Post-translational regulation of KCC2 in the rat hippocampus

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To my mother, Ammi.

"Cling to her feet, because paradise is there" (Prophet Muhammad).

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List of abbreviations

AMPA	2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid
AP-2	Adaptor protein 2
ARMS	Ankyrin rich membrane spanning protein
BDNF	Brain derived neurotrophic factor
BS3	Bis(sulfosuccinimidyl) suberate
$[Ca^{2+}]_i$	Intracellular calcium ion concentration
CaMKII	Ca^{2+}/Calmodulin dependent protein kinase II
CCC	Cation chloride cotransporter
CIP	Cation chloride cotransporter interacting protein
CKB	Brain-type creatine kinase
$[Cl^-]_i$	Intracellular chloride concentration
ClC2	Chloride channel 2
CNS	Central nervous system
CREB	cAMP response element-binding protein
C-terminus	Carboxy-terminus
DAT	Dopamine transporter
DIP	Dynamin inhibitory peptide
E_{Cl^-}	Equilibrium potential of chloride
E_{GABA-A}	Equilibrium potential of $GABA_A$ receptor
Erg4	Early growth response 4
GABA	γ-amino butyric acid
$GABA_AR$	GABA receptor type A
GAT	GABA transporter
GluR	Glutamate receptor
GlyR	Glycine receptor
GlyT	Glycine transporter
GRIP	Glutamate receptor interacting protein
FRS-2	Fibroblast growth factors receptor substrate 2
$[K^+]_o$	extracellular potassium ion concentration
KCC	K^+-Cl^- cotransporter
LSO	Lateral superior olive
MAP	Microtubule associated protein

mGluR	metabotropic glutamate receptor
mZnR	metabotropic zinc-sensing receptor
Na^+-K^+ ATPase	Na^+-K^+ adenosine triphosphatase
NCC	Na^+-Cl^- cotransporter
NGF	Nerve growth factor
NHS	N-hydroxy succiniimide
NKCC	Na^+-K^+-$2Cl^-$ cotransporter
NMDA	N-methyl-D-aspartic acid
NRSE	Neuron-restrictive silencer element
NRSF	Neuron-restrictive silencing factor
N-terminus	Amino-terminus
OSR-1	Oxidative stress response kinase 1
P	Postnatal day
PAM	Protein associated with Myc
PCR	Polymerase chain reaction
PEST	Proline/glutamate/serine/threonine sequences
PKA	Protein kinase A
PKC	Protein kinase C
PMA	4β-phorbol 12-myristate 13-acetate
PNS	Peripheral nervous system
PP	Protein phosphatase
PSD-95	Postsynaptic density protein-95
P75(NTR)	P75 neurotrophin receptor
RCC1	Regulator of chromatin condensation-1
RE-1	Repressor element-1
REST	Repressor element-1 transcription factor
SLC12	Solute carrier family 12
SPAK	Ste20-related proline-alanine-rich-kinase
TM	Trans-membrane
TrkB	Tropomyosin receptor kinase B
USF	Upstream stimulating factor
VGAT	Vesicular GABA transporter
VGCC	Voltage gated calcium channel
WNK	With no lysine

List of original publications

The thesis is based on the following publications which are referred in Roman numerals below and elsewhere in the thesis.

I. Khirug, S. *, Ahmad, F.*, Puskarjov, M., 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(36):12028-12035.

II. Ahmad, F., Coleman, S.K., Kaila, K., Blaesse, P. 2011. Cold-adapted protease enables quantitation of surface proteins in the absence of membrane trafficking. Biotechniques. 50(4):255-257.

III. Puskarjov, M.*, Ahmad, F.*, Kaila, K., Blaesse, P. 2012. Activity-dependent degradation of the K-Cl-cotransporter KCC2 is mediated by calcium-activated protease calpain. J. Neurosci. Under revision.

* denotes equal contribution

The doctoral candidate's contribution:

For Study I, the candidate established and performed all the biochemical experiments and participated in the analysis of the data and writing the manuscript.

For study II, the candidate established and performed all the experiments and analyzed the data and wrote the manuscript.

For Study III, the candidate established and performed most of the biochemical experiments and participated in the analysis of the data and writing the manuscript.

Publications that have been used in other dissertations:

Study I has been used 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).

Abstract

KCC2 is an important K^+-Cl^- cotransporter that along with Na^+-K^+-$2Cl^-$ cotransporter, NKCC1 is largely responsible for the regulation of intracellular chloride concentration in neurons which determines whether the ionotropic GABAergic/glycinergic responses are depolarizing or hyperpolarizing. There are spatiotemporal differences in the intracellular chloride concentration in individual neurons which are attributable to a differential temporal and spatial activation of cation chloride cotransport mediated by KCC2 and NKCC1. Post-translational modulation is a fundamental cellular mechanism for such spatiotemporal regulation of protein activity. This thesis deals with the work that has been ongoing in our laboratory to understand the mechanisms of post-translational regulation of KCC2 cotransport function.

In Study I, we have demonstrated a fast post-translational increase in KCC2 cotransport function in neonatal rat hippocampus after a single seizure episode. This increase of functional KCC2 was caused by an increment in the surface expression of KCC2. Study II deals with the establishment of a modified protease cleavage method for quantitative analysis of surface expression of proteins using a cold-adapted trypsin. This can serve as a fast and reliable procedure and can be easily applied to brain slice preparations as well as cell culture systems. In study II, we have also shown that KCC2 has a low surface expression in the rat hippocampus but a very fast turn-over rate of the plasmalemmal pool. Not surprisingly, modifications in the turn-over rate of the surface pool can be employed as a mechanism to regulate the surface expression of KCC2 and consequently its function. Study III deals with another post-translational strategy to regulate KCC2 function in the rat hippocampus under pathophysiological conditions. While the KCC2 protein is quite stable in the rat hippocampus and has a slow turn-over rate under basal conditions, epileptiform activity and excitotoxicity can induce a rapid calpain-mediated cleavage of KCC2 with a consequent loss of its cotransport function.

1. Review of the literature

1.1 Introduction

Establishment and maintenance of intra- and extracellular ionic homeostasis is essential for normal physiological function of the cell and its appropriate responses to intracellular and extracellular perturbations. Cellular ionic regulation in neurons relies on maintenance of gradients of ionic concentrations across the cell membrane through a properly concerted and coordinated activity of a large number of membrane proteins and receptors (ion transporters and channels) that mediate selective ion fluxes across the membrane. Regulation of chloride homeostasis is critical for several cellular processes like cell volume regulation, cell cycle control, trans-epithelial salt transport, fluid secretion, pH maintenance and inter-neuronal communication (Blaesse et al. 2009, Friauf et al. 2011, Russell 2000). Although Na^+-dependent and Na^+-independent Cl^-/HCO_3^- exchangers (Galanopoulou 2008b, Kaila 1994, Payne et al. 2003) and Cl^- channels like ClC2 (Ben-Ari et al. 2007, Ratte and Prescott 2011, Smith et al. 1995) have also been suggested to contribute to chloride homeostasis in neurons, cation chloride cotransporters (CCCs) (particularly K^+-Cl^- cotransporter isoform 2 (KCC2) and Na^+-K^+-$2Cl^-$ cotransporter isoform 1 (NKCC1) are generally considered major players controlling electrochemical chloride gradient in neurons (Blaesse et al. 2009, Friauf et al. 2011, Mercado et al. 2004, Payne et al. 2003).

CCCs are secondary active transporters (i.e., they use energy from ATP indirectly in the form of K^+ and Na^+ gradients generated by Na^+-K^+ ATPase) that mediate the coupled transport of chloride and cations (K^+ and/or Na^+) down a combined chemical potential difference (see section 1.2.2 and Fig.1). Nine genes in the CCC gene family (solute carrier family 12; SLC12) have been identified: one Na^+-Cl^- cotransporter (NCC), two Na^+-K^+-$2Cl^-$ cotransporters (NKCC1 and 2), four K^+-Cl^- cotransporters (KCC1-4) (Mount and Gamba 2001), a cation chloride cotransporter isoform 9 (CCC9) that transports polyamines and amino acids (Daigle et al. 2009) and a structurally related protein, cation chloride cotransporter interacting protein (CIP) that can modulate the cotransport activity of KCCs and NKCCs (Caron et al. 2000, Wenz et al. 2009). The predicted protein structure of the CCC proteins reveals a membrane topology with a central hydrophobic core containing 12 α-helical transmembrane domains (TM), a hydrophilic amino (N-) and a larger carboxy (C-) terminal domain, both of which are cytoplasmic (Mercado et al. 2004, 2006, Williams et al. 1999).

One of the CCCs, the neuron-specific K^+-Cl^- cotransporter, KCC2, encoded by the SLC12A5 gene, was identified as an essential protein for maintaining a low intracellular

chloride concentration ($[Cl^-]_i$) in adult neurons because of its ability to extrude Cl^- against its electrochemical gradient using the outward K^+ concentration gradient created by the Na^+-K^+ ATP pump (DeFazio et al. 2000, Rivera et al. 1999). Hence, it plays a crucial role in controlling the strength of synaptic inhibition mediated by γ-amino-butyric acid type-A ($GABA_AR$) and glycine (GlyR) receptors, both of which are ligand-gated Cl^- channels (see section 1.2.2.1). Several studies have been targeted to elucidate the molecular and cellular mechanisms controlling KCC2 function. Moreover, recent studies affirming a structural role of KCC2 in spine formation and maintenance (see section 1.2.2.4), only add to the relevance of studies on functional regulation of this key molecule in the brain. Similar to all proteins, gene transcription and protein translation set the first level of regulation of KCC2 function. However, the role of post-translational regulation of KCC2 function has also become an interesting area of research in recent years. These post-translational mechanisms which include protein maturation and intracellular protein trafficking, phosphorylation/dephosphorylation, protein complex assembly and protein degradation, provide a basis for reversible regulation of KCC2 function that allows fast plasticity of inter-neuronal communication.

1.2 Physiology of KCC2

As previously stated, the CCC family includes four KCC isoforms, namely KCC1-4. KCC2 has certain unique features that aids its physiological role as a Cl^- efflux pathway in mature neurons allowing the maintenance of a low $[Cl^-]_i$ required for efficient inhibitory neurotransmission in neural circuits. KCC2 is unique among the KCCs in its exclusive expression in central neurons (see sections 1.2.1 and 1.3.1). Importantly, while KCC2 unlike other KCCs exhibits little or no swelling-induced activation, it is constitutively active under isotonic conditions (see section 1.4.3). This has been attributed to presence of a unique 15-amino acid fragment (called the isotonic domain) within the C-terminus of KCC2 (Mercado et al. 2006), although Bergeron et al. (2006) have proposed that the mere presence of this fragment is not sufficient but rather depends on the context in the whole C-terminal sequence. Unlike other KCCs, KCC2 operates near equilibrium close to the physiological levels of $[Cl^-]_i$ and $[K^+]_o$ and hence has been suggested to be capable of mediating both exit and entry pathways for Cl^- (see section 1.2.2.3). A recent study has also outlined differences in the requirement of cysteine residues in the large extracellular loop for the cotransport function of KCC2 and KCC4 (Hartmann et al. 2010). However, the exact role of the cysteine

residues in modulating KCC2 function or its surface expression and contribution to its unique physiological role in neurons is yet to be identified.

1.2.1 Expression

KCC2 is abundantly expressed in most neurons in the mature central nervous system (CNS), but shows negligible expression in non-neuronal cells like glia and astrocytes, undifferentiated neuronal progenitors and peripheral neurons (Hubner et al. 2001, Li H. et al. 2002, Payne et al. 1996, Rivera et al. 1999, Song et al. 2002, Williams et al. 1999). Interestingly, SLC12A5 gene that encodes KCC2 has been shown to transcribe into two mRNA transcripts by usage of an alternative promoter and the first exon, resulting in the expression of two different isoforms, KCC2a and KCC2b (Uvarov et al. 2007, 2009). Although both isoforms are capable of comparable furosemide-sensitive cotransport activity in heterologous expression systems, expression of KCC2a isoform shows only a modest change during postnatal development, while KCC2b expression increases during development and dominates that of KCC2a in the mature neuronal systems.

In the adult CNS, KCC2 protein is expressed in the spinal cord, brainstem, cerebellum, cortex, and hippocampus (Payne et al. 1996). In the embryonic CNS, KCC2 expression increases differentially in different regions in the brain with time in a more or less caudal-rostral direction which corresponds to the neuronal maturation pattern in the brain (Ikeda M. et al. 2003, Li H. et al. 2002, Rivera et al. 1999, Stein et al. 2004, Wang C. et al. 2002). Hence, KCC2 expression is only detectable in differentiated neurons and not in neuronal precursors (Li H. et al. 2002). Also, there are reports of differences in the expression levels of KCC2 in neurons of the same anatomical regions depending upon their maturation level (Li H. et al. 2002, Rivera et al. 1999, Takayama and Inoue 2010). Animals that are prenatally more developed than rodents have a higher level of KCC2 already at birth (Hyde et al. 2011, Rivera et al. 1999, Vanhatalo et al. 2005). Not surprisingly then, KCC2 has come to be regarded as a marker for neuronal maturation (Li H. et al. 2002, Mikawa et al. 2002, Rivera et al. 1999, 2005, Shimizu-Okabe et al. 2002, Stein et al. 2004).

At the cellular level, KCC2 protein shows a punctate expression at the plasma membrane of both the soma and dendrites of hippocampal and cortical neurons but not in the axon terminals (Bartho et al. 2004, Gulacsi et al. 2003, Gulyas et al. 2001, Hubner et al. 2001, Szabadics et al. 2006, Takayama and Inoue 2006, Zhu L. et al. 2005). KCC2 is also highly expressed in the dendritic spines (Gulyas et al. 2001, Zhu L. et al. 2005) where it is

known to play a structural role in establishment and maintenance of spines (see section 1.2.2.4).

1.2.2 Function

The main physiological role of CCCs is regulation of $[Cl^-]_i$. However, under normal physiological conditions, the direction of net chloride transport is different for the NKCCs and the KCCs in most cells. This is based upon the requirement of CCCs (as secondary active transporters) for the electrochemical gradient of the principle ion (Na^+ or K^+) as the energy source for chloride transport (see Fig.1). While NCCs and NKCCs typically transport chloride into the cell, KCCs typically extrude chloride (Kaila 1994). As transport of chloride into and out of the cell is obligatorily accompanied by the influx and efflux of water, respectively, the CCCs can aid in the regulation of cell volume (MacAulay et al. 2004).

Since KCC2 cotransport is electroneutral i.e. carries K^+ and Cl^- in a stoichiometric ratio of 1:1 in the same direction (Kaila 1994, Payne 1997, Williams and Payne 2004), it has no direct effect on the membrane potential (Farrant and Kaila 2007, Mercado et al. 2004). In absence of any voltage-dependence of its cotransporter activity, KCC2 function is also independent of membrane potential but depends only on the sum of chemical potential differences for K^+ and Cl^- which is set by the outwardly directed K^+ chemical potential maintained by Na^+-K^+ ATPase (Farrant and Kaila 2007, Kaila 1994, Payne 1997). Under normal physiological conditions, KCC2 is near its point of thermodynamic flux reversal point (for equilibrium conditions, see Fig.1), hence it can mediate either net ion efflux or net ion influx depending on small changes in $[Cl^-]_i$ and $[K^+]_o$, (see section 1.2.2.3). In addition to K^+, cations like Rb^+, NH_4^+ and Cs^+ can all serve as substrates for KCC2 cotransport and while kinetics of transport of Rb^+ and NH_4^+ are similar to K^+, Cs^+ transport rate is excessively reduced (Williams and Payne 2004). This can serve as a basis for measurement of KCC2 cotransport function by measuring radioactive Rb^+ fluxes (Payne 1997).

In neurons, regulation of $[Cl^-]_i$ by CCCs is essential for determining the properties of inhibitory neurotransmission which is mediated by Cl^- permeant ion channels $GABA_AR$ and GlyR, although both of these channels are also known to be permeable to HCO_3^- (see sections 1.2.2.1 and 1.2.2.3). KCC2 and NKCC1 are the main CCCs involved in controlling the equilibrium potential of chloride ions (E_{Cl}^-) in neurons (Blaesse et al. 2009, Delpire 2000, Payne et al. 2003). In other words, maintenance of E_{Cl}^- and induction of changes in it are thought to be dependent on opposing actions of chloride extrusion by KCC2

and chloride accumulation by NKCC1. Therefore, these two Cl^- cotransporters directly influence the balance of $[Cl^-]_i$ and depending on their relative activities, set E_{Cl}^- either to hyperpolarizing or depolarizing in a spatio-temporal manner (Fig.1).

