first two postnatal weeks and appears at or near the plasma membrane only by the third week (Zhang et al., 2006). Studies on immature cultured hippocampal neurons have shown that a considerable pool of transport active KCC2 can be recruited within minutes using broad-spectrum kinase inhibitors (Kelsch et al., 2001; Khirug et al., 2005). Examples of this kind suggest that the mere presence of KCC2 protein, even at high levels, does not automatically endow a neuron with efficient Cl^- extrusion. Thus, post-translational modifications are likely to be important in determining the overall kinetics of KCC2-mediated K-Cl cotransport by regulation of the intrinsic ion transport rates and/or the number of plasmalemmal KCC2 molecules (Blaesse et al., 2009).

Work by Kelsch et al. (2001) on cultured hippocampal neurons from rat first suggested that a kinetic activation of KCC2 by tyrosine phosphorylation is required for the increase in neuronal Cl^- extrusion capacity during development (see also Khirug et al., 2005). Along similar lines, later work by Stein and colleagues (2004), utilizing a pan-phosphotyrosine antibody, demonstrated that the amount of tyrosine-phosphorylated KCC2 increased in the mouse cortex between P3 and P30 (Stein et al., 2004). Vale et al. (2005) observed that KCC2 protein was highly expressed in both P1 and P40 neurons of the cochlear nucleus (see also Balakrishnan et al., 2003; Blaesse et al., 2006), whereas the

level of tyrosine-phosphorylated KCC2 was virtually absent at birth and became significantly higher at P40 (Vale et al., 2005). Conversely, threonine residues 906 and 1007 of KCC2 appear to be partially phosphorylated in neonatal mouse brain and dephosphorylated in parallel with brain maturation (Rinehart et al., 2009). A simultaneous substitution of both of these residues to non-phosphorylatable alanines leads to robust activation of KCC2, as seen in HEK-293 cells (Rinehart et al., 2009).

Glycosylation is an important factor in regulation of protein folding, cell surface expression, and function of membrane-expressed glycoproteins (Rasmussen, 1992; Roth, 2002). Although extracellular N-linked glycosylation has been shown to play a decisive role in the membrane expression and function of KCC3 (Ding et al., 2013), KCC4 (Weng et al., 2013) and NKCC1 (Ye et al., 2012), no data exist to establish whether this type of post-translational modification is necessary for KCC2 functions. Nonetheless, a glycosylation pattern of KCC2 of the kind observed in mature neurons does not appear to be alone sufficient for the induction of ion transporter activity (Blaesse et al., 2006; see also Hartmann et al., 2009).

Clustering of KCC2 in the cell membrane (Watanabe et al., 2009; Hartmann et al., 2009; Gauvain et al., 2011; Nardou et al., 2011b) has been suggested to involve KCC2 oligomerization and to regulate its membrane stability, activity, or both (Watanabe et al., 2009; Chamma et al., 2012; see also Blaesse et al., 2006; Hartmann et al., 2009; Uvarov et al., 2009). KCC2 clusters appear to be modulated by phosphorylation mechanisms as loss of KCC2 tyrosine phosphorylation was associated with a more diffuse membrane expression pattern and with shift in E_{GABA-A} towards more depolarized values (Watanabe et al., 2009). Intriguingly, KCC2 clustering was also reported to correlate with the maturation of dendritic spines, with maximal KCC2 clustering observed in mushroom-type spines, intermediate levels in stubby spines and low or no clustering in non-functional filopodia-like dendritic protrusions (Chamma et al., 2012). The main scaffolding protein of GABAergic synapses, gephyrin, forms clusters that are sensitive to (i) phosphorylation state of gephyrin and (ii) to the activity-dependent cleavage of gephyrin by the Ca^{2+}-dependent protease calpain (Tyagarajan et al., 2011; Tyagarajan et al., 2013). While gephyrin has been demonstrated to colocalize or juxtapose with KCC2 in hippocampal and spinal cord neurons (Hübner et al., 2001b; Chamma et al., 2012), further work is needed to assess whether KCC2 clusters functionally associate or are coregulated with gephyrin clusters. Important questions regarding KCC2 clustering are whether such clusters are modulated by neuronal activity e.g. via calpain cleavage, and whether KCC2 clustering requires an interaction of the CTD KCC2 with the cytoskeleton (cf. Li et al., 2007). Deletion of the last 28 amino

acids of the C-terminus (KCC2-Δ1089-1116) results in a membrane-expressed KCC2 protein that does not form clusters (Watanabe et al., 2009). The region 929-1043 of the KCC2 CTD, which is required for KCC2 activity under isotonic conditions, also encompasses two predicted PEST sequences which, among the KCCs, are completely unique to KCC2 (Mercado et al., 2006). This is interesting, because PEST domains can serve to target proteins for calpain-dependent degradation (Rechsteiner and Rogers, 1996; Wang et al., 2003; but see Carillo et al., 1996), although calpain-mediated cleavage of KCC2 has been also speculated to result in functional activation of the cotransporter (Mercado et al., 2004).

There is indication for a very high rate of turnover, i.e. recycling, of KCC2 at the cell membrane (Lee et al., 2007), suggesting that the transporter is subject to continuous kinetic modulation (Blaesse et al., 2009). While the surface half-life of, for instance, $GABA_AR$ subunits is greater than 30 minutes (Thomas et al., 2005), the half-life of membrane-associated KCC2 appears to be strikingly fast at ~5 minutes, as seen in HEK-293 cells (Lee et al., 2007). The work by Lee et al. (2007; 2011) identified serine 940 (S940) located in the C-terminal domain of KCC2 as the main site of direct phosphorylation of KCC2 by protein kinase C (PKC). Using HEK-293 cells, the authors further demonstrated that activation of PKC increases KCC2 cell surface stability and ion transport activity (Lee et al., 2007). Analysis of endogenous KCC2 expressed in cultured rat hippocampal neurons revealed a striking ~300% increase in KCC2 phosphorylation and surface expression within 10 minutes of PKC activation, while inhibition of PKC under basal conditions robustly decreased KCC2 phosphorylation (Lee et al., 2007). In support of that rapid changes in KCC2 membrane expression mediated by mechanisms regulating membrane trafficking, blocking clathrin-dependent endocytosis, was reported to elevate the cell surface levels of KCC2 to ~275% of control values within 45 minutes (Lee et al., 2010; see also Zhao et al., 2008). Such rapid (de)phosphorylation-controlled membrane recycling of KCC2 is likely to permit dynamic post-translational regulation of the relative amount and the intrinsic functional properties of KCC2 molecules located in the plasma membrane and cytosolic vesicles. As a striking example of this, elevation of glutamate levels in dissociated hippocampal neuronal cultures was demonstrated to trigger, through activation of NMDARs, Ca^{2+} and protein phosphatase 1 (PP1)-dependent dephosphorylation of KCC2 at S940, resulting in robust down-regulation of total and membrane-associated KCC2 as well as loss of hyperpolarizing $GABA_A$ responses (Lee et al., 2011; see also Sarkar et al., 2011). How dephosphorylation of S940 leads to degradation of KCC2 protein was not demonstrated. Curiously, as seen in cultured hippocampal neurons, the tyrosine phosphatase inhibitor sodium per-

vanadate was shown to trigger down-regulation of both the total and surface-expressed KCC2 in a manner that was sensitive to the broad-spectrum protease inhibitor leupeptin, which was used at a high concentration of 200 µg/ml (~470 µM; Lee et al., 2010). In spite of the fact that leupeptin is a well-known inhibitor of a number of lysosomal and extra-lysosomal proteases, including the Ca^{2+}-activated calpain (Goll et al., 2003), the authors concluded that the lysosomal pathway was responsible for down-regulation of KCC2 under the pertinent conditions (Lee et al., 2010). It should be noted that leupeptin at high concentrations has been reported to paradoxically result in stimulation of proteolytic activity (Sutherland and Greenbaum, 1983), which further complicates the interpretation of the results obtained under the experimental conditions employed by Lee et al. (2010).

With regard to the post-translational regulation of the structural role of KCC2, only the protein-protein interaction between the CTD of KCC2 and the FERM domain of protein 4.1N has been implicated (Li et al., 2007; Horn et al., 2010). Although NL-2, a postsynaptic adhesion molecule previously implicated in the regulation of GABAergic synaptogenesis (Chih et al., 2005), has been recently suggested to play regulatory role over the structural function of KCC2 in spine formation (see above), the lack of co-immunoprecipitation of KCC2 with NL-2 suggests that this interaction may be indirect (Sun et al., 2013).

2.3.4 THE ROLE OF KCC2 IN CNS PATHOLOGY

In the mature rodent CNS, depolarizing $GABA_AR$-mediated responses are often seen under pathological conditions associated with enhanced neuronal excitation (Cohen et al., 2002; Miles et al., 2012). Deficits in KCC2 expression, often associated with decreased efficacy of GABAergic inhibition and emergence of depolarizing $GABA_AR$-mediated currents that reflect decreased neuronal Cl^- extrusion, have been documented following experimental seizures (Rivera et al., 2002; Pathak et al., 2007; Li et al., 2008; Lee et al., 2010; Barmashenko et al., 2011; Shin et al., 2012; Reid et al., 2013), in models of cerebral ischemia (Papp et al., 2008; Jaenisch et al., 2010; Mao et al., 2012; Dai et al., 2013), traumatic brain injury (Jin et al., 2005; Bonislawski et al., 2007), and neuropathic pain following spinal cord injury or nerve ligation (Coull et al., 2003; Lu et al., 2008; Miletic and Miletic, 2008; Boulenguez et al., 2010; Janssen et al., 2012; Zhou et al., 2012). The clinical relevance of changes in KCC2 expression is also highlighted by reports of decreased levels of KCC2 protein and loss of hyperpolarizing $GABA_AR$-mediated signaling in resected epileptic tissue from human patients with temporal lobe epilepsy (TLE; Palma et al., 2006; Huberfeld et al., 2007; Munoz et al., 2007; see also Cohen et al., 2002; Deisz 2002). Down-regulation of KCC2 and up-regulation of NKCC1 has been implicated in generation of spontaneous

interictal-like activity (i.e. the epileptiform activity occurring between seizures; Huberfeld et al., 2007; Miles et al., 2012).

Under these diverse traumatic situations, KCC2 down-regulation may be related to activation of the TrkB receptor by BDNF (Rivera et al., 2002; Rivera et al., 2004; Coull et al., 2005; Wake et al., 2007; Shulga et al., 2008). Exogenously applied BDNF was shown to down-regulate KCC2 via TrkB receptors in cultured hippocampal neurons (Rivera et al., 2002; Wake et al., 2007). Similarly, following epileptiform activity induced by withdrawal of extracellular Mg^{2+}, the efficacy of Cl^- extrusion was reduced in a BDNF-dependent manner within a few hours in parallel with KCC2 down-regulation of KCC2 mRNA and protein (Rivera et al., 2004; see also Wake et al., 2007). The authors concluded that this effect was attributable to transcriptional changes in KCC2 expression levels (Rivera et al., 2004). However, the possibility that the decreased efficacy of Cl^- extrusion was caused by changes in post-transcriptional mechanisms could not be excluded. Indeed, a study by Wardle and Poo (2003) suggests that BDNF can act on KCC2 function within a time window that is too rapid to be mediated by transcriptional effects. Experiments with animals expressing loss of signaling point mutations in the TrkB receptor demonstrated that both the Shc (src homology 2 domain containing transforming protein) and PLCγ-CREB (phospholipase Cγ-cAMP response element-binding) pathways must be activated to induce down-regulation of KCC2 protein by BDNF or 0-Mg^{2+} (Rivera et al., 2004). Notably, activation of the PLCγ pathway is known to trigger elevations in $[Ca^{2+}]_i$ (Berridge et al., 2000), a necessary condition for rapid down-regulation of KCC2 following intense glutamatergic stimulation (Fiumelli et al., 2005; Lee et al., 2011). In contrast, the activation of the Shc pathway alone via the TrkB receptor enhances KCC2 synthesis under these conditions (Rivera et al., 2004). The above findings by Rivera et al. (2004) point to divergence in the actions of BDNF on regulation of KCC2 via down-stream signaling of the TrkB receptor. Thus, the explanation for how BDNF exerts opposite effects on KCC2 in immature (Aguado et al., 2003; Ludwig et al., 2011) and mature (Rivera et al., 2002; 2004; Coull et al., 2005; Wake et al., 2007; Miletic and Miletic, 2008), or in intact and damaged neurons (Shulga et al., 2008; see also Shulga et al., 2009) may lie within the regulation of molecular cascades down-stream of TrkB.

Knock-down of KCC2 was reported to decrease the resistance of cultured neurons to NMDA-toxicity, whereas overexpression of KCC2 to protect from cell death (Pellegrino et al., 2011). This effect was attributed to K-Cl cotransport function of KCC2, as also overexpression of KCC3, a KCC isoform that does not maintain spines, had a comparable rescuing effect. Moreover, overexpression of the transport-inactive KCC2-Y1087D mutant failed to ameliorate cell death (Pellegrino et al., 2011).

It has been suggested (Huberfeld et al., 2007; Miles et al., 2012) that down-regulation of KCC2 leads to a decrease in the metabolic costs of maintaining cation gradients thereby reflecting an adaptive response to the "energy crisis" (cf. Hansen, 1985) commonly accompanying ischemia and seizure activity (Aiyathurai and Boon, 1989; Berger and Garnier, 1999; Kovac et al., 2012). Such a teleological explanation is probably also valid for the down-regulation of the Na-K ATPase (cf. Pylova et al., 1989; Anderson et al., 1994; Fernandes et al., 1996). Accordingly, work performed on an in *in vitro* model of CNS ischemia has shown that the robust loss of ATP followed by partial recovery during a 3 hour-long reoxygenation period could be completely recovered using either bumetanide or furosemide at concentrations which inhibit both NKCC1 and KCC2 (Pond et al., 2004).

KCC2 in neonatal seizures

Most commonly caused by hypoxic ischemic encephalopathy, hemorrhage, or cerebral infarction, seizures affect ~2% of neonates in intensive care units in Western societies (Bartha et al., 2007; Jensen, 2009; Seshia et al., 2011). Neonatal seizures portend severe neurological dysfunction later in life, with survivors experiencing higher rates of epilepsy (Ronen et al., 2007; Pisani et al., 2012) and motor and cognitive deficits (McBride et al., 2000; Tekgul et al., 2006; Ronen et al., 2007; Painter et al., 2012). Rodent models have revealed that seizures early in development alter synaptic organization and plasticity, and prime cortical neurons to increased seizure-susceptibility later in life (Ben-Ari and Holmes, 2006; Rakhade et al., 2011). Therefore, prompt diagnosis and successful treatment of seizures in neonates is necessary for improving long-term neurologic outcomes.

Standard antiepileptic drugs (AEDs), such as phenobarbital and benzodiazepines, which enhance GABAergic transmission by directly targeting $GABA_A$Rs, are less effective in suppressing seizures in neonates than in adults (Painter et al., 1999; Booth and Evans, 2004). This is not surprising, because the signaling mechanisms and pharmacological properties of neurons in the immature brain are different from those in the adult (Clancy et al., 2001; Avishai-Eliner et al., 2002; Erecinska et al., 2004a). The idea to use bumetanide with the aim to block NKCC1 and thereby to enhance the efficacy of AEDs acting via $GABA_A$Rs, has gained conciderable attention (Dzhala et al., 2005; 2008; 2010; Kilb et al., 2007; Rheims et al., 2008; Mares, 2009; Mazarati et al., 2009; Kahle et al., 2009; Nardou et al., 2009; 2011; Minlebaev and Khazipov, 2011; Wahab et al., 2011; Cleary et al., 2013; Vargas et al., 2013). While this is a prevalent hypothesis in the context of Cl^- regulation in neonatal seizures (for review, see Briggs and Galanopoulou, 2011; Ben-Ari et al., 2012; Löscher et al., 2013; Pressler and Mangum, 2013), the possible changes in

the functional expression of KCC2 have been adressed, surprisingly, by only a few studies (Galanopoulou, 2008; Nardou et al., 2011b).