In addition to its role in maintaining a low $[Cl^-]_i$ for GABAergic hyperpolarizing responses, KCC2 has also been implicated in the regulation of neuronal migration, dendrite outgrowth and formation of excitatory and inhibitory synaptic connections. These functions might dependent on Cl^- efflux activity of KCC2 as well as its ability to interact with cytoskeletal elements (see section 1.2.2.4).

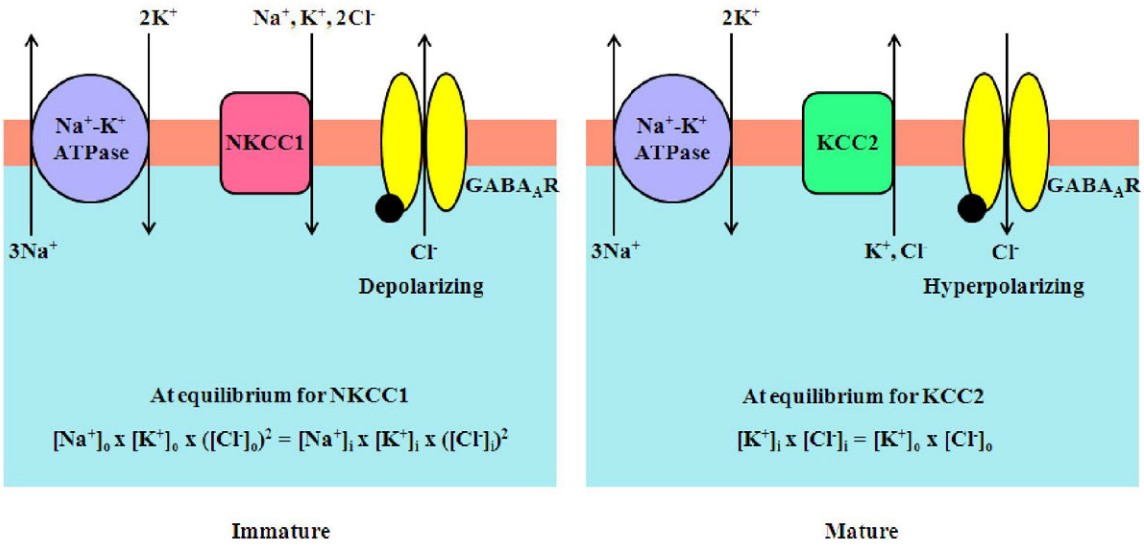

Fig.1. Ion-transport mechanisms underlying $GABA_AR$-mediated responses.

(Left) The dominant cation chloride cotransporter NKCC1 mediates Cl^- uptake in immature neurons, creating a depolarizing E_{Cl}^-. Ionotropic $GABA_AR$ mediates outward directed Cl^- currents. (Right) In mature neurons, KCC2 acts as the principle cation chloride cotransporter and extrudes Cl^-, setting a hyperpolarizing E_{Cl}^-. $GABA_AR$ now allows passage of Cl^- into the cell. Transport by both NKCC1 and KCC2 is fuelled by Na^+ and K^+ gradients generated by the Na^+-K^+ ATPase.

1.2.2.1 KCC2 and $GABA_AR$ hyperpolarizing inhibition

As stated, in the adult mammalian CNS, fast inhibitory neurotransmission is mediated predominantly by $GABA_AR$ and GlyR, which conduct Cl^- down its electrochemical gradient. For hyperpolarizing currents through $GABA_AR$ and GlyR, maintenance of a low $[Cl^-]_i$ (i.e. setting E_{Cl}^- at more negative values than the resting membrane potential) is required so that inward Cl^- flux through these channels can stabilize the membrane potential

at or near the E_{Cl}^-. If E_{Cl}^- is set above the resting membrane potential, currents through $GABA_AR$ and GlyR can be depolarizing and even excitatory as activated $GABA_AR$ and GlyR would allow Cl^- to flow out of the cell (see Fig.1). In developing neurons, GABA is depolarizing (Ben-Ari 2002, Ben-Ari et al. 1989, Cherubini et al. 1991) which is due to a low expression/function of KCC2 and an under-developed chloride extrusion pathway. E_{Cl}^- is set above the resting membrane potential mainly by the predominating chloride influx activity of NKCC1 (Achilles et al. 2007, Brumback and Staley 2008, Kakazu et al. 1999, Sipila et al. 2006, Spitzer 2010, Yamada et al. 2004). As there is a developmental increase in KCC2 expression/function, the relative contribution of chloride extrusion activity of KCC2 in setting the $[Cl^-]_i$ is increased so much so that in mature neurons, E_{Cl}^- is set below the resting membrane potential (Bray and Mynlieff 2009, DeFazio et al. 2000, Hubner et al. 2001, Li H. et al. 2002, Rivera et al. 1999, 2005, Stein et al. 2004, Zhu L. et al. 2008, but see Delpy et al. 2008), causing a shift in GABAergic (and glycinergic) responses from depolarizing to hyperpolarizing (see Fig.1). This shift in GABAergic signaling occurs during early development (first two postnatal weeks in rats). Several studies using different approaches have established a key role of KCC2 in this developmental shift. Consistently, knocking out KCC2 expression (Hubner et al. 2001, Zhu L. et al. 2005), or knocking out KCC2b expression alone (Balakrishnan et al. 2003, Stil et al. 2011, Woo et al. 2002) or knock down of KCC2 expression (Pellegrino et al. 2011, Rivera et al. 1999) prevents or attenuates the shift of E_{GABA-A} from depolarizing to hyperpolarizing. Along similar lines, pharmacological blockade of KCC2 leads to increased $[Cl^-]_i$ levels that can revert the equilibrium potential of $GABA_AR$ (E_{GABA-A}) from hyperpolarizing to depolarizing (DeFazio et al. 2000, Jarolimek et al. 1999, Martina et al. 2001). On the other hand, over-expression of KCC2 in immature neurons leads to decreased $[Cl^-]_i$ levels and a premature shift in E_{GABA-A} from depolarizing to hyperpolarizing (Akerman and Cline 2006, Cancedda et al. 2007, Chudotvorova et al. 2005, Fiumelli et al. 2005, Lee H. et al. 2005, Reynolds et al. 2008). Moreover, mature neurons that have preserved the depolarizing GABA responses or have reduced efficiency of GABA mediated inhibition have been shown to have high levels of $[Cl^-]_i$ in association with low levels of KCC2 expression/function (Banke and McBain 2006, Bartho et al. 2004, Gulacsi et al. 2003, Ikeda M. et al. 2003, Kanaka et al. 2001, Pozas et al. 2008, Toyoda et al. 2005, Ueno et al. 2002, Wang C. et al. 2002, 2005, Vu et al. 2000). In conclusion, it seems like developmental up-regulation of KCC2 expression/function contributes largely to the shift in depolarizing GABA actions to hyperpolarizing. Moreover, changes in KCC2 function in mature neurons is an effective strategy employed by many neuronal systems in inducing

plasticity of inhibitory GABAergic signaling by changes in the $[Cl^-]_i$ (see sections 1.2.2.2 and 1.5).

While the contribution of up-regulation of KCC2 expression/function in the shift of GABA and glycine signaling is well-established, a concomitant down-regulation of NKCC1 function has also been proposed as a contributing factor but has however not been consistently observed (Blaesse et al. 2009). It is relevant to explain the depolarizing and hyperpolarizing actions of GABA based on relative activities of NKCC1 and KCC2 expressed in the plasma membrane in a spatio-temporal manner rather than relying on the absolute expressions of NKCC1 and KCC2 proteins in a neuron (Deisz et al. 2011, Delpy et al. 2008, Gilbert et al. 2007, Marty S. et al. 2002, Vardi et al. 2000, Zhang L. L. et al. 2006). Consistently, distinct sub-cellular localization of KCC2 in soma and dendrites and NKCC1 in axons has been proposed to result in generation of intraneuronal Cl^- gradients and differential GABAergic responses (Banke and McBain 2006, Gavrikov et al. 2006, Jarolimek et al. 1999, Khirug et al. 2008, Szabadics et al. 2006, Vardi et al. 2000, Vu et al. 2000).

Interestingly, both $GABA_AR$ and GlyR can also contribute to inhibitory transmission by shunting mechanism which is independent of the direction of the Cl^- currents (Banke and McBain 2006, Ben-Ari 2002, Farrant and Kaila 2007, Owens and Kriegstein 2002). Indeed, while the depolarization induced by postsynaptic $GABA_AR$ currents can lead to excitation of the neuron, it can also cause shunting inhibition of glutamatergic currents (Lamsa et al. 2000, Palva et al. 2000). Moreover, $GABA_AR$ (and GlyR) is also permeable to HCO_3^- and these HCO_3^- currents can significantly contribute to postsynaptic responses of $GABA_AR$ under certain physiological (Kaila et al. 1987, Kaila 1993, Viitanen et al, 2010) and pathophysiological (Asiedu et al. 2010) conditions in neurons with a reduced Cl^- extrusion capacity. In spite of E_{GABA-A} not being identical to E_{Cl^-} but showing a deviation to more positive values due to permeability to HCO_3^-, E_{GABA-A} is nevertheless mainly set by Cl^- gradient across the neuronal membrane (Blaesse et al. 2009, Farrant and Kaila 2007, Kaila 1994).

1.2.2.2 Neuronal pathologies and KCC2

Synaptic modification in physiological and pathophysiological conditions is critical for both proper functioning of the nervous system and its response to stress and trauma. A decrease in the strength of inhibitory GABAergic transmission has been observed after neuronal trauma and other neuronal pathologies and stresses. An altered intracellular Cl^- homeostasis has been shown to contribute to this decrease in inhibitory GABA signaling (and

even an increase in its depolarizing actions) in several studies (De Koninck 2007, Payne et al. 2003, van den Pol et al. 1996). There are reports of decreased KCC2 expression/function in various nervous system pathologies and diseases like neuronal injury and trauma (Bonislawski et al. 2007, Jin et al. 2005, Nabekura et al. 2002, Toyoda et al. 2003); *in vivo* (Barmashenko et al. 2011, de Guzman et al. 2006, Lee H. H. et al. 2010, Li X. et al. 2008, Pathak et al. 2007, Rivera et al. 2002, Robinson et al. 2010, Shimizu-Okabe et al. 2007, Shulga et al. 2008, but see Mao et al. 2011, Shin et al. 2011, Zhu X. et al. 2012) and *in vitro* (Nardou et al. 2011, Rivera et al. 2004, Wake et al. 2007, Study III) seizure models, and human epileptic tissue (Aronica et al. 2007, Deisz et al. 2011, Huberfeld et al. 2007, Munakata et al. 2007, Munoz et al. 2007, Palma et al. 2006, Shimizu-Okabe et al. 2011); glutamate excitotoxicity (Lee H. H. et al. 2011, Study III); oxidative stress (Wake et al. 2007); ischemic brain injury/oxygen-glucose deprivation (Galeffi et al. 2004, Hershfinkel et al. 2009, Jaenisch et al. 2010, Papp et al. 2008, but see Reid et al. 2000, 2001); Alzheimer-like neurodegenerative pathology (Lagostena et al. 2010); a rodent model of schizophrenia (Liu Y. et al. 2009) and brain tissue from human schizophrenic patients (Hyde et al. 2011); a mouse model of amytrophic lateral sclerosis (ALS) (Fuchs et al. 2010); deafness (Vale et al. 2003); stress (Hewitt et al. 2009, Matrisciano et al. 2010, Sarkar et al. 2011, Tornberg et al. 2005, but see Galanopoulou 2008c); and spinal cord injury-induced neuropathic (Boulenguez et al. 2010, Coull et al. 2003, 2005, Cramer et al. 2008, Hasbargen et al. 2010, Janssen et al. 2011, 2012, Jean-Xavier et al. 2006, Jolivalt et al. 2008, Lu Y. et al. 2008, Miletic and Miletic 2008, Price et al. 2009, but see Tornberg et al. 2005) and peripheral inflammatory pain (Nomura et al. 2006, Zhang W. et al. 2008).

The depolarizing nature of GABA signaling in models of neuronal trauma and pathologies is associated with an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) mainly through Ca^{2+} influx via the voltage gated calcium channels (VGCCs) (Nabekura et al. 2002, Shulga et al. 2008, Toyoda et al. 2003, van den Pol et al. 1996). During development, depolarizing GABA responses promote Ca^{2+} influx through voltage and glutamate gated calcium channels (NMDAR) which contribute to different aspects of neural circuit maturation and refinement including neuronal proliferation and migration, morphological development, and neurite growth and synaptogenesis (Akerman and Cline 2007, Ben-Ari 2002, Ben-Ari et al. 2007, Farrant and Kaila 2007, Fiumelli and Woodin 2007, Galanopoulou 2008b, Ge et al. 2007, Owens and Kriegstein 2002, Represa and Ben-Ari 2005, Wang D. D. and Kriegstein 2009). Depolarizing GABA in immature neurons is also known to induce BDNF expression and release in Ca^{2+}-dependent manner (Berninger et al. 1995, Porcher et al. 2011) which might

also augment the trophic effects of GABA on developing neuronal networks (Owens and Kriegstein 2002, Porcher et al. 2011). Interestingly, hyperpolarizing GABAergic responses in mature neurons do not elicit an increased BDNF secretion but actually depress the latter's expression levels consistent with the hyperpolarizing action of the GABAergic signaling in reducing $[Ca^{2+}]_i$ via inactivation of the L-type VGCCs (Berninger et al. 1995, Porcher et al. 2011).

Along similar lines, premature over-expression of KCC2 and consequent absence of depolarizing glycinergic actions in zebrafish embryos is thought to impair neurogenesis and neuronal differentiation (Reynolds et al. 2008). An up-regulation of functional KCC2 has been found to be necessary to reduce neuronal motility since it acts as a switch in transforming GABA from a motogenic to a stop signal in migrating neurons (Bortone and Polleux 2009). Even in adult neurogenic niches, a similar pattern of GABA signaling is observed (Ge et al. 2006, Reali et al. 2011, Reynolds et al. 2008). In addition, since migrating cells have a higher $[Cl^-]_i$ than settled cells (Bortone and Polleux 2009, Shimizu-Okabe et al. 2002, 2007), a decreased KCC2 function might be involved in migration of neurons in the injured area. A hyperpolarizing to depolarizing shift of GABA signaling and a consequent shift in GABA-mediated BDNF regulation after neuronal trauma and pathology might propel back the surviving neurons into a state of immaturity with greater potential for reformation, re-growth and refinement of neuronal networks after traumatic insults to the mature neuronal networks. Consistently, a number of groups have suggested that a functional decrease in KCC2 and the concomitant shift in GABA responses to depolarizing (or a decrease in its inhibitory hyperpolarizing responses) as well as a change in composition of $GABA_AR$ subunit-composition resembling the development-like state in models of neuronal trauma may be associated with neuronal survival, regeneration and re-establishment of functional connectivity (Cohen et al. 2003, Kahle et al. 2008, Nabekura et al. 2002, Payne et al. 2003, Shulga et al. 2008, 2009, Toyoda et al. 2003, van den Pol et al. 1996). On the other hand, uncontrolled increase in Ca^{2+} influx mediated by depolarizing GABAergic actions in pathophysiological conditions can facilitate activation of neuronal degradation and death; an increased risk of excitotoxicity-induced neuronal degradation; secondary neuronal injury; and pathogenesis of seizure and neuropathic pain (Aronica et al. 2007, Austin and Delpire 2011, Briggs and Galanopoulou 2011, Cohen et al. 2002, 2003, Coull et al. 2005, Fuchs et al. 2010, Galeffi et al. 2000, Jaenisch et al. 2010, Jin et al. 2005, Jolivalt et al. 2008, Kahle et al. 2008, Khalilov et al. 2005, Lee H. H. et al. 2011, Malek et al. 2003, Mao et al. 2011, 2012, Molinaro et al. 2008, Munakata et al. 2007, Nardou et al. 2011,

Pathak et al. 2007, Pellegrino et al. 2011, Pond et al. 2004, Schwartz-Bloom and Sah 2001, Shin et al. 2011, van den Pol et al. 1996).