Working on an *in vitro* preparation composed of two intact interconnected P7-8 rat hippocampi perfused in a tripartite chamber, Nardou et al. (2009) reported that inhibition of NKCC1 with bumetanide did not prevent generation of kainate-induced seizures or propagation of seizures to the contralateral "drug naive" hippocampus. NKCC1 inhibition also failed to prevent the formation of an acute epileptogenic *mirror focus* in the contralateral hippocampus that had not been exposed to propagating seizures originating from the kainate-exposed hippocampus (Nardou et al., 2009). In subsequent studies, Nardou et al. (Nardou et al., 2011a; 2011b) demonstrated that the AED phenobarbital, which prolonges the open-time of $GABA_A$Rs, when applied to the contralateral hippocampus at the onset of propagating seizures, reduced the interictal-like events and prevented the formation of an epileptogenic focus. These results suggest that during the initial few seizure events, GABAergic signaling is efficient enough to prevent epileptogenesis in the rodent hippocampus already by the end of the first postnatal week, despite the still relatively immature level of Cl^- extrusion capacity at this age (Khirug et al., 2005; Tyzio et al., 2007; 2008). In the studies by Nardou et al. (2011a; 2011b), phenobarbital was rendered seizure-aggravating after a number of ictal-like events due to a progressive increase in $[Cl^-]_i$. Work by Dzhala et al. (2010) suggested that the progressive accumulation of Cl^- in P5-7 mice results from the up-regulation of NKCC1. However, Nardou et al. (2011b) showed that epileptic mirror foci are formed at this age also in NKCC1 KO mice. Importantly, the authors also demonstrated that seizures eventually lead to down-regulation of KCC2 function that, in the epileptic mirror neurons, was paralleled by internalization of KCC2 from the cell surface into the cytosol (Nardou et al., 2011b).

Three episodes of neonatal kainate-induced status epilepticus (3KA-SE), each elicited at P4-P6, were demonstrated to result in a premature appearance of hyperpolarizing $GABA_A$R-mediated signaling at P9, instead of P14 in CA1 pyramidal neurons and paralleled by increased KCC2 immunoreactivity in the CA1 region, as observed at P10 (Galanopoulou, 2008). However, these effects were reported to be specific to male rats only as similar levels of KCC2 immunoreactivity were observed in female control animals at P10 as in males who had received three daily kainate injections. Moreover, 3KA-SE at P4-6 in the female pups was associated with a transient *depolarizing* shift in E_{GABA-A} at P8-13 that was attributed to increased functional expression of NKCC1 during this period (Galanopoulou, 2008). In contrast, gender-related differences in the effects

of the $GABA_AR$ agonist isoguvacine were observed neither in the hippocampal CA3 region, the thalamus, nor the amygdala at P4-6 (Glykys et al., 2009). Regardless, the consequences of seizures on the functional expression of KCC2 in neonatal rats of either sex in the *immediate postictal period* were not assessed in the study by Galanopoulou (2008), where data was collected a minumum of ~five days after the first seizure episode. Similarly, in the study by Nardou et al. (2011b) a minimum of 5 hours had passed since the first kainate-triggered ictal event before internalization of KCC2 was assessed and observed. From the stand point of potential therapeutic interventions involving pharmacological modulation of KCC2 function, assessment of the immediate effect of seizures on KCC2 expression is of interest.

KCC2 in temporal lobe epilepsy

Mesial temporal lobe epilepsy (TLE), with seizure onset in the structures of the medial temporal lobe, notably the hippocampus, is the most common type of partial epilepsy refractory to AEDs (Semah et al., 1998; Tatum, 2012). It is also the most commonly occurring type of acquired epilepsy in adult humans (Semah et al., 1998; Wiebe, 2000; Engel, Jr., 2001). The causes of TLE are likely to be complex and patients often present with a precipitating injury, such as birth trauma, head injury, febrile seizures, and meningitis, typically taking place during early childhood (Blumcke et al., 2002). A hallmark of TLE is sclerosis of the hippocampus with neuronal loss in the regions of CA1 and CA3/4, but to a lesser extent in CA2 (Blumcke et al., 2002). It is, however, unclear whether this is a cause or a consequence of seizures (Jefferys, 1999; Blumcke et al., 2002). It is also far from clear, to which extent the processes that contribute to *epileptogenesis* (i.e. the gradual transformation of non-epileptic tissue to one spontaneously generating seizures that takes place before the first spontaneous seizure occurs) overlap with those at play during *ictogenesis*, the rapid process of initiation and propagation of a seizure in time and space (Pitkänen and Lukasiuk, 2011; Löscher et al., 2013). For instance, there is some indication that the sclerotic loss of hippocampal cells may actually hinder epileptogenesis (Milward et al., 1999). Thus, an *a priori* interpretation that any observed change in the epileptic substrate reflects pathological progression, may lead to failure in recognizing intrinsic adaptive processes.

The most effective treatment for drug-resistant TLE symptoms to date is resection of the ictogenic brain regions (Engel, Jr., 2001). This has enabled *ex vivo* studies aimed at elucidating the mechanisms of seizure generation in humans. Such work has demonstrated that the *in vitro* correlates of interictal activity may in part be attributable to depolarizing $GABA_AR$-mediated signaling of a population of subicular pyramidal neurons downstream of the sclerotic CA1 region (Cohen et al., 2002; see also

Köhling et al., 1998; Benini et al., 2011). This depolarization was later shown to be associated with a reduction of KCC2 expressing cells or down-regulation of KCC2 mRNA and protein expression in the CA1-subiculum intersection and subiculum proper (Huberfeld et al., 2007; see also Palma et al., 2006; Munoz et al., 2007). Interestingly, the observed interictal activity was dependent on NKCC1 function, as inferred by its sensitivity to low concentrations of bumetanide (Huberfeld et al., 2007). While increased NKCC1 mRNA in the subiculum has been reported (Palma et al., 2006), others have observed increased NKCC1 immunoreactivity in the CA2 region but not in the subiculum (Sen et al., 2007). While an acute damage-induced depolarizing shift in E_{GABA-A} is likely to result from post-translational modification of CCCs and down-regulation of the Na-K ATPase, the consolidation of this effect may well involve long-term genomic regulation of KCC2 expression (Löscher et al., 2013). From the above studies performed on chronically epileptic TLE tissue it is impossible to conclude to which extent the observed changes in KCC2 expression are related to the epileptogenic or the ictogenic processes, and to which extent they are a result of genomic vs post-translational regulation.

Rodent models of TLE suggests that the time course for significant changes in both KCC2 protein and mRNA expression levels may under certain conditions take place on a timescale of few hours. For example, following rapid (all stimulations within a day) hippocampal kindling, KCC2 mRNA levels in the mouse dentate gyrus (DG) decreased to ~55% by 2 hours and to ~30% by 6 hours after the last stimulation, when also a general decrease in KCC2 immunostaining was observed in the CA1, CA3 and DG regions. By 24 hours after kindling, partial recovery of KCC2 mRNA and immunostaining was observed (Rivera et al., 2002). Rapid decrease of total and plasmalemmal KCC2 protein in the time course of 1-2 hours has been reported in the mouse hippocampus following status epilepticus (SE) induced by the muscarinic acetylcholine receptor (mAChR) agonist pilocarpine. This rapid effect observed *in vivo* correlated with increased tyrosine phosphorylation and was suggested to be induced by increased degradation of KCC2 (Lee et al., 2010; see also Takkala and Woodin, 2013). Others using the pilocarpine model in rats, have reported decreased levels of KCC2 mRNA and protein, paralleled by positive E_{GABA-A} shifts in the CA1, CA3, DG, and the subiculum for up to two weeks post-SE during the latent period (Pathak et al., 2007; Barmashenko et al., 2011). At least in the DG, by the time spontaneous seizures begin to appear (2-8 weeks after SE), KCC2 total protein levels recover and the capacity of DGCs to extrude Cl^- substantially improves (Pathak et al., 2007). An important question to be adressed by future studies, is whether the initial acute loss of DGC dendritic spines followed by neo-spinogenesis, which

starts approximately two weeks after pilocarpine-induced SE (Isokawa, 1998; Isokawa, 2000; see also Thind et al., 2010), involves specific changes in the expression of ion transport-independent KCC2 functions. It appears that expression of KCC2 after SE displays regional variation as, unlike the apparent recovery of KCC2 expression in DG (Pathak et al., 2007; see also Rivera et al., 2002), significantly decreased KCC2 immunoreactivity in the perirhinal cortex is observed upto 4-5 months following pilocarpine SE (Benini et al., 2011).