However there are certain key aspects of GABAergic signaling that have to be considered before ascertaining the physiological relevance of KCC2 down-regulation and a decreased GABAergic inhibition in pathological conditions. GABAergic synapses of mature neuronal circuits operate very close to their switch point. The consequences of GABAergic signaling in a neuronal network is quite complicated and context-dependent in relation to the resting membrane potential, temporal and spatial aspects of excitatory inputs, the heterogeneity of GABAergic neurons and the inter- and intra-neuronal differences in intracellular Cl^- concentration as well as activity-mediated fast changes in the chloride homeostasis and an increase in the physiological significance of its HCO_3^- conductance (Ben-Ari and Holmes 2005, Cossart et al. 2005, De Koninck 2007, Farrant and Kaila 2007, Ikeda M. et al. 2003, Martina et al. 2001, Marty A. and Llano 2005, Owens and Kriegstein 2002, Prescott et al. 2006, Rivera et al. 2005, Staley and Mody 1992, Staley et al. 1995, Tyagarajan and Fritschy 2010, Viitanen et al. 2010, Wright et al. 2011). In addition, the ability of KCC2 to reverse its direction of cotransport under conditions of high extracellular K^+ (see section 1.2.2.3) that accompany increased neuronal excitability in models of neuronal pathologies, makes it difficult to predict whether a functional down-regulation of KCC2 is beneficial or detrimental with certainty. Moreover, the effect of structural interaction of KCC2 with the dendritic cytoskeletal proteins (see section 1.2.2.4) might add an increased facet in the pathological consequences of KCC2 degradation in neuronal trauma and excitotoxicity.

Nevertheless, there are both reports that suggest that a down-regulated KCC2 function and a depolarizing GABAergic signaling might aid the survival and regeneration of neurons as well as those that suggest that KCC2 down-regulation might contribute to a decreased survival and susceptibility of neurons after trauma and other pathologies. Wake et al. (2007) found a decreased viability of cells over-expressing KCC2 in a model of H_2O_2-induced neuronal oxidative stress. While co-application of $GABA_AR$ agonists during H_2O_2 incubation was shown to exacerbate neuronal death, GABA antagonists increased the cell viability (Wake et al. 2007). Maintenance of an increased $[Cl^-]_i$ by an up-regulation of NKCC1 in the injured sensory neurons (which lack KCC2) has been shown to contribute to neuronal regeneration of these injured neurons (Pieraut et al. 2007, 2011). Recently, Pellegrino et al. (2011) have demonstrated that suppression of KCC2 expression and function increased the susceptibility of neurons to oxidative stress and excitotoxicity, while over-expression of functional KCC2 could rescue the neuronal resistance, suggesting that

maintenance of functional KCC2 is essential for neuronal survival after excitotoxic insults. In a model of excitotoxic insult, reduced expression of KCC2 by BDNF-pretreatment rendered $GABA_AR$ agonists neurotoxic which otherwise were neuroprotective in control neurons (Molinaro et al. 2008). An increase in the extracellular Cl^- concentration abolished the neurotoxic effect of the $GABA_AR$ agonists in the BDNF-pretreated neurons, while down-regulation of KCC2 expression prevented the neuroprotective action in controls neurons imposed on with an excitotoxic insult (Molinaro et al. 2008). Neuroprotective action of topiramate against neuronal death in a cerebral ischemia model of brain injury was associated with increased GABAergic inhibitory transmission including an increased expression of KCC2 (Mao et al. 2012). Interestingly, post-traumatic hippocampal neurons become dependent on BDNF trophic support for their survival and this dependence is attributed to depolarizing GABAergic actions that induce intracellular Ca^{2+} increase and cause an upregulation of pan-neurotrophin receptor p75(NTR) (Shulga et al. 2008, 2009, 2012). While recovery from neuronal injury coincides with the recovery of KCC2 expression and function and at this stage the trophic support of BDNF for neuronal survival is no longer required (Shulga et al. 2008), a functional block of NKCC1 can be neuroprotective and abolishes the dependency of the injured neurons for BDNF (Shulga et al. 2012). Probably, KCC2 down-regulation and a decrease in the inhibitory GABAergic signaling have multifaceted consequences on the neuronal system that depend on the type, severity and stage of the trauma as well as on the neuronal type involved.

1.2.2.3 Reversibility of KCC2 cotransport

In addition to the activity-dependent decrease of hyperpolarizing GABAergic signaling by functional down-regulation of KCC2 and a consequent increase in $[Cl^-]_i$, KCC2 has also been proposed to result in substantial decrease in the strength of inhibitory GABAergic signaling by acting as a chloride uptake mechanism in event of increased extracellular K^+ associated with heightened neuronal activity (Kaila 1994, Vinay and Jean-Xavier 2008). This property of KCC2 derives from operation of its cotransport function near equilibrium close to the physiological levels of $[Cl^-]_i$ and $[K^+]_o$ (Farrant and Kaila 2007, Payne 1997, Payne et al. 2003, Rivera et al. 2005). Hence, during periods of high neuronal activity, KCC2 can function as a net K^+-Cl^- influx pathway, thereby reducing the extracellular K^+ levels while at the same time increasing intracellular Cl^- levels leading to a positive shift in E_{GABA-A} and a compromised inhibition (Cordero-Erausquin et al. 2005, Kakazu et al. 2000, Payne et al. 2003). The reversibility of KCC2 cotransport has been

verified experimentally (DeFazio et al. 2000, Hershfinkel et al. 2009, Jarolimek et al. 1999, Kakazu et al. 2000, Viitanen et al. 2010), and may explain activity-dependent decline of inhibitory signaling (Ling and Benardo 1995, Thompson and Gahwiler 1989), whereby repetitive GABAergic transmission results in a biphasic response of $GABA_AR$ to GABA (hyperpolarization followed by a prolonged depolarization). In addition, due to a reduced Cl^- driving force in response to high frequency stimulation, depolarizing HCO_3^- currents through $GABA_AR$ and associated K^+ transients will also aid in this kind of diminishment of inhibitory signaling (Cordero-Erausquin et al. 2005, Kaila et al. 1993, 1997, Viitanen et al. 2010).

1.2.2.4 Structural roles of KCC2

Presence of KCC2 in dendrites and soma which are the main targets of inhibitory inputs (Chattopadhyaya et al. 2004, Szabadics et al. 2006) are explainable by the chloride extrusion function of KCC2 necessary for maturation of inhibitory GABAergic synapses (see section 1.2.2.1). What was intriguing however, was a high KCC2 expression in dendritic spines (Gulyas et al. 2001) which are major postsynaptic targets of glutamatergic inputs (Freund and Buzsaki 1996). Only recently however a cotransport independent structural role for KCC2 in spine formation and development of excitatory synapses, based upon its interaction with protein 4.1N that acts as a scaffold for cytoskeletal interaction with trans-membrane proteins, has been proposed (Li H. et al. 2007). Hence, spine maturation was altered (observable as long, irregularly branched and highly motile dendritic protrusions) and functional excitatory synapses were reduced in KCC2-deficient mice and these abnormalities could be rescued by expression of transport inactive KCC2 mutant (Li H. et al. 2007). On the other hand, suppression of KCC2 expression in mature neurons reduced the efficiency of excitatory neurotransmission by changes in AMPA receptor (AMPAR) membrane distribution in a cotransport independent manner (Gauvain et al. 2011). Premature expression of KCC2 has been shown to cause alterations in dendritic growth and branching, reduced synaptogenesis and impaired neuronal differentiation and maturation (Akerman and Cline 2006, Cancedda et al. 2007, Lee H. et al. 2005, Reynolds et al. 2008) which has been attributed to a premature decrease in $[Cl^-]_i$ and a consequent reduction in GABA depolarization. However, Horn et al. (2010) have implicated a structural role of KCC2 in inducing changes in the cytoskeletal arrangements in the morphological alterations observed upon premature KCC2 expression. They concluded that previous studies based their inferences on involvement of cotransport activity of KCC2 using a transport-inactive mutant (C568A) of KCC2 (Cancedda et al. 2007, Reynolds et al. 2008) which also had a reduced

affinity for 4.1N (Horn et al. 2010). Along similar lines, pre-mature over-expression of KCC2 resulted in a permanent increase in spine density in cortical neurons (Fiumelli et al. 2012). Since the effect could be replicated by over-expression of both the transport inactive N-terminal deleted KCC2 as well as the C-terminal domain of KCC2 but not the C568A KCC2 mutant, the authors proposed an ion-transport independent structural role of KCC2 in excitatory synaptogensis (Fiumelli et al. 2012). However, the structural role of KCC2 cannot alone explain the alterations in neuronal differentiation and maturation in systems with prematurely over-expressed KCC2 based on studies that implicate depolarizing GABA effects which in turn depend on a reduced KCC2 cotransport function (Akerman and Cline 2006, Bortone and Polleux 2009) and studies showing that a reduced excitation in immature neurons by over-expression of inward-rectifier K^+ channel $K_{ir}2.1$ can mimic the effects of premature KCC2 over-expression (Cancedda et al. 2007). Hence, the effects observed in the studies with premature KCC2 over-expression on excitatory signaling seem to involve both its transport-dependent function and its ability to physically interact with 4.1N to change the cytoskeletal elements (Medina and Chudotvorova 2006). Interestingly, a functional blockade of NKCC1 cotransport and hence a lack of depolarizing GABAergic signaling during a critical period has recently been shown to induce changes in the morphology, density and branching of dendrites (Wang D. D. and Kriegstein 2008, 2011) which might also be explained by a proposed transport independent role of NKCC1 in embryo morphogenesis (Walters et al. 2009).

Recently, endogenous KCC2 has been proposed to regulate synaptogenesis even during embryonic stage when its expression is very low. Indeed, deletion of SLC12A5 causes an increased GABAergic and glutamatergic synaptogenesis and increased postsynaptic currents (Khalilov et al. 2011). Although the mechanism by which KCC2 can regulate synaptogenesis in the embryonic stage when it is thought to be incapable of K^+-Cl^- cotransport in most neurons was not studied, the authors proposed that either the structural role of KCC2 or the cotransport function of KCC2 in a sub-population of neurons could be important (Khalilov et al. 2011).

A physical interaction of KCC2 and 4.1N may actually have a more far-reaching corollary than glutamatergic synaptogenesis alone. As shown for several plasma membrane proteins that interact with cytoskeletal elements (Bennett and Baines 2001, Denker and Barber 2002), such an interaction can serve as a functional and/or organizational protein-protein interaction-mediated regulatory mechanism for KCC2 itself and hence may contribute to GABAergic synaptogenesis (Akerman and Cline 2006, Chudotvorova et al. 2005) both

structurally and functionally. Importantly, presence of a K^+-Cl^- cotransport inactive KCC2 early in the development (Balakrishnan et al. 2003, Blaesse et al. 2006, Lohrke et al. 2005, Milenkovic et al. 2007, Vale et al. 2005) may be explained by the morphogenic and structural role played by KCC2 in synapse formation and refinement. Thus, premature expression of KCC2 facilitates formation of functional GABAergic synapses by increasing the density of $GABA_A$Rs (Akerman and Cline 2006, Chudotvorova et al. 2005). Although a decreased $[Cl^-]_i$ level mediated by KCC2 chloride extrusion has been proposed to be the basis of this developmental maturation of GABAergic synapses (Akerman and Cline 2006, Chudotvorova et al. 2005), a structural role of KCC2 as an interacting partner with cytoskeletal elements and $GABA_A$R (Huang Y. et al. 2012) might be interesting to examine. Interestingly, a cotransport independent structural role of KCC2 modulation of the cytoskeletal elements has been found to promote migration and metastasis of cancerous cells (Wei et al. 2011).

In conclusion, KCC2 seems to affect excitatory synaptogenesis directly and independent of the depolarizing GABA-mediated effects (Andang and Lendahl 2008, Gauvain et al. 2011, Horn et al. 2010, Li H. et al. 2007). Hence, because of its involvement in maturation of both inhibitory and excitatory neurotransmission, KCC2 might act as a synchronizing factor for functional development of excitatory and inhibitory neurotransmission (Akerman and Cline 2006, 2007, Ben-Ari 2002, Kanold and Shatz 2006, Li H. et al. 2007).

1.3 Regulation of KCC2 protein synthesis

Since KCC2 is a key molecule involved in the developmental shift of GABA signaling in the CNS (see section 1.2.2.1), it is under tight translational regulation during early development and indeed, a number of factors have been implicated in regulation of KCC2 protein synthesis during development. Developmental up-regulation of KCC2 function in rodent cortical and hippocampal neurons is mainly under transcriptional control. However, there is strong evidence for post-translational regulation of KCC2 function in triggering the GABA shift as well. This and the next sections outline the factors that regulate KCC2 function by translational and post-translational mechanisms.

1.3.1 Regulation of KCC2 expression by transcription factors

A number of transcriptional regulatory sites have been identified in the KCC2 encoding SLC12A5 gene that are thought to be critical for the control of KCC2 expression. These include an E-box element, a dual repressor element-1 (RE-1) site and an early growth

response 4 (Egr4) element. As discussed above, KCC2 has a neuron-specific expression (see section 1.2.1) which was initially attributed to a neuron-restrictive silencer element (NRSE) or repressor element-1 (RE-1) in intron 1 of the SLC125A gene that binds to a silencing factor restrictive to neurons (neuronal restrictive silencing factor, NRSF; also known as repressor element-1 transcription factor, REST complex) and hence does not allow the transcription of genes in non-neuronal cells (Karadsheh and Delpire 2001). Other studies have confirmed a REST-mediated transcription inhibition of the SLC12A5 gene via the RE-1 (Uvarov et al. 2005, Yeo et al. 2009), however the absolute dependence of a RE-1 and REST interaction for the neuron-specific expression of KCC2 is uncertain (Uvarov et al. 2005). Instead of the RE-1 site, a 1.4-kb promoter region upstream of the NRSE-containing intron 1 has been argued to be sufficient in driving the neuron-specific expression pattern of KCC2 (Uvarov et al. 2005). A transcription factor, early growth response protein 4 (Erg4) that binds to SLC12A5 regulatory sequences and positively regulates KCC2 expression, may serve as a contributing factor in specifying the neuron-restricted expression of KCC2, since Egr4 is highly enriched in neurons (Uvarov et al. 2006). Based upon a similar temporal expression pattern of Egr4 and KCC2, the former might also regulate the developmental increase in KCC2 protein (Uvarov et al. 2006). The dual RE-1–REST complex interactions have also been implicated in the up-regulation of KCC2 during postnatal development (Yeo et al. 2009). Lastly, the E-box in the SLC12A5 promoter has also been demonstrated to influence its transcription by interactions with the upstream stimulating factors (USF proteins) (Markkanen et al. 2008).

In addition to the transcription factors that can regulate production of KCC2 mRNAs, the stability of KCC2 mRNAs itself has also been recently shown to be modulated by microRNAs which are non-coding RNA molecules that can regulate gene expression (Bartel 2004). MicroRNA-92 was found to physically interact with KCC2 mRNA and reduce its translation (Barbato et al. 2010). Since microRNA-92 is developmentally down-regulated, it might constitute another cellular mechanism employed in a tight regulation of KCC2 protein expression during development (Barbato et al. 2010).

Lastly, steroid hormones that can act as transcription factors have been shown to regulate the expression of KCC2. Male sex hormone testosterone was found to increase KCC2 expression during development in both sexes while 17β-estradiol decreased KCC2 expression only in neurons with a depolarizing GABAergic signaling in a Ca^{2+}-dependent manner (Galanopoulou 2006, 2008a, Galanopoulou and Moshe 2003). The mechanisms

however, have not been deduced for these hormonal regulations of KCC2 translational changes, although transcription factor cAMP response element-binding protein (CREB) has been implicated (Galanopoulou 2006). Another hormone triiodothyronine (physiologically active form of thyroid hormone thyroxin) which is an amino acid derivative can up-regulate KCC2 functionally during development most probably by post-translational modifications (Friauf et al. 2008), as well as in injured mature neurons (Shulga et al. 2009). Interesting, in adult neurons, thyroxin has been shown to down-regulate KCC2 expression (Shulga et al. 2009).

1.3.2 Regulation of KCC2 expression by neurotrophic factors

Neurotrophic factors play crucial roles in the development of the CNS. They have widespread effects on factors governing CNS development such as neuronal survival, neuronal precursor cell differentiation, neurite outgrowth, synaptogenesis and maturation of neuronal function (Huang E. J. and Reichardt 2003). Hence, the role of neurotrophic factors in regulating KCC2 expression during development is but expected, considering the essential role KCC2 plays in maturation of inhibitory signaling and in structural organization of synapses.

The contribution of brain-derived neurotrophic factor (BDNF) in regulating the expression of KCC2 has been well documented both in the developing and mature neurons. The involvement of BDNF in the developmental up-regulation of KCC2 protein has been proposed by a number of studies (Aguado et al. 2003, Carmona et al. 2006, Ludwig et al. 2011a, 2011b, Rivera et al. 2004, Yeo et al. 2009). Both BDNF and neurturin contribute to an expressional increase in KCC2 transcription through the action on transcription factor Erg4 (Ludwig et al. 2011a, 2011b). BDNF has also been shown to remove the inhibition of REST complex on the dual RE-1 site present in the SLC12A5 gene (Yeo et al. 2009). In addition to BDNF, other trophic factors that act via tyrosine kinase receptors, such as insulin like growth factor-1 (Kelsch et al. 2001) and nerve growth factor (NGF) (Lagostena et al. 2010) have also been implicated in functional and expressional regulation of KCC2 during development. Interestingly, trophic actions of depolarizing GABA that can cause a Ca^{2+} influx through VGCCs in immature neurons have been implicated in the developmental up-regulation of KCC2 expression (Ganguly et al. 2001, Kriegstein and Owens 2001, Leitch et al. 2005). However, this view has been refuted by others (Ludwig et al. 2003, Pfeffer et al. 2009, Sipila et al. 2009, Titz et al. 2003, Wojcik et al. 2006).

As opposed to up-regulatory action of BDNF on KCC2 expression in immature neurons, in the adult brain, BDNF was shown to have an opposite effect of rapid down-regulation of KCC2 function (Huang Y. et al. 2012, Wardle and Poo 2003) and a more sustained down-regulation of its mRNA and protein (Molinaro et al. 2008, 2009, Rivera et al. 2002, 2004). Interesting, both positive and negative regulation of KCC2 expression by BDNF have been shown to be dependent on tropomyosin receptor kinase B (TrkB) signaling (Huang Y. et al. 2012, Rivera et al. 2004, Wardle and Poo 2003). Rivera et al. (2004) have proposed that the opposing effects of BDNF in the developing vs. the mature brain are due to activation of different downstream signaling cascades by TrkB receptors. Interestingly, like its role in up-regulation of KCC2 during development, BDNF can cause an expressional and functional up-regulation of KCC2 in injured mature neurons (Boulenguez et al. 2010, Shulga et al. 2008).

1.4 Post-translational regulation of KCC2 function

Recent studies are just beginning to elucidate the role of post-translational regulation in controlling KCC2 function both in its developmental up-regulation (Balakrishnan et al. 2003, Blaesse et al. 2006, Friauf et al. 2008, Hartmann et al. 2009, Kelsch et al. 2001, Khirug et al. 2005, Lohrke et al. 2005, Milenkovic et al. 2007, Rinehart et al. 2009, Vale et al. 2005) and its down-regulation in neuropathological conditions (Fiumelli et al. 2005, Hewitt et al. 2009, Jin et al. 2005, Lee H. H. et al. 2010, 2011, Sarkar et al. 2011, Vale et al. 2003, Study III). Like most other proteins, functional regulation of KCC2 at the post-translational level can be attributed to modifications in its interaction with self/other proteins, sub-cellular targeting, changes in phosphorylation states and protein degradation. These categories have been discussed separately. However, the division is purely aesthetic and there is no reason to assume them to be independent of each other. Actually, there are examples where two or more strategies may be inter-related (see below).

For KCC2, the large intracellular C-terminal domain harbors the major sites of regulation and conformational stability determinants, the latter not being independent of regulatory protein-protein interactions (Adragna et al. 2004, Bergeron et al. 2006, Strange et al. 2000). Consistently, cotransport function of KCC2 is lost on modifications in the C-terminal domain (Casula et al. 2001, Strange et al. 2000). Although KCC2 cotransport is also abolished upon deletion of its N-terminus (Li H. et al. 2007), relatively little is known how the N-terminal domain of KCC2 affects its cotransport function. Some important proposed

post-translational mechanisms that are thought to regulate KCC2 cotransport have been summarized at the end of section 1.4.4.1 (See Fig. 2).

1.4.1 Regulation of KCC2 activity by protein-protein interaction

Regulation of function of a protein by modulation in its interaction with other proteins is a common phenomenon in cellular biology. The protein-protein interactions can affect the function of a protein in several ways. They can modulate the sub-cellular distribution of the protein by acting as a scaffolding and/or stabilizing factor in relation to its accessibility to accessory proteins, substrates and cofactors and aid in formation of physical complexes or functionally-coupled protein units for the proper functioning in a cellular micro-environment. Protein-protein interactions can also act as a means of allosteric modulation of the protein function. Even though the large intracellular C-terminal domain of KCC2, based upon conservation of its sequence across species, is thought to play a key role in maintaining the functional conformation of KCC2 and in mediating protein-protein interactions that can regulate KCC2 cotransport function, very little is known about how other proteins interact with it to modulate its function.

KCC2 was found to co-localize with $GABA_AR$ receptors (Belenky et al. 2008, Gulacsi et al. 2003, Williams et al. 1999, Vu et al. 2000) and gephyrin, which clusters $GABA_AR$ and GlyR (Gulyas et al. 2001, Tyagarajan and Fritschy 2010), however evidence for a physical interaction between KCC2 and $GABA_AR$ was provided only recently by co-immunoprecipitation (Huang Y. et al. 2012). Brain-type creatine kinase (CKB) has been shown to physically interact with KCC2 and to enhance its chloride extrusion activity (Inoue et al. 2004, 2006). Although there is no evidence that CKB directly phosphorylates KCC2 or influences its total protein or surface expression, it might regulate KCC2 indirectly by its proposed role in regeneration of ATP necessary for ion-driving force of KCC2 for K^+-Cl^- cotransport (see section 1.2.2.1). Moreover, since CKB does not seem to change the global ATP levels in the cells, its indirect 'thermodynamic action' on KCC2 function (Payne et al. 2003) might represent its ability to change local ATP levels (Inoue et al. 2004, 2006). Indeed, the formation of functionally coupled units operating in small micro-environments in the cell membrane seems quite a successful cellular mechanism for maximal utilization of resources, as has also been shown in a study dealing with physical interactions between KCC2 and Na^+-K^+ ATPase α2 subunit and its consequences (Ikeda K. et al. 2004). The α2 isoform was found to play a crucial role in Cl^- homeostasis, not compensated for by the α1 and α3 isoforms. Moreover, like the KCC2-CKB interaction discussed above, the functional interaction of

Na^+-K^+ ATPase α2 with KCC2 did not depend on changes in the latter's protein levels but might have affected KCC2 function thermodynamically by changes in the local K^+ gradient (Ikeda K. et al. 2004). Although it is the α3 isoform that has a characteristic neuron-specific expression (Cameron et al. 1994), the α2 isoform is known to be expressed in embryonic neurons and speculated to be an important modulator of neuronal activity in the neonatal brain (Moseley et al. 2003). Moreover, young hippocampal neurons have been shown to express all three Na^+-K^+ ATPase α subunit isoforms (Cameron et al. 1994). Another noteworthy point is that local K^+ gradients are short-lived and would be expected to affect KCC2 cotransport only slightly. Nevertheless, in view of the similarities between interaction of KCC2 with CKB and Na^+-K^+ ATPase α2, formation of a physically linked and functionally coupled unit comprising KCC2, $GABA_AR$/GlyR, CKB and Na^+-K^+ ATPase α2 subunit might be speculated for a tight regulation of Cl^- homeostasis that is required for the efficient inhibitory GABA/glycine signaling. A developmental regulatory mechanism might then replace the role played by Na^+-K^+ ATPase α2 subunit by that of α3 subunit whose expression dominates over that of α2 isoform in adult neurons. Such a developmentally regulated transition in α2 and α3 mRNA expression has been observed in rat heart muscles between postnatal days, P7 and P14 (Orlowski and Lingrel 1988).

Not surprisingly then, functional tyrosine-phosphorylated KCC2 has been found to exist in lipid rafts, functional micro-domains on the plasma membrane (Watanabe et al. 2009). This is in consistent with studies that demonstrate localization of two key components of inhibitory signaling, $GABA_AR$ and Na^+-K^+ ATPase in lipid rafts micro-domains (Dalskov et al. 2005) and the role of these micro-domains in maintenance of inhibitory synapses as well as dendritic spines (Hering et al. 2003). However, another study has proposed that functional KCC2 is located in the non-raft fraction of the membrane and that the state of membrane-distribution is a key factor for up-regulation of KCC2 function during development (Hartmann et al. 2009).

CCCs have been shown to form homo- and hetero-dimers (Blaesse et al. 2006, Casula et al. 2001, Gerelsaikhan and Turner 2000, Parvin et al. 2007, Pedersen et al. 2008, Simard et al. 2007), although the functional relevance of their oligomerization is obscure. Direct evidence supporting the existence of oligomeric KCC2 in native tissues came from the observations in rat brainstem tissue (Blaesse et al. 2006). While immature neurons harbored monomeric inactive KCC2, in more mature neurons, active KCC2 was shown to exist as a mixture of dimers, trimers, and tetramers (Blaesse et al. 2006, but see Uvarov et al. 2009). Although no direct evidence was provided regarding the contribution of the oligomeric state

of KCC2 to its physiological function, the synchronicity between the increase in the proportion of oligomeric KCC2 and the activation of KCC2 function suggested changes in KCC2 oligomerization state might serve as a mechanism regulating maturation of GABAergic neurotransmission in lateral superior olive (LSO) neurons (Blaesse et al. 2006). Tyrosine phosphorylation of KCC2 that activated KCC2 by changing its localization to lipid raft domains has also been suggested to increase the oligomerized state of KCC2 (Watanabe et al. 2009). Recently, CIP1 (see section 1.1) was found to physically interact with KCC2 and activate it by heteromer formation in a heterologous expression system (Wenz et al. 2009). However, the mechanism of activation was not studied.

Regulator of chromatin condensation (RCC1) domain of protein associated with Myc (PAM) has been demonstrated to physically interact with the C-terminal domain of KCC2 and increase the latter's cotransport activity (Garbarini and Delpire 2008). The interaction of PAM with KCC2 was speculated to activate KCC2 by changes in KCC2 sub-cellular localization, as a scaffolding factor for mediating interaction with other proteins/kinases or membrane trafficking (Garbarini and Delpire 2008). Along similar lines, a di-leucine motif in the C-terminus of KCC2 has been implicated in a physical interaction with clathrin-binding adaptor protein-2 (AP-2) that allocates the functional pool of KCC2 for a constitutive endocytotic recycling pathway (Zhao et al. 2008). As discussed below (section 1.4.2), changes in the rate of insertion and/or retrieval of surface expressed KCC2 can serve as an important regulatory format for modulating the cotransport function of KCC2. Absence of an analogous di-leucine endocytotic motif in NKCC1 might serve as a useful cellular contrivance for differential regulation of chloride extrusion and influx mechanisms in the neurons (Zhao et al. 2008).

Finally, although interaction of KCC2 and cytoskeletal associated protein 4.1N has been proposed to be relevant for structural development of glutamatergic synapses in spines (see section 1.2.2.4), it might serve a role for cytoskeletal docking of functional KCC2 itself at inhibitory synapses on appropriate domains of the plasma membrane and/or regulation of its cotransport activity by direct mechanical effects of cytoskeletal elements. Such functional/organizational regulations are not uncommon for other ion channels and transporters (Bennett and Baines 2001, Denker and Barber 2002).

1.4.2 Regulation of KCC2 activity by changes in surface expression, trafficking and recycling

Changes in activity of plasmalemmal proteins by regulation of their plasma membrane expression can be thought of as a strategy analogous to the regulation of cytosolic proteins by modulating the latter's sub-cellular distribution and their accessibility to substrates. However, as in the latter case, the mere presence of a protein at the cell surface does not necessarily imply activity of the protein. Nevertheless, surface trafficking is clearly one of the initial steps of the cellular mechanism associated with functional activation of membrane proteins. While there is no conclusive data regarding a direct implicit kinetic modulation of KCC2 cotransport function and most studies on regulation of KCC2 activity have not distinguished between the contribution of changes in the expression of KCC2 in the plasma membrane and contribution of its kinetic regulation (Blaesse et al. 2009), some of the reports on KCC2 functional modulation have shown an apparent association of plasma membrane expression with its K^+-Cl^- cotransport function (Boulenguez et al. 2010, Lee H. H. et al. 2007, Rivera et al. 2004, Sarkar et al. 2011, Wake et al. 2007, Zhang L. L. et al. 2006, Study I).

Developmental up-regulation of KCC2 protein causes a shift from depolarizing to hyperpolarizing $E_{GABA\text{-}A}$ in the rat hippocampus in the second week after birth (see section 1.2.2.1). However, in many studies, the expression of total KCC2 protein has been found to be higher than what would be expected based upon the KCC2 functional up-regulation during development (Balakrishnan et al. 2003, Blaesse et al. 2006, Friauf et al. 2008, Kelsch et al. 2001, Khirug et al. 2005, Vale et al. 2005, Zhang L. L. et al. 2006). Hence, it has been proposed that there is a presence of inactive KCC2 in developing neurons that requires post-translation activation. However, these studies, by no means, belittle the essential role of transcriptional effects on KCC2 in mediating a developmental increase in KCC2 function, but indicate that there is probably enough non-functional KCC2 protein in developing neurons that can be activated in an allostatic manner (Sterling 2003).

Even in juvenile (postnatal day, P19-22) rats, only a small fraction of total KCC2 protein (20%) is expressed in the plasma membrane of hippocampal neurons (Study II) at a developmental stage when hippocampal neurons have a hyperpolarizing $E_{GABA\text{-}A}$ (Khirug et al. 2005), and KCC2 mRNA (Rivera et al. 1999, Wang C. et al. 2002) and protein (Stein et al. 2004, Zhu L. et al. 2008, but see Dzhala et al. 2005) expression comparable to adult rats. In heterologous over-expression systems up to 50% of total KCC2 can be membrane expressed (Lee H. H. et al. 2007). The observation of presence of a small fraction

of surface expressed KCC2, combined with the observation that at basal levels, the membrane pool of KCC2 is highly dynamic (Lee H. H. et al. 2007, Rivera et al. 2004, Zhao et al. 2008), suggest that KCC2 function can be dynamically regulated by changes in its surface expression pattern by alterations in the rate of insertion and/or removal from the plasma membrane (Zhao et al. 2008, Lee H. H. et al. 2007, 2010). Regulation of membrane protein activity by retrieval and insertion into the plasma membrane seems to be a general cellular physiological scheme and has been implicated in synaptic plasticity both at inhibitory (Collingridge et al. 2004, Jacob et al. 2008, Vithlani et al. 2011) and excitatory (Collingridge et al. 2004, Greger and Esteban 2007, Groc and Choquet 2006) synapses.

For KCC2, most studies have unfortunately concentrated on the changes in surface expression without attending to the actual mechanisms (changes in rates of insertion or endocytosis of surface KCC2) responsible (Blaesse et al. 2009, Chorin et al. 2011). However, Lee H. H. et al. (2007) did try to address the mechanism of increased surface expression of KCC2 mediated by protein kinase C (PKC) and showed it to be a consequence of a decreased rate of internalization of surface expressed KCC2 (for a similar study that has recently been carried out on the effects of BDNF on $GABA_AR$ in developing neurons; see Porcher et al. 2011). Zhao et al. (2008) have identified the critical role of a di-leucine motif in the C-terminus of KCC2, capable of interacting with clathrin-dependent endocytosis machinery via clathrin-binding adaptor protein-2 (AP-2), in the constitutive endocytosis of surface expressed KCC2. In contrast to Rivera et al. (2004) and Lee H. H. et al. (2010), they showed that the endocytosed KCC2 is not degraded by lysosomal pathway but rather is recycled back to the plasma membrane via a constitutive endosomal endocytotic-recylcing pathway (Zhao et al. 2008). However, to date, no study has addressed the relevance of changes in the insertion rate of KCC2 in the membrane which would be expected to have an inverse effect to the changes in the rate of endocytosis (see also Ortiz 2006 for NKCC2). Unfortunately, there are biochemical methods to quantify the re-insertion of previously endocytosed membrane proteins (Ehlers 2000, Porcher et al. 2011, Zhao et al. 2008) but no biochemical method to analyze the *de novo* insertion of new membrane proteins. We have recently developed a biochemical assay for determining the total rate of insertion of membrane proteins (unpublished data).