Disease-associated changes in the expression of CCCs leading to reemergence of depolarizing or even excitatory $GABA_AR$-mediated signaling, typical to immature neurons, have been proposed to reflect neuronal dedifferentiation and 'recapitulation of a developmental programme' (Cohen et al., 2003). Teleologically, this might serve the purpose of re-establishing plasticity and promote *de novo* targeting of neurons during damage-related rewiring (Nabekura et al., 2002; Cohen et al., 2003; Payne et al., 2003; Toyoda et al., 2003; Rivera et al., 2005). Importanly, the timing of the critical period for plasticity, at least in the visual cortex, appears to depend on the level of GABAergic activity (Hensch and Fagiolini, 2005; Hensch, 2005a; Hensch, 2005b). In support, intriguing correlations between reactivation of critical periods for neural plasticity (Hensch, 2005a) and decreased levels of GABA in the visual cortex (Arckens et al., 2000) and of KCC2 protein in the CA1 and the basolateral amygdala (Karpova et al., 2011) have been reported.

Down-regulation of KCC2 may also prevent the generation of the large extracellular K^+ transients during intense activation $GABA_ARs$, e.g. during seizures or any type of high frequency stimulation of neurons, leading to substantial electrogenic uptake of Cl^- driven by efflux of HCO_3^- which is replenished by carbonic anhydrase-catalyzed hydration of CO_2 (Kaila et al., 1997; Ruusuvuori and Kaila, 2013). Recent work has shown that the HCO_3^--driven intraneuronal accumulation of Cl^- can activate extrusion of Cl^- by KCC2 (Viitanen et al., 2010), thereby giving rise to a K^+ efflux that accounts for the increase in $[K^+]_o$ which results in non-synaptic depolarization of the membrane potential. Such a pathophysiological increase in $[K^+]_o$ can lead to a vicious cycle comprising a further depolarization of both the membrane potential and E_{GABA-A}, and to cellular swelling that enhances proepileptic ephaptic signaling (Jefferys, 1995; Somjen, 2002; Miles et al., 2012; Löscher et al., 2013). Taken together, the above conciderations underscore the fact that it is by no means a trivial question whether, and when, pathophysiological changes in CCC expression and function are causes of epileptogenesis, or adaptive, seizure suppressing consequences of epilepsy.

3 AIMS

The major aim of this Thesis was to investigate the effects of glutamatergic signaling on KCC2 expression, and *vice versa*, during brain development.

The specific aims were to:

- Investigate the effects and underlying mechanisms of neonatal seizures on KCC2 expression during the immediate postictal period (I and II)

- Identify the proximal mechanisms responsible for rapid down-regulation of KCC2 under conditions of enhanced glutamatergic activity (III)

- Assess the functional role of KCC2 in spinogenesis *in vivo* (IV)

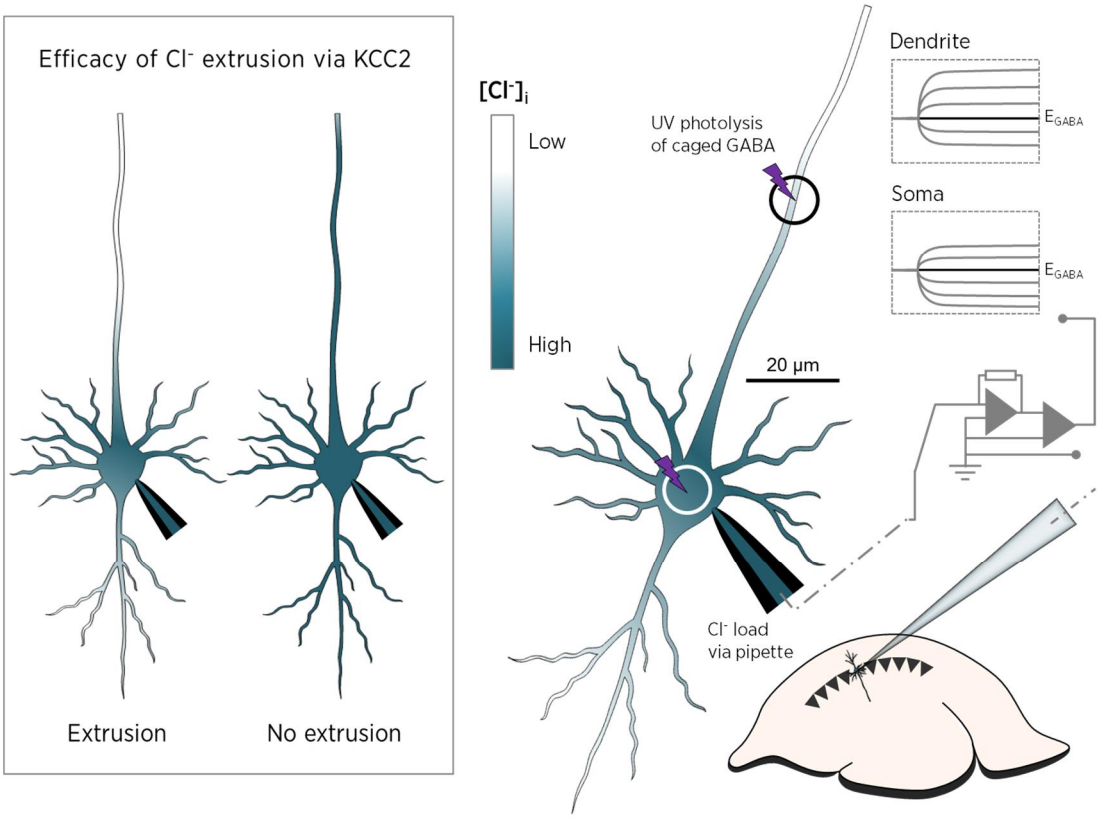

Figure 4. An electrophysiological method to quantitatively assess the efficacy of KCC2-mediated Cl^- extrusion in pyramidal neurons. A defined Cl^- load was imposed via a patch pipette to clamp the somatic Cl^- concentration ($[Cl^-]$) to that of the filling solution of the patch pipette. As a result of net dendritic Cl^- extrusion by KCC2, a declining somato-dendritic $[Cl^-]$ gradient is formed from the soma along the dendrite (indicated as lightening of turquoise from the soma to the distal dendritic parts). A spot of UV light was positioned consecutively at the somatic and the dendritic location for local GABA uncaging to evoke $GABA_AR$-mediated currents for determination of their reversal potential in the voltage clamp mode of whole-cell patch clamp recordings. Black and white circles indicate the diameters and the relative locations of the 10 ms-long uncaging flashes. Dendritic E_{GABA-A} more negative than the somatic E_{GABA-A} indicates the presence of an effective Cl^- extrusion mechanism and the value of the bumetanide-insensitive difference of these, ΔE_{GABA}, is taken as a quantitative measure of KCC2-mediated Cl^- extrusion.

4 METHODS

For detailed description of the materials and methods used in this Thesis work, the reader is referred to the original publications (I-IV). An overview of the methodology is provided below:

The electrophysiological methods comprised visually guided whole-cell, cell-attached, and field potential recordings in hippocampal and neocortical slices prepared acutely from neonatal and juvenile rats and mice. Spike activity of intact single neurons was monitored using cell-attached current clamp in the 0-Mg^{2+} *in vitro* model of epileptiform activity. Whole-cell voltage clamp was used to study KCC2 functionality under conditions of a defined Cl^-load imposed via the patch pipette (see Fig. 4). To determine $E_{GABA\text{-}A}$, UV-laser photolysis of caged GABA was used to evoke $GABA_AR$-mediated currents. Field potential recordings were performed to monitor kainate- and 0-Mg^{2+}-induced activity in slices. Induction of neonatal seizures in transgenic mice lacking BDNF and their WT littermates was done using the proconvulsant kainic acid. Assessment of behavioral seizures was performed using video recordings. Confocal and epifluorescence microscopy was used to trace dendrites of dye-filled pyramidal neurons and to identify neurons transfected using *in utero* electroporation.

5 RESULTS

5.1 A SINGLE SEIZURE EPISODE LEADS TO RAPID FUNCTIONAL ACTIVATION OF KCC2 IN THE NEONATAL RAT HIPPOCAMPUS (I)

The findings of the present study show that immediately following kainate-induced neonatal SE in P5-7 rats, the furosemide-sensitive Cl^- extrusion capacity of CA1 pyramidal neurons is significantly enhanced, as seen in *ex vivo* slices following a 1 hour recovery period. This effect is paralleled by similar increases in not only the surface expression of both of the KCC2a and KCCb splice variants but also of the Na-K ATPase, which generates the thermodynamic driving force for K-Cl cotransport. The increase in the Cl^- extrusion capacity did not correlate with an increase in the total KCC2 protein level, indicating that the low level of KCC2 protein is not a limiting factor for functional activation of KCC2 in hippocampal pyramidal neurons at this age in the rodent brain. In corroboration with the idea that the functional up-regulation of KCC2 is due to kainate-induced increase in neuronal activity and not kainate *per se*, a brief application of kainate to naive hippocampal slices similarly led to enhanced KCC2 function and surface expression, but these effects were not seen when neuronal activity was blocked with TTX. To gain further insight into the underlying mechanisms of KCC2 activation, experiments with the dynamin-inhibitory peptide (DIP) demonstrated that an up-regulation of KCC2 function similar to that following kainate-induced activity could be evoked by an acute block of clathrin-dependent endocytosis. Both the kainate-induced up-regulation of Cl^- extrusion and of KCC2 surface expression were fully blocked by the kinase inhibitor K252a, suggesting a potential involvement of the Trk family of receptor tyrosine kinases in mediating the effect of KCC2 activation following neonatal seizures. Although K252a, a potent non-specific inhibitor of Trk kinases (Knusel and Hefti, 1992), has been shown to prevent TrkB-mediated down-regulation of KCC2 (Rivera et al., 2002; 2004), the possibility that its effects observed herein may be mediated through inhibition of other kinases than TrkB, e.g. PKC (cf. Kase et al., 1986; Lee et al., 2007), cannot be excluded. Thus, further experimental evidence is required to test the hypothesis that the functional up-regulation of KCC2 after neonatal seizures is dependent on BDNF-TrkB signaling.

5.2 BDNF IS REQUIRED FOR ACTIVITY-DEPENDENT BUT NOT CONSTITUTIVE UP-REGULATION OF KCC2 DURING HIPPOCAMPAL DEVELOPMENT (II)

To investigate the involvement of the BDNF-TrkB signaling in the functional up-regulation of KCC2 following a single neonatal seizure episode, suggested by the effect of the Trk inhibitor K252a in study I, homozygous BDNF KO ($BDNF^{-/-}$) mice were used in the present study. Strikingly, the seizure-induced enhancement of neuronal Cl^- extrusion capacity observed previously in P5-7 rats and now also in P6 WT ($BDNF^{+/+}$) mice, was strongly impaired in P6 $BDNF^{-/-}$ mice. Accordingly, compared to the WT littermates, significantly less KCC2 was incorporated into the plasma membrane in $BDNF^{-/-}$ pups following kainate-induced status.

Identical levels of total hippocampal KCC2 protein and of Cl^- extrusion capacity of CA1 pyramidal neurons were observed in $BDNF^{-/-}$ and WT mice at P13-14. This provides strong support for the conclusion that the observed impairment in functional activation of KCC2 is not a result of a general impairment in the developmental up-regulation of KCC2 as a result of lack of BDNF.

In order to investigate the time course of the functional enhancement of KCC2 following neonatal seizures, the window for assessment of KCC2 function was extended from the 1 hour after slicing recovery up to 5 hours. Interestingly, in WT P6 mice, a progressive decay in the efficacy of Cl^- extrusion was observed after the initial up-regulation. The total level of KCC2 protein in hippocampal slices prepared from kainate-injected animals was significantly decreased by the 4^{th} hour following recovery from slicing. Both the down-regulation of KCC2 function and total protein could be prevented by incubating the slices with MDL-28170, an inhibitor of the Ca^{2+}-activated protease calpain.

5.3 ACTIVITY-DEPENDENT CLEAVAGE OF KCC2 MEDIATED BY CALCIUM-ACTIVATED PROTEASE CALPAIN (III)

The main finding of this study was that KCC2 is subject to calpain-mediated cleavage following intense NMDAR activation. Treatment of slices with a Ca^{2+}-ionophore (ionomycin), NMDA, or 0-Mg^{2+} conditions, resulted in a robust calpain inhibitor-sensitive loss of full-length KCC2 protein, as seen by immunoblotting with a polyclonal antibody raised against aa residues 929-1045 of the KCC2 C-terminal domain. An antibody raised against 1-100 aa of KCC2 showed a truncated N-terminal fragment of KCC2, with a molecular weight ~100 kDa, following *in vitro* cleavage with recombinant calpain. Importantly, the proteasome inhibitor lactacystin had no influence on loss of KCC2 protein triggered by NMDAR activation. Interictal-like activity induced by Mg^{2+} withdrawal led to a significant reduction in surface-expressed KCC2, and to complete inactivation of KCC2-mediated Cl^- extrusion that could be prevented by inhibiting calpain activity.

Another important finding of this study was that both KCC2 total protein and Cl^- extrusion function remain stable for several hours in the absence of mRNA translation induced with emetine or cycloheximide. Accordingly, no rapid change in total KCC2 protein was observed in the presence of the broad-spectrum protease inhibitor leupeptin. Together these observations suggest the turn-over of total cellular KCC2 protein under control conditions is low due to a slow basal degradation rate, which is in direct contrast to what was suggested previously (cf. Rivera et al., 2004). Notably, robust down-regulation of KCC2 protein could be still induced by NMDAR stimulation under full block of mRNA translation by cycloheximide.

In summary, the obtained data suggest that rapid down-regulation of KCC2 function is unlikely to result from diminished *de novo* synthesis of KCC2. It also appears that rapid down-regulation and functional inactivation of KCC2 under conditions of elevated intracellular Ca^{2+} levels, typical to a wide range of CNS insults, is a result of calpain-mediated cleavage.

5.4 AN ION TRANSPORT-INDEPENDENT ROLE FOR KCC2 IN DENDRITIC SPINOGENESIS *IN VIVO* (IV)

This study set forth to investigate whether expression of KCC2 is sufficient for the induction of functional dendritic spines during the brain growth spurt. Utilizing the powerful technique of introducing genetic material to a subset of cortical neurons at a defined time period during CNS development provided by *in utero* electroporation (IUE), neuronal precursors of rat neocortical layer 2/3 pyramidal neurons where electroporated at E17.5 with rat KCC2-FL and enhanced GFP plasmids. Contrary to a previous report using a similar approach (Cancedda et al., 2007), impairments in either dendritic length or the number of branch points were observed neither at P10, P15, or P90 following overexpression of KCC2 using IUE. The only obvious methodological difference with regard to the above-mentioned study is that in the present work, in order to ensure complete visualization of dendritic arbors, all neurons where filled post-hoc with Lucifer-yellow, whereas in the other study dendritic morphology was assessed based on the GFP signal alone. Overexpression of KCC2 was, however, associated with a persistently increased spine density of both apical and basal dendrites of layer 2/3 pyramidal neurons at P10, P15, and P90. Importantly, the increased density was seen in spines in a wide-range of head diameters, indicating that overexpression of KCC2 was associated with the induction of synaptically active spines. This notion was corroborated by the observation of significantly higher frequency of mEPSCs recorded from KCC2 overexpressing neurons compared to neighboring non-transfected control neurons solely expressing native KCC2.

Based on the previous work by Li et al. (2007), which implicated a structural, ion transport-independent role for KCC2 in the maintenance of functional dendritic spines, the ability of the N-terminally deleted KCC2-ΔNTD to induce spines was assessed. As expected on the basis of the results by Li et al. (2007) this KCC2 variant, which is devoid of Cl^- extrusion function was observed to induce a comparable level of spines as the full-length KCC2-FL. Unexpectedly, the mere C-terminal domain of KCC2 (KCC2-CTD) was able to induce spines with wide range in diameter, similarly to the KCC2-FL and the KCC2-ΔNTD.