Lastly, studies that have indicated the presence of non-functional KCC2 in immature neurons requiring post-translational stimulation (Balakrishnan et al. 2003, Blaesse et al. 2006, Kelsch et al. 2001, Khirug et al. 2005, Milenkovic et al. 2007, Zhang L. L. et al. 2006), might point to an activation step that allows KCC2 to enter a dynamic functional pool

positioned in close proximity with the plasma membrane that is capable of constant recycling in and out of the plasma membrane. Alternatively, the activation step might allow KCC2 already positioned near the plasma membrane to enter the dynamic recycling functional pool. Presence of such a low expression in the plasma membrane and a functional pool of membrane proteins docked in vesicles near the plasma membrane and undergoing continuous recycling have been observed for NKCC2 (Ortiz 2006). Hence, immunohistochemical techniques used to affirm surface expression of KCC2 should be used with caution since the resolution between actual membrane proteins and proteins in transport vesicles near the membrane is not optimal (see discussion in Vale et al. 2005). This also suggests requirement of a biochemical technique for reliable quantification of surface expressed proteins that can be distinguished from proteins that are not expressed on the membrane but are in close proximity or physically linked to the membrane components (see section 1.7 and Study II).

1.4.3 Regulation of KCC2 activity by kinases and phosphatases

There are several predicted putative phosphorylation sites for Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), protein kinase A (PKA), protein kinase C (PKC) and tyrosine kinases in the KCC2 molecule and most are located in the large intracellular C-terminal domain (Adragna et al. 2004, Song et al. 2002). Like other intracellular phosphorylation/dephosphorylation cascades that take part in rapid regulation of protein function, phosphorylation/dephosphorylation of KCC2 acts at the protein level in absence of changes in its expression pattern. Evidently, coordination of these cellular phosphorylation/dephosphorylation events which are critically linked to short-term regulation of KCC2 activity would be expected to ensure that neurons respond to different physiological and pathological conditions rapidly and precisely, given the critical role KCC2 plays in establishing and maintaining the inhibitory signaling of GABA and glycine in the CNS (see sections 1.2.2.1, 1.2.2.2 and 1.5). Recent studies have been directed to identify the protein kinases/phosphatases that are directly involved in regulation of KCC2 function. So far there is data confirming a direct role of tyrosine kinases/phosphatases, PKC and protein phosphatase 1-protein phosphatase 2A (PP1-PP2A) in regulation of KCC2 function (discussed below).

Studies on regulation of KCC2 function by tyrosine kinases and particularly at Y1087 residue of KCC2 have produced conflicting data. Exogenous activation of a cytoplasmic tyrosine src kinase had been previously shown to activate KCC2 in cultured neurons, although no evidence for a direct KCC2 phosphorylation was provided (Kelsch et al,

2001). In line with this study, Y1087 has been suggested to be indispensable for surface expression and function of KCC2 in a heterologous system (Strange et al. 2000). Regulation of the tyrosine phosphorylation state of KCC2 has also been observed in models of neuronal stress. For instance, exposure of neurons to oxidative stress and induction of epileptic activity by exposure to an extracellular solution lacking Mg^{2+} or BDNF rapidly increased the net rate of tyrosine dephosphorylation of KCC2 in a Ca^{2+}-dependent manner, which preceded a later phase of a reduction in the total levels of KCC2 mRNA and protein. This fast decrease in tyrosine phosphorylation was observed concomitantly with a rapid decrease in the chloride extrusion capacity of KCC2 which was ascribed to a decreased surface expression of KCC2 (Wake et al. 2007). However, the exact site of phosphorylation in the KCC2 molecule was not identified. Recently, phosphorylation of Y1087 has been reported to change the localization of plasmalemmal KCC2 in the membrane and cluster them in lipid rafts on the cell membrane and this change was shown to be associated with an increased KCC2 function (Watanabe et al. 2009). In contradiction however, Lee H. H. et al. (2010) have shown that phosphorylation of tyrosines at 903 and 1087 mediated by src family of tyrosine kinases decreases the cell surface stability of KCC2 by enhancing their lysosomal degradation in a pilocarpine-induced seizure model. Along similar lines, tyrosine phosphorylation has been implicated in neuronal injury-induced degradation of KCC2 (Shulga et al. 2012). The contradictory results of tyrosine kinase-mediated regulation might be explained by employment of different heterologous systems for KCC2 expression and/or the indirect modification by the kinases at different steps of cellular cascades.

A serine at position 940 in KCC2 was proposed to be important in interaction with 4β-phorbol 12-myristate 13-acetate (PMA)-induced effectors, most likely, PKC and PKC-mediated actions were found to decrease KCC2 function (Bergeron et al. 2006). Henceforth, S940 was indeed shown to be a target of a dynamic phosphorylation-dephosphorylation cycle mediated by PKC and PP1 activity, critical for functional regulation of KCC2 (Lee H. H. et al. 2007, 2011, Sarkar et al. 2011). However, in contradiction to Bergeron et al. (2006), PKC was shown to cause a functional increase of KCC2 by a direct phosphorylation at S940 in KCC2. The functional increase coincided with an increase in the surface expression of KCC2 which in turn, depended on an increased stability of KCC2 expressed in the plasma membrane caused by a decreased internalization of surface expressed KCC2 (Lee H. H. et al. 2007, see also Study II). Accordingly, in an NMDA-induced model of neuronal trauma, phosphorylation of KCC2 at S940 was decreased by activation of PP1 mediated by Ca^{2+} influx through NMDAR (Lee H. H. et al. 2011). This fast decrease in S940

phosphorylated KCC2 was concomitantly paralleled by a fast reduction in both total and surface protein levels of KCC2. Stress-induced decrease in KCC2 function has also been demonstrated to be mediated by a dephosphorylation step of S940 (Sarkar et al. 2011). In agreement with role of PKC phosphorylation of S940 of KCC2 in its functional up-regulation, activation of group I metabotropic glutamate receptors (mGluR1s) has been shown to rapidly increase KCC2 chloride extrusion activity in a PKC-dependent manner (Banke and Gegelashvili 2008).

However, consistent with the studies of Bergeron et al. (2006), another study has proposed a PKC-mediated rapid down-regulation of KCC2 function (Fiumelli et al. 2005). Neither of these studies has specified a direct role of PKC in KCC2 phosphorylation. Hence, it may be possible that the PKC mediated down-regulation of KCC2 function in these cases were not due to a direct phosphorylation on KCC2. There are similar contradictory reports of PKC-mediated activation and inhibition of NKCC1 activity which have been proposed to be a result of indirect multistep regulation by PKC (Russell 2000). Interestingly, Brumback and Staley (2008) suggested that changes in Na^+-K^+ ATPase activity are responsible for activity-induced changes in E_{GABA-A} that were observed by Fiumelli et al. (2005) and attributed to KCC2 (Woodin et al. 2003). Since PKC-mediated increase in KCC2 function has been shown to involve a direct phosphorylation of KCC2 (Lee H. H. et al. 2007, 2011, Sarkar et al. 2011), it is the most conceivable scheme concerning the direct modulation of KCC2 activity by PKC.

Only one study has shown a PKA-mediated functional modulation of KCC2 (Ouardouz and Sastry 2005). However, it fell short of describing any direct phosphorylation of KCC2. Surprisingly, inhibitors against both PKA and protein phosphatase could prevent the observed functional modulation of KCC2 (Ouardouz and Sastry 2005).

With no lysine (WNK) kinases and their downstream Ste20-related proline-alanine-rich-kinase/oxidative stress response kinase 1 (SPAK/OSR1) are thought to play an important role in controlling electrolyte homeostasis by regulation of the activity of a number of ion transporters, particularly the CCCs which are important regulators of cell volume (Delpire and Gagnon 2008, Kahle et al. 2005, 2006, MacAulay et al. 2004). There have been many studies on cell volume-dependent regulation of KCC2 function by WNKs but none of them have provided evidence for a direct phosphorylation of KCC2. Also, most of the studies have utilized heterologous non-neuronal systems. It is important to realize that while stimulation of NKCC1 activity after cell shrinkage in neurons has been documented (Schomberg et al. 2003), no such cell volume-mediated regulation of KCC2 activity has been

observed. Moreover, KCC2 expression is restricted to neurons and is thought to have a specific physiological function of maintaining a low $[Cl^-]_i$ in isotonic conditions in neurons (see section 1.2.2.1). An absent (Payne et al. 1996) and modest (Song et al. 2002, Strange et al. 2000) activation of KCC2 cotransport function in hypotonic conditions in heterologous systems indicates an obscure physiological role of swelling-induced KCC2 activity in neurons (Williams et al. 1999, also see discussion in Song et al. 2002). While neurons are relatively resistant to swelling in response to osmotic changes due to absence of aquaporin expression, the mechanisms of cell swelling induced by hypotonia and neuronal swelling induced by intense activity are different (Blaesse et al. 2009, Payne et al. 2003) and hence data from cell volume mediated-regulation of heterologously expressed KCC2 under conditions of hypotonia cannot be extrapolated to physiological regulation of KCC2 function in situations of activity-induced neuronal swelling. Although KCC2 was proposed to limit dendritic swelling in response to glutamatergic excitation based upon its expression near the excitatory synapses (Gulyas et al. 2001), it was later shown to be structurally important for spine formation (see section 1.2.2.4). Indeed, while hypotonicity-induced activity of KCCs depends upon serine-threonine phosphatses, the constitutive isotonic activity of KCC2 was found to be independent of them (Mercado et al. 2006, Song et al. 2002). Hence, cell volume-dependent modulation of KCC2 in heterologous systems may not be as relevant in the physiological regulation of KCC2 in the neuronal micro-environment as for other hypotonicity-activated KCC isoforms (Kahle et al. 2008, Mercado et al. 2004), particularly KCC3, which has been shown to be involved in neuronal volume regulation based upon knock-out studies (Boettger et al. 2003, Byun and Delpire 2007) and KCC1, based upon its expression in nervous system and its ability for housekeeping cell-volume regulatory function (Gillen et al. 1996, Kanaka et al. 2001). Interestingly, the presence of a SPAK binding motif and several potential threonine and serine phosphorylation sites in KCC2a but not in KCC2b (Uvarov et al. 2007) might indicate differential cell-volume based regulation of the two KCC2 isoforms.

Nevertheless, in the context of cell volume-dependent regulation, serine-threonine phosphorylation is thought to reciprocally increase the cotransport function of NCC and NKCC1-2 and decrease the transport function of KCCs (Gagnon et al. 2006, Kahle et al. 2006, Rinehart et al. 2009, 2011). Thus, WNK3 reciprocally regulates the chloride efflux (by inhibition of KCC1 or KCC2) and influx (by activation of NKCC1) in neurons (Kahle et al. 2005) and may be important in the physiology of circadian variations of GABA signaling in the CNS (Belenky et al. 2010, Kahle et al. 2006). WNK4 and its binding partner SPAK can

also inhibit chloride extrusion activity of KCC2, while reciprocally acting as a positive regulator of NKCC1 function (Gagnon et al. 2006, Garzon-Muvdi et al. 2007). Recently, WNK2-mediated phosphorylation has been implicated in decreasing KCC2 function and increasing NKCC1 function (Rinehart et al. 2011). Although the authors showed an abundant WNK2 expression in the brain, the indirect evidence for KCC2 modulation relies on studies in *Xenopus* heterologous expression system. Interesting, while WNK2 phosphorylation caused an increased surface expression of NKCC1, changes in KCC2 surface expression were not addressed (Rinehart et al. 2011). Rinehart et al. (2009) have identified two phosphorylation sites which are conserved among all KCC isoforms and can be modulated during development and by physiological perturbations and contribute to functional regulation of KCCs. Thus, KCC3 was found to be dephosphorylated at T991 and T1048 in response to hypotonicity and this caused a cooperative activation of KCC3. The homologous threonine phosphorylation sites in KCC2, T906 and T1006, were shown to be developmentally regulated i.e. neonatal brain had phosphorylated KCC2 while the adult brain lacked it. Based upon the finding that phosphorylation states of in KCC3 is sensitive to WNK1 and PP1 and PP2A and leads to modulation of the intrinsic transport function of KCC3 without affecting the surface expression, cotransport function of KCC2 was proposed to be regulated in an analogous manner. However, direct involvement of any kinase or phosphatase in regulation of the phosphorylation status of T906 and T1006 in KCC2 was not addressed (Rinehart et al. 2009).

There is some indirect evidence that KCC2 function is positively regulated by PP1-PP2A under hypotonic conditions. This is based upon data that inhibition of PP1-PP2A abolishes swelling-induced chloride extrusion activity of KCC2 but does not seem to have any effect on the constitutive chloride extrusion function of KCC2 (Mercado et al. 2006). But other reports suggest that protein phosphatases can induce changes in KCC2 function in isotonic conditions. Hence, unhindered activity of PP1-PP2B by expression of inactive forms of WNK4 kinase was suggested to increase KCC2 activity in isotonic conditions (Garzon-Muvdi et al. 2007). Recently, NMDA and Ca^{2+} induced activation of PP1 was shown to dephosphorylate S940 residue in KCC2 in the glutamate excitotoxicty model of trauma (Lee H. H. et al. 2011). Thus while PKC mediated phosphorylation increased the surface stability of KCC2 (Lee H. H. et al. 2007), PP1-mediated dephosphorylation decreases surface stability of KCC2 which is ultimately degraded (Lee H. H. et al. 2011). However, the how dephosphorylation of S940 leads to degradation of KCC2 protein was not demonstrated.

In conclusion, it seems likely that KCC2 function is dynamically regulated by a differential activation of specific protein kinases and phophatases in distinct neuron types and under distinct conditions.

1.4.4 Regulation of KCC2 activity by protein degradation

As previously described, KCC2 expression levels show an activity-dependent decrease in various pathological and physiological conditions (see section 1.2.2.2). Although there are observations of degradation of KCC2 at both mRNA and/or protein levels in these studies, the relative contributions of transcriptional, post-transcriptional and post-translational regulations of KCC2 function have remained obscure. Recent studies have advocated for post-translational regulation of KCC2 function (including protein degradation) that could account for the fast decrease in many of these models (Fiumelli et al. 2005, Lee H. H. et al. 2011, Wake et al. 2007, Study III), suggesting that regulation of KCC2 at the level of translation takes effect only at a later stage. Moreover, there seem to be two phases of functional decrease of KCC2 at the protein level – an initial rapid phase that might involve reduced surface expression of KCC2 followed by a delayed and sustained phase that most probably involves protein degradation (Kitamura et al. 2008, Lee H. H. et al. 2011, Wake et al. 2007, Wardle and Poo 2003) that might consolidate the initial effect.

Some studies suggested a fast degradation of surface expressed and total KCC2 under basal conditions (Lee H. H. et al. 2010, Rivera et al. 2004). Hence, it was thought that the decrease in KCC2 protein amount was an indirect effect of KCC2 mRNA degradation. However, using experimental blockade of both protein synthesis as well as protein degradation, we show that in brain slice preparations, KCC2 protein is much more stable than previously thought (Study III). This seems a bit surprising since unlike other KCCs, KCC2 C-terminal domain harbors two predicted PEST (proline/glutamate/serine/threonine) sequences (Mercado et al. 2006) which are thought to signal degradation of a protein. However, as opposed to an active PEST domain, PEST domains that are latent can serve as signals for regulated degradation of proteins (Rechsteiner and Rogers 1996, Rogers et al. 1986). This suggests that PEST sequences in KCC2 might be latent and activated in pathophysiological conditions by post-translational modifications like phosphorylation/dephosphorylation for its lysosomal degradation (Lee H. H. et al. 2010).

Interestingly, a number of studies have implicated Ca^{2+} influx through activated NMDAR in activity-dependent decrease of KCC2 expression/function (Kitamura et al. 2008, Lee H. H. et al. 2011, Liu Y. et al. 2009, Wang W. et al. 2006). In Study III, we have shown

that KCC2 can indeed be down-regulated by calpain-mediated proteolysis (see next section). In summary, proteolysis/degradation of KCC2 protein by calpain or lysosomal activity might function as a fast mechanism of changing neuronal chloride homeostasis in various physiological and pathophysiological states.