To shed further light on the underlying mechanism of KCC2-mediated spinogenesis, effects of the KCC2 mutant KCC2-C568A, which is devoid not only of the ion transport function but also of the interaction with the actin cytoskeleton via protein 4.1N (cf. Horn et al., 2010), were assessed. Importantly, overexpression of this KCC2 mutant had no effect on spine density or spine head diameter. Together with data obtained using KCC2-ΔNTD and KCC2-CTD, this result suggests that KCC2-induces spines via an ion transport-independent function mediated by its C-terminal domain that most likely involves an interaction with the cytoskeleton via 4.1N.

6 DISCUSSION

6.1 STUDY I

The main finding of the present study is that a single neonatal seizure episode triggers a fast increase in neuronal Cl^- extrusion capacity which leads to a large hyperpolarizing shift in E_{GABA-A} and to the development of a native somato-dendritic E_{GABA-A} gradient in rat hippocampal CA1 pyramidal neurons at P5-7. The post-seizure value of E_{GABA-A} is close to that seen in mature neurons. Notably, an approximately 2-fold activity-dependent increase in the plasmalemmal protein pool with no change in total KCC2 protein was observed by two different biochemical approaches, biotinylation and cleavage of surface proteins. The increase in surface KCC2 may not fully account for the increase in Cl^- extrusion efficacy since additional changes in the intrinsic turnover rate of the transporter may take place in parallel, but it is likely to play a role in the functional effects observed in the present studies. That fast changes in membrane trafficking of KCC2 have a significant effect on K-Cl cotransport obtained further support from the result that inhibiting endocytosis in single pyramidal neurons by adding the endocytosis blocker DIP into the patch pipette led to an increase in the Cl^- extrusion capacity. These results are striking as they demonstrate that the efficacy of neuronal Cl^- extrusion by KCC2 is not rate-limited by the total expression level of the transporter protein at this early developmental stage; and that KCC2 functionality can be precociously enhanced in an activity-dependent manner close to the level that is seen after the developmental shift in E_{GABA-A}.

To the best of my knowledge, no prior study has investigated the immediate consequence of a single neonatal seizure episode on the capacity of hippocampal pyramidal neurons to extrude Cl^-. The work by Nardou et al. (2011b) using two isolated interconnected hippocampi demonstrated that following 15 three minute-long kainate (400 nM) applications at 20 minute intervals to one of the hippocampi led to emergence of spontaneous ictal events in the drug-naive contralateral hippocampus and loss of Cl^- extrusion capacity paralleled by internalization of KCC2 in CA3 pyramidal neurons. In the present work a *single* application of kainate (300 nM for 10 minutes) to hippocampal slices from neonatal rats of comparable age led to enhanced surface expression of KCC2 in the CA1 region and an up-regulation of the neuronal Cl^- extrusion capacity within minutes after the ictal event. An identical up-regulation of KCC2 was observed *in vivo* following a single kainate-induced SE. Thus, it appears that the initial response to a neonatal ictal event is enhancement of KCC2 surface expression and consequent neuronal Cl^- extrusion capacity. However, aggravation of seizures leads to down-regulation of KCC2. An interesting hypothesis for future work is that the initial functional enhancement of KCC2 acts as an intrinsic anti-ictogenic or an antiepileptogenic

mechanism in the neonatal hippocampus. The gradual increase in KCC2 expression during neuronal maturation has often been considered as an *explanation* of the parallel developmental shift in E_{GABA-A} (Ben-Ari et al., 2007). An important conclusion from the present work is that immature hippocampal neurons are capable of undergoing a large precocious negative shift in E_{GABA-A}, based on a more efficacious utilization of their low total KCC2 protein pool. However, fast post-translational effects such as those described in the present work obviously do not exclude long-term effects based on enhanced KCC2 protein synthesis. Indeed, the work by Galanopoulou (2008) on a similar neonatal seizure model, but involving three episodes of SE each with one day interval, demonstrated that an increase in KCC2 expression leads to sustained KCC2 activation, which apparently takes place after the initial post-translational effect described in the present work. In future work it would be of interest to determine whether such long-term changes in KCC2 protein expression take place already after a single episode of neonatal SE, after the time window of the present experiments.

In view of the tight functional and structural coupling of KCC2 and the α2 subunit of the Na-K ATPase (cf. Ikeda et al., 2004), it is interesting that the up-regulation of the membrane expression of KCC2 was paralleled by a quantitatively similar up-regulation of the Na-K ATPase, the ion pump which ultimately provides the thermodynamic driving force for K-Cl cotransport mediated by both KCC2a and KCC2b. It would be interesting to investigate whether KCC2 physically interacts with the Na-K ATPase also through the α3 subunit, as the expression of this subunit of the Na-K ATPase appears to be neuron-specific while that of α2 is observed in both glia and neurons (Cameron et al., 1994). While the available data indicate that the KCC2b isoform is required for the developmental shift in E_{GABA-A} (see section 2.3.1) little is known about the functional significance of KCC2a (Uvarov et al., 2007; 2009). Nevertheless, the parallel activity-dependent up-regulation of the two isoforms is of interest because of their capability of heterodimerization (Uvarov et al., 2009). Recent work suggests that heterodimerization of KCC2 with other KCCs might be a way of regulating KCC2 trafficking and function (Ding et al., 2013; see also Wenz et al., 2009).

The similarities of the fast increase in the Cl^- extrusion capacity paralleled by the increase in the surface expression of KCC2 observed between the *in vivo* and *in vitro* models in the present study are consistent with the idea that the two neonatal seizure models have common underlying molecular mechanisms of KCC2 regulation in response to epileptiform activity. The block of the activity-induced activation of KCC2 by K252a in the *in vitro* model is interesting in light of the previous finding that K252a blocks the activity-induced down-regulation of KCC2 in mature neurons (Rivera et al., 2004). A similar age-

dependent difference in the direction of the effect induced by a signaling cascade has been described for the action of BDNF on $GABA_A$ currents, which are enhanced by BDNF in immature and suppressed in mature neurons (Mizoguchi et al., 2003). Based on the K252a effect *in vitro*, BDNF-TrkB signaling is a promising candidate mediating the activation of KCC2 *in vivo*.

6.2 STUDY II

This study not only mechanistically elaborates on the findings of Study I, but demonstrates that the *developmental* up-regulation of KCC2 can take place in the absence of BDNF. The results obtained using full homozygous BDNF KO mice demonstrated that the fast functional up-regulation of KCC2 following a single neonatal seizure is dependent on BDNF signaling. In Study I, enhanced surface expression of KCC2 was observed in parallel with functional enhancement of the Cl^- extrusion capacity. Similarly, in this Study BDNF KO mice exhibited reduced levels of KCC2 compared to WT littermates, suggesting that disrupted KCC2 trafficking in BDNF KO mice may account for the lack of enhancement of Cl^- extrusion following neonatal seizures. Interestingly, work on trafficking of $GABA_A$Rs has identified BDNF-TrkB signaling as an important regulatory (Brunig et al., 2001). Given that the seizure-induced up-regulation of both KCC2 function and surface expression were completely prevented by the Trk inhibitor K252a (Study I), and in the BDNF KO mice, it appears that this signaling pathway may be important also for regulation of KCC2 trafficking.

Impairments in the developmental up-regulation of functional KCC2 protein, which have been
suggested to accompany changes in BDNF-TrkB signaling (Aguado et al., 2003; Carmona et al., 2006; Ludwig et al., 2011b), are an unlikely reason for the observed impairment activation of KCC2 following neonatal seizures, because KCC2 total protein levels were indistinguishable between BDNF KO and WT littermates at P14. Endogenous BDNF has been demonstrated to mediate fast activity-dependent down-regulation of KCC2 in older animals (Rivera et al., 2002; Rivera et al., 2004). Thus, it appears that activation of the BDNF-TrkB pathway may have opposite effects on KCC2 function depending on the stage of brain development. Intriguingly, recent work has demonstrated qualitatively different responsiveness of mouse brain TrkB receptors to BDNF before and after ~P12 (Knüsel et al., 1994; Di Lieto et al., 2012).

An interesting observation made in Study II is, that following a single neonatal seizure, down-regulation of KCC2 ensues after the initial functional up-regulation. In Study I, where changes in KCC2 induced by the neonatal seizure where examined during the early period, no change in the total KCC2 protein pool was observed. However, extending the experimental time window to 4 hours revealed progressive down-regulation of Cl^- extrusion capacity that was paralleled

by significant calpain-dependent reduction of the total KCC2 protein pool. It was not investigated whether the presently observed down-regulation of KCC2 protein and transport capacity is mediated by TrkB. Given the sensitivity of KCC2 down-regulation to inhibition of BDNF-TrkB signaling (Rivera et al., 2002; Rivera et al., 2004) and activation of calpain by BDNF (Zadran et al., 2010a; Zadran et al., 2010b), an interesting hypothesis for future work is that the down-regulation of KCC2 observed herein is mediated by TrkB.

6.3 STUDY III

The key finding of this study is that KCC2, a protein instrumental in the development and plasticity of GABAergic hyperpolarizing inhibition and in the maturation and function of dendritic spines and glutamatergic synapses, is a substrate of the Ca^{2+}-activated protease calpain and is down-regulated following calpain activation. The validity of the present findings was extended by a parallel study (Zhou et al., 2012) carried out independently of this Thesis. This work, consistently the present one, demonstrated that down-regulation of KCC2 following pathological activation of NMDARs is attributable to calpain activation but not to decreased KCC2 mRNA levels in spinal cord neurons of adult rats (Zhou et al., 2012).

Calpains constitute a family of intracellular non-lysosomal cysteine proteases that are activated by Ca^{2+} in the cell cytosol at neutral or slightly alkaline pH (Zhao et al., 1998; Goll et al., 2003). In contrast to relatively promiscuous degradative proteases, such as many of the lysosomal proteases (Cuervo and Dice, 1998), calpains regulate cellular processes by partially truncating a restricted set of substrates, conferring them post-translational protein modifications, which can also serve as signals for protein degradation (Wu and Lynch, 2006; Croall and Ersfeld, 2007; Zadran et al., 2010a). There are so far 14 known members in the calpain family of proteases, out of which two isoforms, calpain-1 and calpain-2, are particularly abundant in the CNS (Croall and Ersfeld, 2007). These two CNS isoforms of calpain do not differ with respect to their substrate preference but rather in their sensitivity to the level of $[Ca^{2+}]_i$. While calpain-1, also known as µ-calpain is activated in the low µM range of $[Ca^{2+}]_i$, calpain-2 is activated only at high >0.4 mM $[Ca^{2+}]_i$ thereby earning its eponymous alias, m-calpain (Baudry et al., 2013). The other main difference between the two major CNS calpain isoforms is that calpain-2 can bypass the Ca^{2+} requirement and be activated by BDNF-signaling via phosphorylation by the MAPK/ERK kinase (Zadran et al., 2010a; Zadran et al., 2010b). In practice, it is however very difficult to specifically attribute a given effect to either of the two isoforms as known calpain inhibitors target both calpain-1 and 2 with a similar potency (Goll et al., 2002; Baudry et al., 2013). The use of RNA interference to specifically suppress one or the other calpain isoform is not feasible in acute

preparations, such as the brain slice, as both of these calpain isoforms have a half-life of several days (Zhang et al., 1996). In the present work, direct calpain-mediated cleavage of KCC2 was demonstrated using recombinant calpain-2. However, given the above considerations, both isoforms may well be responsible for the observed KCC2 down-regulation under the present conditions (see also Zhou et al., 2012). Thus, here they are collectively referred to as 'calpain'.

Constitutive calpain cleavage of synaptic proteins is part of normal CNS function (Amini et al., 2013), and activation of calpain by excitatory amino acid signaling confers selective activity-dependent cleavage of its substrates (Wu and Lynch, 2006; Liu et al., 2008; Baudry et al., 2013). Thus, the role of calpain-mediated signaling is pivotal in regulation of neuronal plasticity. Hyper-activation of calpain under pathophysiological conditions, including experimental seizures (Bi et al., 1996; Sierra-Paredes et al., 1999; Fujikawa, 2005; Sharma et al., 2009), is, however, often associated with neurotoxicity (Liu et al., 2008). Increased calpain expression has been reported in hippocampal and cortical tissue of patients with drug-resistant TLE (Feng et al., 2011; Das et al., 2012). Several studies have shown neuroprotective effects of calpain inhibitors in various models of CNS pathology (Markgraf et al., 1998; Zhang et al., 2003; O'Hanlon et al., 2003; Yu et al., 2008; Nimmrich et al., 2008; Granic et al., 2010), indicating that calpain-mediated cleavage of proteins can be detrimental. In line with this, one of the calpain inhibitors used in the present work, MDL 28170, has been reported to reduce pyramidal cell death in the CA1 of the hippocampus in a rodent model of TLE (Araujo et al., 2008). Thus, the novel interaction between KCC2 and calpain, first reported herein, may be of clinical interest.

The present data acquired using cycloheximide, emetine, and leupeptin all point to a rather low basal turnover of KCC2. It should be noted here that, because of the several days long half-life of calpain (Zhang et al., 1996), it is unlikely that the observed stability of KCC2 under arrested *de novo* translation is due to reduced calpain levels in the present experimental time window of four to five hours. However, previous data obtained with leupeptin in neuronal cultures (Lee et al., 2010) and analysis of the KCC2 surface pool in hippocampal slices (Rivera et al., 2004) indicated a high turnover rate. The data of the present work demonstrate that activation of calpain can lead to fast changes in KCC2 protein levels. While the study by Rivera et al. (2004) reported a constant decrease (0.2%/min) in the total KCC2 protein pool under control conditions (i.e., in standard physiological solution) in biochemical experiments, the KCC2 protein level in the present study remained stable for at least 5 hours after recovery under control conditions as well in the absence of protein synthesis. The difference between previous and present data is most likely attributable to non-optimal

slice preparation and maintenance conditions used in the biochemical assays by Rivera et al. (2004) which affected $[Ca^{2+}]_i$ levels and, consequently, calpain activity (see also discussion section in the original publication, Study III). While the methodological approach used by Rivera et al. (2004) did not permit analysis of protein turnover *per se*, and the data demonstrated a high degradation rate of KCC2 under the conditions used for the biochemical experiments, this paper is often referred to (e.g. Uvarov et al., 2006; Yang et al., 2010) as evidence for fast down-regulation of KCC2 to take place at the transcriptional level. The present work shows that such conclusions are unwarranted. Indeed, recent work by Pan and colleagues demonstrated that the chronic down-regulation of KCC2 protein triggered by enhanced NMDAR activation was not associated with changes at the level of KCC2 mRNA (Zhou et al., 2012).

In view of the low turnover rate of the total KCC2 protein observed presently, studies reporting fast functional changes in the efficacy of KCC2-mediated Cl^- extrusion caused by changes in gene expression warrant re-interpretation. For example, Yang et al. (2010) reported that changes in E_{GABA-A} taking place within 30 minutes of high frequency stimulation of rat CA1 pyramidal neurons may be blocked by employing KCC2 antisense oligonucleotides through the recording pipette. Surprisingly, however, the authors failed to replicate the antisense effects using a high 100 µM concentration of bumetanide (Yang et al., 2010), which is known to block K-Cl cotransport (Payne, 1997; Delpire et al., 2009; Nardou et al., 2011b).

The present data clearly show that an enhanced degradation rate of KCC2 is required to rapidly reduce KCC2 protein levels. The calpain-mediated cleavage of KCC2 presented here might be one of the major mechanisms responsible for an enhanced degradation of KCC2, while changes at the transcriptional level (e.g., Rivera et al., 2002; Huberfeld et al., 2007) are involved in long-term effects. Work by Rivera et al. (1999) demonstrated that the KCC2 antisense approach to block KCC2 synthesis results in severely decreased total levels of KCC2 protein in organotypic hippocampal cultures after 8 hours of antisense application. In view of the present data, this suggests that down-regulation of KCC2 attributable to synthesis deficits takes place roughly 5-8 hours after arrest of KCC2 translation. It would be interesting to further investigate, whether changes in the phosphorylation state of KCC2, which also are known to trigger enhanced degradation of KCC2 (Lee et al., 2010; 2011; Kahle et al., 2013), are involved in the regulation of calpain-mediated KCC2 cleavage.