1.4.4.1 Calpain and KCC2

Calpains are calcium-activated cysteine endoproteases that act at neutral pH (Croall and DeMartino 1991, Goll et al. 2003, Saido et al. 1994). Out of a dozen, two calpain isoforms – calpain-1 (μ-calpain) and calpain-2 (m-calpain) are abundantly expressed in neurons and localize in both soma and synaptic terminals (Liu J. et al. 2008a, Zadran et al. 2010a). While calpain-1 requires 1-20 μM Ca^{2+} for activation, calpain-2 needs Ca^{2+} in mM concentration ranges for activation *in vitro* (Goll et al. 2003, Liu J. et al. 2008a, Zadran et al. 2010a). Since an increase of $[Ca^{2+}]_i$ concentration to μM ranges is associated with conditions of hyperexcitability because of opening of glutamate and voltage gated calcium channels, calpain-1 has been shown to be activated in response to excessive exposure to glutamate or agonists of glutamatergic receptors. Interestingly, interaction of both calpains with phospholipids in sub-membrane cellular regions can decrease the calcium requirement for activation (Chakrabarti et al. 1996, Saido et al. 1994, Shao et al. 2006) and might result in their localized and short activation. Indeed, controlled activation of calpain-1 and 2 by interaction with phospholipids in a sub-membrane microenvironment near the source of calcium influx might explain why many calpain substrates are membrane expressed or associated proteins and might be critical for calpain-mediated regulation of synaptic signaling of neuronal networks (Liu J. et al. 2008a). Recently, calpain-2 has been shown to be activated by BDNF-TrkB-MAPK signaling independent of $[Ca^{2+}]_i$ (Zadran et al. 2010b).

Calpains have been implicated in regulation of widespread neuronal functions like neural development and gene transcription; synaptic plasticity and structural stability of synapses; neurotransmitter release and vesicular trafficking (Goll et al. 2003, Liu J. et al. 2008a, 2008b, Lynch G. and Baudry 1984, Shiosaka 2004, Tomimatsu et al. 2002, Vanderklish et al. 1995, Wu H. Y. and Lynch 2006, Zadran et al. 2010a, 2010b). Interestingly, calcium overload-induced unregulated and hyperactive calpain-mediated proteolysis has been proposed to contribute to the mechanisms of neuronal death (Liu J. et al. 2008a, Wu H. Y. and Lynch 2006) observed in models of excitotoxicity (Bano et al. 2005, Liu J. et al. 2008b, Siman and Noszek 1988, Siman et al. 1989, Xu et al. 2007), and pathogenesis of excitotoxicity-related pathologies like ischemic (Bartus et al. 1994, 1995,

Bevers and Neumar 2008, Lee K. S. et al. 1991, Schafer et al. 2009) and traumatic brain injuries (Kampfl et al. 1997, Pike et al. 2001, Posmantur et al. 1997), seizures (Araujo et al. 2008, Bi et al. 1996a, 1997, Feng et al. 2011, Holopainen 2008, Xu et al. 2007) and neurodegenerative diseases like Alzheimer's disease (Lee M. S. et al. 2000, Liu F. et al. 2005, Raynaud and Marcilhac 2006, Saito et al. 1993). However, several reports suggest that calpain might have complex roles aiding both toxic and protective aspects of pathophysiology of neurodegeneration, probably depending on the kind, severity and stage of insult (Faddis et al. 1997, Lynch D. R. and Gleichman 2007, Neumar et al. 2003, Pike et al. 1998, Wang K. K. 2000, Wu H. Y. and Lynch 2006).

As a regulatory protease, not surprisingly, only a relatively small number of proteins have been identified as substrates for calpain. These include cytoskeletal proteins, transcription factors, membrane receptors, ion channels, and transporters (Goll et al. 2003, Liu J. et al. 2008a, Lynch D. R. and Gleichman 2007). Moreover, calpain-cleavage results in limited fragmentation of proteins even under *in vitro* conditions (Goll et al. 2003, Saido et al. 1994). Calpain-mediated fragmentation can either modulate, activate or abolish the substrate protein's original function (Kishimoto et al. 1989, Saido et al. 1994) or the fragments generated might acquire novel functions such as regulation of gene transcription (Lynch D. R. and Gleichman 2007).

Of the various physiological processes regulated by calpain, particularly interesting are those involved in maintenance and plasticity of synaptic signaling. Calpain proteolyzes and regulates the function of proteins involved in structural, functional and regulatory aspects of glutamatergic synaptic signaling. Hence, cytoskeletal and associated proteins like spectrin (Seubert et al. 1989, Siman et al. 1984), ankyrin-rich membrane spanning protein (ARMS) (Wu S. H. et al. 2010), microtubule associated protein (MAP) (Sandoval and Weber 1978, Siman and Noszek 1988) as well as proteins that function as ion channels and as mediators of intracellular signaling in response to extracellular cues like NMDAR (Bi et al. 1998b, Guttmann et al. 2001), AMPAR (Bi et al. 1998a, 1996b) and mGluR subunits (Xu et al. 2007), and their anchoring proteins like glutamate receptor-interacting protein (GRIP) (Lu X. et al. 2001) and postsynaptic density protein-95 (PSD-95) (Lu X. et al. 2000) have been shown to be calpain targets. In addition, kinases and phosphatses like PKC (Kishimoto et al. 1989), calmodulin-dependent protein kinase II (CamKII) (Hajimohammadreza et al. 1997) and calcineurin (Liu F. et al. 2005) that can modify glutamatergic signaling are also cleaved by calpain. Evidently, previous studies have concentrated on the effects of calpain activation on excitatory glutamatergic synaptic

signaling. Only recently have studies shown that calpain can also regulate synaptic signaling at GABAergic synapses by modulation of the presynaptic (Gomes J. R. et al. 2011) as well as postsynaptic (Kawasaki et al. 1997) machineries. Hence, a vesicular GABA transporter (VGAT) was shown to be a calpain substrate in models of ischemic injury, glutamate-excitotoxicity and seizure-like activity (Gomes J. R. et al. 2011). Similarly, glycine transporter 2 (GlyT2) has also been shown to be a calpain substrate (Baliova et al. 2004). Gephyrin, a critical scaffolding protein for the postsynaptic $GABA_AR$ clustering (Jacob et al. 2005, Tyagarajan and Fritschy 2010) was shown to be regulated by calpain-mediated cleavage (Kawasaki et al. 1997). In Study III, we have shown that KCC2 is a target of calpain proteolysis *in vitro* as well as in models of excitotoxicity and seizure-like activity. Interestingly, calpain cleavage of KCC2 *in vitro* does not seem to require the presence of its PEST domains (unpublished data). This is not surprising since the absolute requirement of PEST domains in calpain-mediated degradation of PEST-containing proteins is obscure (Carillo et al. 1996, Rechsteiner and Rogers 1996, Tompa et al. 2004). Indeed, calpain-recognition seems to require disordered secondary and tertiary structural determinants rather than the primary structure of proteins alone (Stabach et al. 1997, Tompa et al. 2004). Alternatively, the latent PEST domains in KCC2 might be activated by phosphorylation/de-phosphorylation of nearby residues (Lee H. H. et al. 2011) for recognition by calpain.

Since KCC2 is a protein that has an important functional role in GABAergic signaling as well as a structural role in glutamatergic signaling (see sections 1.2.2.1 and 1.2.2.4), the observation that KCC2 is a calpain target (Study III) has physiological consequences for both inhibitory and excitatory signaling in the neuronal networks. Importantly, calpain cleaves KCC2 in the C-terminal region (unpublished data) that has been shown to interact with protein 4.1N which is critically important in cytoskeletal organization at synapses (see section 1.2.2.4). Moreover, as stated above, calpain-2 can be activated by BDNF-TrkB-MAPK signaling in neurons and rearrangements in the actin cytoskeletal at synapses by BDNF-calpain signaling (Bramham 2008, Glading et al. 2004, Zadran et al. 2010b) might employ proteolysis of KCC2 and modulation of its structural role as one of the strategies. The link between calpain and KCC2 might also be speculated to serve as one of the mechanisms employed by BDNF in regulation of KCC2 function and hence regulation of synaptic signaling at inhibitory synapses under pathophysiological conditions (see sections 1.2.2.2, 1.3.2 and 1.5).

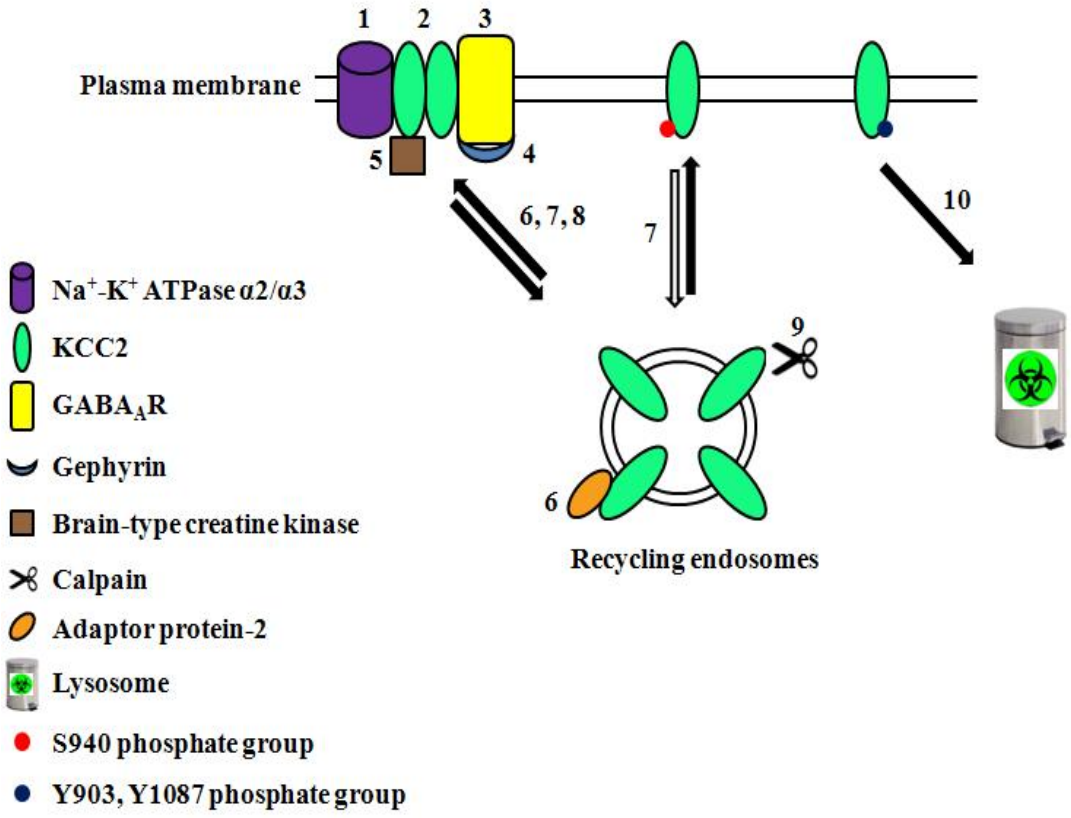

Fig.2. Known post-translational mechanisms affecting KCC2 cotransport.

A number of mechanisms can regulate KCC2 function including protein-protein interactions, phosphorylation as well as protein degradation. The mechanisms have been described in detail in the text. The key for the various molecules used is shown on the left. Open arrow denotes a reduced rate of retrieval from the membrane. The numbers in the figure represent the citations from which the data is derived from. These are as follows-1: Ikeda K. et al. 2004, 2: Blaesse et al. 2006, 3: Huang Y. et al. 2012, 4: Gulyas et al. 2001, 5: Inoue et al. 2006, 6: Zhao et al. 2008, 7: Lee H. H. et al. 2007, 8: Study II, 9: Study III, 10: Lee H. H. et al. 2010.

1.5 Activity-dependent regulation of KCC2 in developmental and pathophysiological regulation of Cl^-

Some parts of this section have been discussed elsewhere in this thesis, but have been included here to summarize the topic. Several studies have shown an activity-dependent modulation of KCC2 function/expression and the consequent changes in inhibitory neurotransmission mediated by $GABA_AR$ signaling which may have a profound effect on the overall synaptic homeostasis (Fiumelli and Woodin 2007). Interestingly, the changes in

KCC2 function mediated by activity seem to be dependent on the developmental status of the neurons (Wright et al. 2011).

Depolarizing actions of GABA (and glycine) in immature neurons have been proposed to induce KCC2 expression in a Ca^{2+}-dependent manner and accelerate the developmental shift in GABA signaling from depolarizing to hyperpolarizing (Bray and Mynlieff 2009, Galanopoulou and Moshe 2003, Galanopoulou et al. 2003, Ganguly et al. 2001, Leitch et al. 2005, Shibata et al. 2004). Interestingly, the shift in GABAergic signaling paralleled by an up-regulated KCC2 expression was accelerated by maternal separation as well as recurrent seizures in immature male rats and was $GABA_AR$-dependent (Galanopoulou 2008c). Even a single episode of status epilepticus can rapidly increase the functional expression of KCC2 in the plasma membrane without changing its protein levels (Study I). However, other studies have demonstrated a lack of effect of GABA mediated depolarizing synaptic transmission on KCC2 developmental up-regulation (Ludwig et al. 2003, Pfeffer et al. 2009, Titz et al. 2003, Wojcik et al. 2006). The importance of compensatory changes in maintaining synaptic homeostasis (Kilman et al. 2002, Liu G. 2004, Marty S. et al. 2002, Mody 2005, Turrigiano and Nelson 2004, Tyagarajan and Fritschy 2010, Wang D. D. and Kriegstein 2008, 2011) should not be ignored after disallowing GABA-mediated depolarization and subsequent Ca^{2+} transients in immature neurons. Indeed, knocking out or pharmacological block of NKCC1 that causes a block of GABAergic depolarization, leads to an increased excitability of glutamatergic signaling and has little effect on KCC2 developmental up-regulation (Sipila et al. 2009). KCC2 up-regulation may require only sub-threshold membrane depolarization that is sufficient to open low-threshold L-type VGCCs and cause an increase in $[Ca^{2+}]_i$ (Bray and Mynlieff 2009, Fiumelli and Woodin 2007).

There are reports that have implicated excitatory glutamatergic inputs in the developmental up-regulation of KCC2 in immature neurons (Kanold and Shatz 2006, Liu Y. et al. 2009, Shibata et al. 2004, but see Ganguly et al. 2001, Ludwig et al. 2003, Titz et al. 2003). In addition to synaptic activation of glutamatergic ionotropic receptors and the consequent depolarization ensued by it, activation of group I metabotropic glutamatergic receptors (mGluR1s) have also been shown to rapidly up-regulate KCC2 function in a PKC-dependent manner (Banke and Gegelashvili 2008). Recently, vesicular Zn^{2+} which is released along with glutamate has been demonstrated to increase the surface expression and activity of KCC2 via metabotropic zinc-sensing receptor (mZnR) in a Ca^{2+}-dependent manner in hippocampus of juvenile (P12-15) mice (Chorin et al. 2011). Spontaneous nicotinic

cholinergic activity has also been suggested to up-regulate KCC2 during development and contribute to the shift in GABA signaling (Liu Z. et al. 2006).

Another approach to test whether neuronal activation promotes maturation of inhibitory GABAergic transmission via an up-regulation in KCC2 function/expression is by studying the effects of sensory deprivation on the GABAergic shift. Cochlear ablations prior to onset of hearing (Shibata et al. 2004), rearing of turtles in dark (Sernagor et al. 2003) and removal of synaptic inputs from the brainstem by spinal cord-transection (Jean-Xavier et al. 2006) have all been shown to prevent the developmental GABA switch in the respective sensory centers.

Whether or not GABA is required for its own depolarizing to hyperpolarizing shift and whether direct effects of depolarizing GABA or a consequent downstream action of neurotrophins (see below) is the actual trigger is debatable, especially considering that blocking GABA depolarization in immature neurons only delays the GABAergic switch and does not abolish it. However, what is certain is that sources of depolarization in the immature neurons do play an important role in maturation of inhibitory neurotransmission (Gaiarsa 2004, Gubellini et al. 2001), in part by postsynaptic mechanisms of an increase in KCC2 expression/function (Fiumelli and Woodin 2007, Shibata et al. 2004). However, since depolarization-induced Ca^{2+} transients (Katz and Shatz 1996) as well as neurotrophic factors (Gottmann et al. 2009) affect almost all developmental programs in neurons, it is difficult to examine whether their effect on the up-regulation of KCC2 function/expression is direct and specific.

Nevertheless, neurotrophic factors, in particular, BDNF have been suggested to play a critical role in developmental KCC2 up-regulation (see section 1.3.2). BDNF shows an increased activity-dependent synthesis and release by GABA mediated depolarization in a Ca^{2+}-dependent manner in developing neurons (Berninger et al. 1995, Marty S. et al. 1996, Porcher et al. 2011) but a reduced release by hyperpolarizing GABA transmission in more mature neurons (Berninger et al. 1995, Obrietan et al. 2002, Porcher et al. 2011). Since kainate-induced seizures have been shown to increase the expression of BDNF in developing rats (Kornblum et al. 1997, Yokoi et al. 2007), the kainate-induced increase in the total (Galanopoulou 2008c) and plasma membrane expressed (Study I) protein levels of KCC2 might be mediated by BDNF-TrkB signaling (unpublished data). However in mature neurons, BDNF depresses KCC2 expression/function (see above). This is somewhat similar to the proposed age-specific effects of GABA on KCC2 expression. While $GABA_AR$ agonists increase the expression of KCC2 in immature (male) neurons of substantia nigra that exhibit

depolarizing GABA currents, they decrease KCC2 expression in (female) substantia nigra neurons that already have hyperpolarizing GABA responses at a similar age (Galanopoulou 2007, 2008c, Galanopoulou and Moshe 2003, Galanopoulou et al. 2003).

In mature neuronal networks, activity-dependent mechanisms reduce GABAergic inhibitory responses in physiological and pathophysiological states by depressing KCC2 function/expression (see section 1.2.2.2). Periods of high frequency activity like postsynaptic spiking alone (Fiumelli et al. 2005) or coupled with GABAergic transmission (Balena et al. 2010, Ormond and Woodin 2011, Woodin et al. 2003) can lead to fast reduction in KCC2 function in Ca^{2+}-dependent manner, most likely by changes in its surface expression which is manifested as a decrease in inhibitory GABA signaling (Fiumelli et al. 2005, Woodin et al. 2003).

In addition to spiking activity, glutamatergic activity regulates $[Cl^-]_i$ by changes in expression/function of KCC2 and thus contributes to efficiency of inhibitory GABAergic signaling. Hence, induction of long term potentiation (LTP) in hippocampal neurons (Liu Y. et al. 2009) and application of glutamate or repetitive activation of glutamatergic neurons (Kitamura et al. 2008) have been shown to induce a decrease in KCC2 expression and function in an NMDA-dependent Ca^{2+}-mediated manner. In pathological glutamate excitotoxicity (Lee H. H. et al. 2011, Study III) and during seizures (see section 1.2.2.2), KCC2 function undergoes a fast decrease.

As stated above, neurotrophins like BDNF have also been proposed to decrease KCC2 expression and function in mature neuronal networks in an activity-dependent manner in physiological and pathophysiological conditions (Boulenguez et al. 2010, Coull et al. 2005, Huang Y. et al. 2012, Mao et al. 2011, Miletic and Miletic 2008, Molinaro et al. 2008, Rivera et al. 2002, 2004, Wake et al. 2007, Wardle and Poo 2003, Zhang W. et al. 2008). Since BDNF in mature neurons has been shown to be released in an depolarization-dependent Ca^{2+}-mediated manner (Binder and Scharfman 2004, Gottmann et al. 2009, Kovalchuk et al. 2004, Kuczewski et al. 2009), pathophysiological neuronal hyperactivity might decrease KCC2 expression/function indirectly via BDNF activation of TrkB (Mao et al. 2011, Rivera et al. 2004, Wake et al. 2007, Wardle and Poo 2003). Similar to its age-dependent action on KCC2 expression/function, BDNF potentiates depolarizing GABAergic signaling in immature neuronal networks but suppresses hyperpolarizing signaling in mature ones (Brunig et al. 2001, Mizoguchi et al. 2003, Porcher et al. 2011). Recently, it has been proposed that BDNF can potentiate the inhibitory GABAergic responses only in the absence of KCC2 and in conditions of high $[Cl^-]_i$ (Huang Y. et al. 2012). Interestingly, the reappearance of KCC2 after

neuronal trauma has also been shown to depend on BDNF-TrkB-mediated signaling, reminiscent of the developmental up-regulation of KCC2 (Boulenguez et al. 2010, Shulga et al. 2008, 2012). Hence, BDNF signaling may be a common regulatory mechanism for a functional decrease of KCC2 during aberrant neural activity (Wright et al. 2011).

1.6 Neonatal seizures and KCC2

Various lines of evidence have correlated epilepsy with an altered chloride homeostasis. KCC2 down-regulation and the associated decrease of GABAergic inhibition (or increase of GABAergic excitation) has been implicated in the pathogenesis of seizures (Kahle et al. 2008, Payne et al. 2003). In addition, deficiency of KCC2 leads to increased propensity to hyperexcitability and seizure-like activity (Hekmat-Scafe et al. 2006, Hubner et al. 2001, Khalilov et al. 2011, Riekki et al. 2008, Stil et al. 2011, Tornberg et al. 2005, 2007, Woo et al. 2002, Zhu L. et al. 2008). Both *in vitro* and *in vivo* epilepsy models in rodents and human epileptic tissue have a reduced KCC2 expression associated with an impaired GABAergic inhibition (see section 1.2.2.2). However, it is not really clear if this down-regulation is actually a consequence or a cause of epilepsy i.e. whether it is the primary event contributing to seizure pathogenesis or a secondary event that is a seizure-suppressive mechanism (Kahle et al. 2008, Miles et al. 2012).

Depolarizing GABA responses in immature neurons are due to a higher $[Cl^-]_i$ in presence of a robust NKCC1-mediated chloride influx and absence of KCC2-mediated chloride extrusion (see section 1.2.2.1). Since GABA acts as the major inhibitory drive in the nervous system, depolarizing actions of GABA result in a suppressed inhibition of neuronal circuits and at the same time can cause their excessive excitation via membrane depolarization. Consequently, pathogenesis of neonatal seizures is different from seizures in the adult animals and depolarizing GABAergic signaling is thought to aid the generation of seizures in the developing brain (Ben-Ari 2006, Ben-Ari and Holmes 2005, 2006, Briggs and Galanopoulou 2011, Dzhala and Staley 2003, Dzhala et al. 2010, Galanopoulou 2008b, Galanopoulou and Moshe 2009, Glykys et al. 2009, Kahle and Staley 2008, Khalilov et al. 2005, Khazipov et al. 2004, Nardou et al. 2011).

However, as previously stated, both depolarizing and hyperpolarizing $GABA_AR$ currents can act as inhibitory signals by shunting membrane conductance, $GABA_AR$ activation that mediates depolarizing currents can either be excitatory or inhibitory in developing neurons (see section 1.2.2.1). Moreover, immature hippocampal neurons have a wide variation in $[Cl^-]_i$ depending on cell type and size and hence differential responses to

GABA signaling are possible depending on whether $GABA_AR$ Cl^- fluxes are directed inwardly or outwardly (Dzhala et al. 2010, Tyzio et al. 2007). Hence, there are several contradictory reports of pro-convulsant and anti-convulsant effects of GABAergic signaling in developing animals (Ben-Ari et al. 2007, see also Table 1 in Dzhala et al. 2010) . An analogous effect of $GABA_AR$ agonists in protective vs aggravating effect for immature vs. mature neurons on ischemia- and excitotoxicity-induced neuronal injury has also been underlined (Schwartz-Bloom and Sah 2001).

Pharmacological enhancement of $GABA_AR$ currents that has been used as a strategy to subdue seizures in adults have often been found to be ineffective to a large extent in controlling seizures in neonates and can even potentiate them especially recurrent seizures that progressively increase $[Cl^-]_i$ in absence of a robust Cl^- extrusion mechanism in the immature neurons (Briggs and Galanopoulou 2011, Dzhala et al. 2005, 2008, 2010, Glykys et al. 2009, Kahle and Staley 2008). Consequently, antagonists against NKCC1-mediated chloride influx that ultimately reduce the depolarizing drive of $GABA_AR$ activation have been shown to reduce neonatal seizures both alone and in conjugation with $GABA_AR$ agonists (Briggs and Galanopoulou 2011, Dzhala et al. 2005, 2008, 2010, Glykys et al. 2009, Kahle and Staley 2008, Mazarati et al. 2009, Nardou et al. 2009, 2011). However, other studies have suggested that cotransport function of NKCC1 prevents hyperexcitability of immature neuronal circuits and pharmacological blockade of NKCC1 function results in hyperexcitability and increased risk of seizure pathogenesis (Kilb et al. 2007, Zhu L. et al. 2008).

Little is known how KCC2 cotransport function is modulated during seizure episodes in developing neurons and what consequences this has on its pathogenesis. Recurrent seizures shift the GABA signaling from hyperpolarizing to depolarizing in developing (female) neurons, which attain hyperpolarizing GABAergic signaling much before male pups (Galanopoulou 2008c). Interestingly, recurrent seizure episodes in immature (male) neurons with depolarizing GABAergic responses of identical age resulted in an up-regulated KCC2 expression and a shift in GABAergic signaling to hyperpolarization (Galanopoulou 2008c). Thus, translational effects of recurrent seizures on KCC2 function are different in neurons with hyperpolarizing and in those with depolarizing GABAergic responses (Galanopoulou 2007). In addition to this expressional increase of KCC2 that causes a depolarizing shift in GABA signaling in immature (male) neurons after triple seizure episodes, we have shown that a short episode of seizure is sufficient to rapidly increase the activity of KCC2 by inducing changes in its plasma membrane expression levels without

changing its total expression levels in immature (male) neurons (Study I). This fast and transient post-translational effect on KCC2 function is abolished by kinase inhibitor K252a (Study I) and is mediated by BDNF-TrkB signaling (unpublished data). Such an increase in KCC2 function by post-translational mechanisms in developing neurons has also been proposed to explain the regulation of KCC2 function by thyroid hormones (Friauf et al. 2008). Since, depolarizing $GABA_AR$ have been implicated in generation of seizures in the neonatal rat hippocampus, a fast increase in KCC2 plasma membrane expression and K^+-Cl^- cotransport can act as a protective mechanism against ictogenesis in neonatal hippocampus and may explain the resistance of developing animals to seizure-induced cell loss and subsequent seizures (Ben-Ari and Holmes 2006, Galanopoulou 2007, Galanopoulou and Moshe 2009, Galanopoulou et al. 2002, Holopainen 2008, Scantlebury et al. 2007).

Although Study I deals with regulation of KCC2 function in a model of neonatal seizure, the observations made throw some light on activity-mediated post-translational regulation of KCC2 function in general. A number of studies have proposed the existence of non-functional KCC2 protein in immature neurons, the activation of which being dependent on diverse signals like changes in phosphorylation states and/or indirect effects mediated by protein kinases (Balakrishnan et al. 2003, Fiumelli et al. 2005, Kelsch et al. 2001, Khirug et al. 2005, Vale et al. 2005, Woodin et al. 2003, but see Stein et al. 2004), oligomerization (Blaesse et al. 2006), plasma membrane expression (Gulyas et al. 2001, Zhang L. L. et al. 2006) and localization in non-raft domains (Hartmann et al. 2009). Since exogenous over-expression of KCC2 in immature neurons has been shown to result in hyperpolarizing GABA responses (Chudotvorova et al. 2005, Lee H. et al. 2005), the mechanisms of KCC2 post-translational modification and trafficking thought to activate KCC2 function during maturation would be expected to be present in the developing neurons before the endogenous up-regulation of KCC2 function. This is consistent with the finding that kinetics of developmental KCC2 up-regulation are different in slices and cultures and KCC2 can be rapidly activated by a global kinase inhibition in immature cultures but not in immature slices (Khirug et al. 2005), implying that *in vivo* KCC2 molecules are already phosphorylated (for comparison of tyrosine phosphorylation status of KCC2 during development in cultures and *in vivo*, refer to Kelsch et al. 2001 and Stein et al. 2004). Hence, it seems likely that even when only a small fraction of KCC2 is actually surface expressed (Study II), the immature neurons have enough KCC2 that can be activated via post-translational mechanisms for a rapid increase in the chloride extrusion capacity comparable to mature neurons (Study I).

1.7 Biochemical techniques to quantitate surface expressed protein

As discussed previously, for most cell-surface active proteins including transporters like KCC2, regulation of their surface expression serves as an important modulator of their function (see section 1.4.2). Hence, biochemical analysis of absolute and relative surface expressed proteins is a relevant area of interest for membrane protein biochemists. The traditional method of quantifying proteins expressed on the plasma membrane has been centrifugation-based sub-cellular fractionation (Heynen et al. 2000, Jones and Matus 1974, Nielsen et al. 2005, Wolfe et al. 1971). It has however, lost much of its significance since it results in preparations of crude plasma membrane fractions that are almost always cross-contaminated with intracellular membranes (Huber et al. 2003, Lund et al. 2009).

For long, biotinylation has served as the method of choice for quantitative analysis of surface expressed proteins for cells in suspension (Elia 2008, Luna 2001, Wisniewski 2011) and also for brain slice preparations (Gutlerner et al. 2002, Heynen et al. 2003, Thomas-Crusells et al. 2003). The technique relies on covalent labeling of proteins in the plasma membrane by membrane-impermeant chemically-modified biotin (vitamin H) derivatives. Although depending upon the conjugated functional group, biotin derivatives can also be covalently linked to carbohydrate moieties and thiol groups in a protein (Elia 2008, Luna 2001), the most commonly used derivatives of biotin for surface labeling are N-hydroxysucciniimide (NHS) esters that label surface expressed proteins at the primary amino groups of lysyl residues on accessible extracellular sites at physiological pH in solutions lacking primary amines (Luna 2001). Subsequently, biotin-labeled proteins that represent the surface expressed proteins can be purified by utilizing the extremely sturdy non-covalent interaction between biotin and avidin/streptavidin (Green 1975, Wilchek and Bayer 1990). However, there are several drawbacks of this technique. The purification step for biotin-labeled proteins can be both time consuming as well as error-prone. Absolute quantification of the ratio of the plasma membrane pool of the protein of interest by Western blotting requires loading the biotinylated and internal proteins on different lanes (Boudreau and Wolf 2005) and is complicated because of an absence of a single protein that can serve as a loading control for both the biotinylated and the internal protein fractions (Study II). Another potentially problematic complication is the contamination of labeled surface expressed protein fraction after biotinylation of intact cells with cytoplasmic proteins; mainly those involved in cell signaling, cytoskeletal organization and vesicular trafficking (Scheurer et al. 2005, Yu et al. 2006), probably because of their strong association with membrane proteins

which persists even in presence of strong detergents used in solubilizing membrane proteins. The problem of contamination might also be aggravated by non-existence of an idealized membrane-impermeable characteristic of common commercial biotin reagents even at low temperatures (Peirce et al. 2004, Yu et al. 2006) which may be due to presence of a plasma membrane receptor for biotin (Vesely et al. 1987). In addition, presence of an endogenous biotinidase that can catalyse both removal of attached biotin and its transfer to another molecule, is another potentially detrimental factor in using biotin to characterize the surface expression of proteins (Hymes and Wolf 2000).

Another method that employs covalent modification of surface expressed proteins utilizes a membrane-impermeant reactive molecule bis(sulfosuccinimidyl) suberate (BS3) (Mattson et al. 1993, Staros 1982). BS3 is a primary amine reactive homo-bifunctional cross-linker that can form covalent amides with lysyl residues of proteins at physiological pH and in aqueous solutions lacking primary amines (Staros 1982). It has been used for quantification of surface expressed proteins both in cell cultures (Archibald et al. 1998, Hall and Soderling 1997a, 1997b, Hall et al. 1997) and in brain slices (Boudreau and Wolf 2005, Boudreau et al. 2007, Grosshans et al. 2002a, 2002b). However, BS3 cross-linking of surface proteins can lead to formation of excessively high molecular weight covalent complexes which do not reliably enter polyacrylamide gels and resolve properly and hence are difficult to quantitate (Grosshans et al. 2002b, Liang et al. 2007). Hall and Soderling (1997b) have suggested a shortened reaction time for BS3 cross-linking as a solution to limit the excessive covalent cross-linking, but this could lead to a reduced efficiency of the reaction.

Finally, molecular shaving of cell surface by use of proteases like trypsin and chymotrypsin has also been employed for quantitative analysis of the surface pool of membrane proteins in cell cultures (Hall and Soderling 1997a, 1997b, Olsen et al. 2006) and in brain slice preparations (Grosshans et al. 2002a, 2002b). Like the biotin derivatives and BS3, the proteases are membrane-impermeant and inaccessible to the cytoplasmic proteins (Grosshans et al. 2002b), and can be used in physiological solutions and at physiological pH. However, unlike with biotinylation and BS3 cross-linking, the physiological temperature requirement for progression of the proteolytic cleavage has been a significant drawback in its utilization in quantitative analysis of surface expressed proteins since the trafficking of membrane proteins is not blocked during the entire incubation time (Boudreau and Wolf 2005, Grosshans et al. 2002b). Hence, surface expression of proteins like KCC2 with a highly dynamic endocytotic-recycling trafficking rate (Lee H. H. et al. 2007, Rivera et al. 2004, Zhao et al. 2008) can be over-approximated leading to misinterpretations (Grosshans et al.