Several key proteins for GABAergic and glycinergic signaling, such as the GABA transporter GAT1 (Baliova et al., 2009), vesicular inhibitory amino acid transporter VIAAT (Gomes et al., 2011), GABA synthesizing enzyme GAD65 (Buddhala et al., 2012) glycine transporters GlyT1 and GlyT2 (Baliova et al.,

2004; Baliova and Jursky, 2005) are all cleaved by calpain. Thus, the present finding of calpain-mediated KCC2 down-regulation suggests a more general role for calpain in the regulation of GABAergic and glycinergic neurotransmission. This idea gains further support from the finding that gephyrin, a key protein for clustering of both $GABA_A$Rs and GlyRs, is also a calpain substrate (Tyagarajan et al., 2011; Tyagarajan et al., 2013). An important hypothesis for future work is that calpain plays a determining role in the erosion of inhibition (cf. Trevelyan et al., 2007) that takes place in response to ictal activity and contributes to AED resistance.

As mentioned above, in addition to activity-induced elevation in intracellular Ca^{2+}, calpain-2 is also activated by BDNF signaling (Zadran et al., 2010b). Seizure-induced up-regulation of BDNF-TrkB signaling (Rivera et al., 2002), activity-dependent endogenous release (Rivera et al., 2004) as well as exogenous application of BDNF (Rivera et al., 2002; Wake et al., 2007) have been demonstrated to result in rapid impairment of KCC2 expression and function, demonstrating a critical role for BDNF-TrkB signaling in down-regulation of KCC2. Thus, it would be of interest to test whether the BDNF-triggered activation of calpain plays a role in the BDNF-induced down-regulation of KCC2.

In conclusion, this work shows that fast, activity-dependent down-regulation of KCC2 is attributable to calpain-mediated cleavage of the protein, and not to suppression of KCC2 gene expression. It thereby identifies a new molecular mechanism for the fast regulation of KCC2 involved in synaptic plasticity under physiological and pathophysiological conditions.

6.4 STUDY IV

The main finding of this study was that overexpression of KCC2 induces the formation of functional dendritic spines. In humans, the most intensive period for synaptogenesis takes place between the third trimester of pregnancy and the first few postnatal years (Huttenlocher and Dabholkar, 1997; Petanjek et al., 2011). In rodents, the peak period of synaptogenesis is limited to a time window between the second and fourth postnatal week (Juraska, 1982; Micheva and Beaulieu, 1996; De Felipe et al., 1997). The intimate temporal correlation between the developmental up-regulation of KCC2 expression and synaptogenesis in rodents and humans (see section 2.3.2) points to a possible role for KCC2 in synapse formation. The major novel finding of the present study is that overexpression of KCC2 leads to increased dendritic spinogenesis under *in vivo* conditions. Previous work on cultured neurons has shown that maturation of dendritic spines fails when neurons are deprived of KCC2 expression before or at the onset of spinogenesis (Li et al., 2007; Gauvain et al., 2011) and that overexpression of KCC2 is able to rescue spine development when neurons are transfected with KCC2 before the onset of spine formation (Li et al., 2007; see

also Sun et al., 2013). Notably, in cultured neurons expression of endogenous KCC2 is observed already before formation of synapses (see Ludwig et al., 2003).

In the present study, a striking increase in the density of spines over a wide range of head diameters, which was paralleled by increased frequency of mEPSCs, was observed throughout the observed first three postnatal months in the rat somatosensory cortex following *in utero* electroporation of neurons at E17.5. In view of the minor effect of suppression of KCC2 expression starting *after* the onset of synaptogenesis on the density and morphology of spines in cultured hippocampal neurons and Purkinje cells *in vivo* (Gauvain et al., 2011; Seja et al., 2012), the present data suggest that KCC2 may play a role in the induction of synaptogenesis. Testing whether suppression of KCC2 expression after the peak period of spinogenesis affects the maintenance of spines, is, however, needed to further assess the differential role of KCC2 in induction versus maintenance of dendritic spines in neocortical pyramidal neurons.

Notably, the present study did not demonstrate any effect of premature expression of KCC2 or the of IUE procedure *per se* on the dendritic length or branching of cortical layer 2/3 pyramidal neurons. This is in striking contrast with a previous report (Cancedda et al., 2007), which suggested that precocious termination of depolarizing GABAergic signaling through overexpression of KCC2 impairs the morphological maturation of layer 2/3 pyramidal neurons. Our data is, however, in agreement with the study by Pfeffer et al. (2009) who observed no morphological alterations in hippocampal neurons from $NKCC1^{-/-}$ mice which displayed normal dendritic length, branching, and spine synapse density despite a much reduced depolarizing action mediated by $GABA_A$Rs.

The present work also demonstrates that not only is the ion transport function of KCC2 not needed for spine induction but there is apparently no necessity for membrane association of KCC2, as similar spine-induction as with KCC2-FL was observed with KCC2-CTD, which is predicted (cf. Payne et al., 1996) to contain no membrane spanning sequences. Unlike in the present *in vivo* experiments, work *in vitro* has reported that when expressed before the onset of glutamatergic synaptogenesis, KCC2-CTD exerts dominant-negative effects on spine maturation (Li et al., 2007), but not when expressed at later time points (Gauvain et al., 2011). Similarly, no dominant-negative effects of the KCC2 mutant KCC2-C568A (cf. Pellegrino et al., 2010) were observed presently on spine density or spine head distribution of layer 2/3 pyramidal neurons.

A recent study reported of the existence of several previously unrecognized alternative KCC2 transcripts in both human adult and fetal brain, including truncated KCC2 variants (Tao et al., 2012). Interesting questions for future work are whether there are alternative KCC2 proteins that endogenously dissociate between ion transport and spine

formation and whether truncated KCC2 or KCC2 proper (see also Adusei et al., 2010; Hyde et al., 2011; Tao et al., 2012) contribute to disease states, such as schizophrenia (Glausier and Lewis, 2012), autism spectrum disorders, fragile X (De Rubeis et al., 2012), and epilepsy (Wong and Guo, 2012), in which pathological changes in dendritic spines have been implicated.

7 CONCLUSIONS

The main findings of Study I are that (i) the immediate postictal effect of a neonatal seizure on KCC2 in the hippocampi of P5-7 rats is a robust post-translational activation of the transporter function of KCC2 close to the mature level of Cl^- extrusion, and (ii) the low total level of KCC2 protein in the P5-7 hippocampus is not a limiting factor for functional up-regulation of KCC2 during this stage in development. Functional up-regulation of KCC2 in response to seizures enhances the ability of neurons to cope with the increased intracellular Cl^- loads associated with seizure activity and may represent an endogenous first-in-line anti-ictogenic mechanism of the neonate brain by keeping the reversal potential of $GABA_AR$-mediated responses more negative than the action potential firing threshold.

The results of Study II expand on the mechanism of the functional activation of KCC2 as an immediate response to a neonatal seizure. The results obtained in this study using mice with full genetic disruption of BDNF expression, together with the data on the effects of Trk receptor kinase inhibition in Study I, strongly indicate that intact BDNF-TrkB signaling is necessary for the precocious functional up-regulation of KCC2 to take place in response to neonatal seizure. Study II also reports that BDNF-signaling is not a requirement for the canonical up-regulation of KCC2 during development. Together, these findings highlight the importance of BDNF-signaling in fast post-translational regulation of KCC2.

The results of Study III indicate that down-regulation of KCC2 total protein and neuronal Cl^- extrusion capacity is unlikely to be attributed to a deficit in synthesis of KCC2 protein, and demonstrate that under conditions of elevated $[Ca^{2+}]_i$ the enhanced degradation and functional inactivation of KCC2 is a consequence of KCC2 cleavage by the Ca^{2+}-activated protease calpain.

Study IV reports an ion transport-independent role for KCC2 in the genesis of dendritic spines *in vivo*, and demonstrates that expression of KCC2 is sufficient for the induction of functional glutamatergic spines during the brain growth spurt.

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