2002b, Study II). Importantly, several other membrane proteins important in neurotransmission have been shown to undergo a constitutive, dynamic endocytosis-reinsertion cycles at basal conditions, including $GABA_AR$ (Kittler and Moss 2003, Porcher et al. 2011), AMPAR subunits glutamate receptors 2/3 (GluR2/3) (Malinow and Malenka 2002, Malinow et al. 2000), dopamine transporter (DAT) (Holton et al. 2005, Loder and Melikian 2003) and GABA transporter (GAT) (Deken et al. 2003). Taking advantage of previous reports of utilization of homologous enzymatic proteins from species thriving at extreme temperatures for biotechnologically relevant themes (e.g., see Vosberg 1989 for the use of DNA polymerase from *Thermus aquaticus* in the polymerase chain reaction or PCR), we utilized a cold-adapted trypsin from cod fish (Jonsdottir et al. 2004) to address this issue and successfully quantified the absolute surface expression of both AMPAR subunit GluR4 and KCC2 at low temperatures when trafficking of membrane proteins is inhibited (Study II).

Unlike GluR2 and GluR3 which have a shorter intracellular C-terminal chain and whose surface expression is dynamically regulated by a constitutive endocytotic-recycling pathway, the surface pools of GluR1 and GluR4 are more stable and do not show a similar constitutive recycling under basal conditions which is thought to be because of differential interaction with binding partners for their longer carboxy tails of GluR1 and 4 (Bredt and Nicoll 2003, Gomes A. R. et al. 2003, Greger and Esteban 2007, Groc and Choquet 2006, but see Ehlers 2000). Using the property of a reduced membrane recycling rate of GluR4, we showed that while quantification of surface expression of proteins like GluR4 is not compromised when the proteolysis is carried out at mammalian physiological temperatures, it can indeed be misleading for proteins like KCC2.

2. Aims of the study

The hypothesis tested in this study was whether the stability of total and plasmalemmal KCC2 protein levels in the rat hippocampus is regulated by post-translational mechanism.

The specific aims were as follows:

1) To analyze the effects of a seizure episode on KCC2 function in neonatal rat hippocampus and the underlying mechanism.
2) To design a reliable method for quantitative analysis of surface expressed proteins.
3) To assess the stability of the total and surface expressed KCC2 protein levels and to understand the mechanism involved in the fast down-regulation of KCC2 function in pathophysiological states of excitotoxicity and hyperexcitability.

3. Experimental procedures

3.1 Preparation and maintenance of slices

Hippocampal slices from P5-7 and P15-22 rats (Wistar) were used. The animals were anesthetized with halothane (Sigma), decapitated and the brains were removed and immersed in ice-cold sucrose-based cutting solution containing (in mM): 87 NaCl, 2.5 KCl, 0.5 $CaCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 7 $MgCl_2$, 50 sucrose and 25 D-glucose, equilibrated with 95% O_2 and 5% CO_2. 600 µm horizontal or 400 µm coronal acute brain slices were cut using a Vibratome 3000 (Vibratome). The slices were allowed to recover at 34°C in standard physiological solution containing the following (in mM): 124 NaCl, 3 KCl, 2 $CaCl_2$, 25 $NaHCO_3$, 1.1 NaH_2PO_4, 2 $MgSO_4$, and 10 D-glucose, equilibrated with 95% O_2 and 5% CO_2, pH 7.4 for 1 hour.

3.2 Induction of neonatal seizures

Seizures were induced in male P5-7 rat pups with a single intraperitoneal injection of kainate (2 mg/kg body weight; Tocris Bioscience). An identical volume of 100 µl saline was injected in control rats. Rats were injected in pairs, with a 1 hour interval between the littermates and a randomized order of saline and kainate injections. Behavioral seizures progressed from an initial stage of immobility, followed by bouts of scratching behavior, hyperactivity, ataxia, and isolated myoclonic jerks, into tonic or tonic-clonic seizures. Continuous behavioral monitoring was performed by video recording to define the onset of seizures (the initial stage of immobility). Slices containing the hippocampus were prepared after a 1 hour seizure period. Slices from saline-injected rats were prepared 1.5 hours after the injection.

3.3 Induction of seizure-like activity *in vitro*

Seizure-like activity was induced by a 10 minutes incubation of horizontal hippocampal slices (600µm) in 300 nM kainate in physiological solution (in mM): 124 NaCl, 4 KCl, 1.3 $CaCl_2$, 25 $NaHCO_3$, 1.1 NaH_2PO_4, 1.3 $MgSO_4$, and 10 D-glucose, equilibrated with 95% O_2 and 5% CO_2, pH 7.4 at the experimental temperature of 34°C. After 10 min, the slices were transferred to physiological solution with 1 µM TTX (Tocris Bioscience) added to block neuronal activity and 10 µM bumetanide to block NKCC1. In biochemical experiments, a 5 minutes kainate application followed by a 5 minutes washout with

physiological solution was repeated up to six times. Some slices were pre-exposed to 200 nM K252a for 30 min before exposure to kainate plus K252a.

3.4 Western blot analysis

Hippocampal slices or CA1 regions isolated from them were homogenized in RIPA buffer (150 mM NaCl, 1% Triton-X, 0.5% deoxycholic acid, 0.1% SDS and 50 mM Tris-Cl, pH 8.0) with a protease inhibitor mixture (Roche). Proteins were separated by SDS-PAGE. Gel loading was performed in Laemmli buffer containing 80mM Tris-HCl, 2% SDS, 10% glycerol, 5.3% β-mercaptoethanol and 2% bromophenol blue. Proteins were then transferred onto nitrocellulose membranes (PerkinElmer) electrophoretically in transfer buffer containing 25 mM Tris, 192 mM glycine and 10% methanol, pH 8.3. Membranes were blocked in TBST/milk (20 mM Tris, 150 mM NaCl, 0.1% Tween-20 and 5% nonfat dry milk, pH 7.5) for 1 hour at room temperature. Incubation with the respective antiserum diluted in TBST/milk was performed overnight at 4°C with agitation. The primary and secondary antibodies used for quantitative analysis of the blots have been described in the respective original publications included in this thesis. After washing the blots in TBST to remove unbound primary and secondary antibodies, the immunoreactive signals were detected using an enhanced chemiluminescence kit (Pierce) and an LAS-3000 documentation system (Fujifilm). Quantification of the chemoluminescent signals was performed by Advanced Image Data Analysis imaging software (Raytest). All measurements were within the linear range of the sensitivity of the camera. After quantification, the representative images included in the figures were optimized for brightness and contrast using Paint Shop Pro X (Corel) and Microsoft Powerpoint.

3.5 Surface biotinylation

Test and control hippocampal slices were labeled with 100 µM biotin (Biotinamidohexanoyl-6-aminohexanoic acid N-hydroxysuccinimide ester; Sigma) in standard physiological solution on ice for 1 hour with continuous equilibration with 95% O_2 and 5% CO_2. The reaction was quenched by washing the slices with 1 M glycine or 100 mM lysine in standard physiological solution for 10 minutes on ice. Hippocampal slices or isolated CA1 regions were homogenized and the biotinylated proteins were purified on immobilized streptavidin agarose (Sigma) and eluted in SDS-PAGE sample buffer at 75°C for 10 minutes. The eluent from the streptavidin column (biotinylated proteins) and the

supernatant (unlabeled internal proteins) were separated on SDS-PAGE and the immunoblot analysis was performed as described above.

3.6 Trypsin cleavage of surface proteins

Hippocampal slices (400 µm) were treated with cod trypsin (2 U/ml in standard physiological solution with continuous oxygenation) on ice for 1 hour. After a wash step (10 minutes in standard physiological solution on ice), the hippocampal slices were homogenized and the immunoblot analysis for the proteins in the homogenate was performed as described above.

Human embryonic (HEK) cells over-expressing the protein of interest were washed with ice cold phosphate buffered saline (PBS) on ice. The incubation with 2 U/ml cod trypsin was carried out on ice in PBS for 1 hour. The reaction was stopped by the addition of protease inhibitor cocktail (Roche). Cells were then homogenized in RIPA buffer and the immunoblot analysis was performed as described above.

3.7 *In vitro* calpain cleavage assay

Rat brain homogenate was diluted to about 1-2 mg/ml of protein in a final volume of 500 µl (as assessed by a BioRad DC protein assay kit; BioRad). Rat calpain-2 (m-calpain) (CalBiochem) was added at a concentration of 20 mg/ml in calpain assay buffer (100 mM HEPES, 50 mM NaCl, 0.1% Triton-X, 20 mM $CaCl_2$ and 20 mM dithiothreitol, pH 7.4). The cleavage reaction was stopped after 10, 20, or 30 minutes by adding 0.1 M EDTA and protease inhibitors (Complete Mini EDTA free protease inhibitor mixture; Roche). In some experiments, calpain-2 was pre-incubated with 100 mM MDL 28170 for 5 minutes.

3.8 Ionomycin assay of calpain activation

To directly raise $[Ca^{2+}]_i$, coronal rat brain slices bathed in standard physiological solution were treated with 50 µM ionomycin (Ascent), a Ca^{2+} ionophore, at 34°C for 4 hours. Slices treated with 30 µM MDL 28170 in addition to ionomycin were pre-incubated with MDL 28170 for 30 min.

4. Results and Inferences

4.1 Study I

The results and inferences obtained from data collected during Study I are as follows:

1) In spite of a low protein expression of KCC2 in P5-7 rats which have depolarizing GABAergic responses, a single seizure event induced by kainic acid that has been used as a model for temporal lobe epilepsy (cf. Ben-Ari and Cossart 2000) culminated into an increased surface expression and chloride extrusion function of KCC2 both *in vivo* and *in vitro*. This was observed as a shift in E_{Cl^-} to hyperpolarizied levels, similar to what is seen in hippocampal neurons from mature rats. This indicates that KCC2 has a low plasma membrane expression under basal conditions (see Study II) and that the total protein levels in developing rats are sufficiently high to set off hyperpolarizing GABAergic responses and a somato-dendritic Cl^- gradient comparable to adults. Hence, blocking of endocytosis of KCC2 which has a fast endocytosis-recycling rate (cf. Lee H. H. et al. 2007, Study II) by dynamin inhibitory peptide (DIP) caused a similar effect on the chloride driving force of $GABA_AR$.
2) Since the above shift in depolarizing to hyperpolarizing GABAergic responses were observed in absence of any increase in KCC2 expression but rather depended on an increased surface expression, it implies that post-translational regulation of KCC2 may also play a role in the developmental shift in the GABA switch. However, the part played by a developmental increase in KCC2 protein levels cannot be overlooked since the protein levels in P16 rat hippocampus are not similar to those in P5-7 rats, although an increase in spine density during development might also be reflected in the amount of KCC2. On the other hand, translational up-regulation of KCC2 can consolidate the effects of an increased KCC2 plasma membrane expression/function by post-translational mechanisms during development in an activity-dependent manner.
3) Interestingly, analogous to the signaling mediated by neurotrophic factors implicated in the developmental increase of KCC2 protein expression (see section 1.3.2), the fast post-translational increase in the KCC2 surface expression and function could be blocked by the kinase inhibitor K252a and was dependent on BDNF-TrkB signaling (unpublished data).
4) Although Na^+-K^+ ATPase α2 subunit has a predominantly glial expression, it is known to be expressed in embryonic neurons (cf. Moseley et al. 2003) as well as in young hippocampal neurons (cf. Cameron et al. 1994) and might function as a

thermodynamic energy source for the KCC2-mediated cotransport in the neonatal brain (see section 1.2.2.1). The increased KCC2 plasma membrane expression and Cl^- extrusion function, paralleled by an increased surface expression of Na^+-K^+ ATPase α2 subunit might contribute to an intrinsic anti-epileptogenic mechanism responsible for a high resistance of immature neuronal circuits to seizures by increasing GABAergic hyperpolarizing inhibition. In neonatal brain, KCC2a and KCC2b show similar expression patterns and levels, largely co-localize, form heteromers and have comparable cotransport activity (cf. Uvarov et al. 2007, 2009). Not surprisingly then, surface expression of both KCC2 isoforms in neonatal rat CA1 neurons was affected in a similar manner after the single seizure episode.

4.2 Study II

The results and inferences obtained from data collected during Study II are as follows:

1) The problems encountered during quantitative analysis of plasma membrane expression of proteins by established methods like sub-cellular fractionation, biotin-labeling and BS3-mediated cross-linking are highly suggestive of using at least two different biochemical procedures for the same (see section 1.7). Hence, an existing protocol of molecular shaving of surface expressed proteins to quantitate their expression was modified to be operational at low temperatures so that it could be conveniently used for membrane proteins that have a short plasmalemmal half life and a high recycling rate. Using a mammalian protease, we showed that molecular shaving of surface expressed proteins at mammalian physiological temperatures can lead to erroneous results for plasmalemmal proteins like KCC2 which have a fast recycling rate. On the other hand, surface analysis of plasmalemmal proteins like AMPAR subunit GluR4 that have stable surface expression can be carried out at mammalian physiological temperatures.
2) We established the cod-trypsin catalyzed proteolysis for quantitative analysis of surface expression of proteins at low temperatures. As a proof of principle, we studied the kinetics of cod-trypsin-mediated cleavage of surface expressed proteins for both KCC2 and GluR4. Further, we demonstrated that the technique can be used for both qualitative (using a mutant of GluR4, R507K that is incapable of being targeted to the membrane (cf. Coleman et al. 2009)) and quantitative (plasma membrane expression of KCC2 in control slices and slices treated with a PKC activator (cf. Lee H. H. et al. 2007)) analysis of surface expressed proteins.

3) Finally, we showed that expression of KCC2 in the plasma membrane is indeed very low (15-20% of total KCC2 protein) and that it has a fast recycling rate under basal conditions that can be regulated by post-translational mechanisms (cf. Lee H. H. et al. 2007, Study I) to eventually alter the surface expression and the function of the cotransporter.

4.3 Study III

The results and inferences obtained from data collected during Study III are as follows:

1) The major surprising conclusion from these data was that under basal conditions KCC2 protein exhibits a high stability and a low turn-over rate. Previous studies indicated that KCC2 protein has a very high turn-over rate (cf. Rivera et al. 2004, Lee H. H. et al. 2010). However, our experimental data based upon selective blockade of both protein synthesis and protein degradation via the lysosomal pathway supported a stable KCC2 protein expression in P15-21 rat hippocampus. Interestingly, Zhao et al. (2008) also showed that even when the stability of plasmalemmal KCC2 was low under basal conditions, the endocytosed proteins was not degraded but rather recycled back to the membrane.
2) KCC2 harbors PEST sequences that have been implicated in constitutive as well as conditional protein degradation (see section 1.4.4). Indeed, while we showed that KCC2 protein and function levels are quite stable under basal conditions, we also demonstrate that KCC2 protein and function can be down-regulated by proteases-mediated cleavage/degradation. Hence, we demonstrated that KCC2 can be cleaved by calpain both *in vitro* and under pathophysiological conditions of excitotoxicity as well as epileptiform activity. The latent PEST domains in KCC2 might be speculated to be activated for regulated proteolysis by phosphorylation/dephosphorylation mechanisms (cf. Lee H. H. et al. 2010, 2011).
3) Establishment of KCC2 as a target of calpain-catalyzed cleavage is particularly interesting as KCC2 function and hence GABAergic signaling can consequently be altered by calpain. Along similar lines, although we did not show this, structural aspects of KCC2 in both GABAergic and glutamatergic synaptic signaling might be expected to experience amendments in a calpain-dependent manner.

In summary, the studies included in this thesis underline the importance of post-translational regulation of KCC2 function mediated by changes in the plasmalemmal expression levels as

well as protein proteolysis in the rat hippocampus. In addition, an improved method for quantitative analysis of surface expressed proteins at low temperatures when protein trafficking is halted, was also designed. Future studies would be targeted at the cellular mechanisms responsible for changes in the surface pool of KCC2 and to develop a methodology to quantify the insertion of highly mobile membrane proteins like KCC2 into the plasma membrane.

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