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## Chapter 7

### Transport-dependent and independent functions of KCC2 at excitatory synapses

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#### [NON PRINT ITEMS]

**Abstract:** This chapter explores non-canonical functions of the neuronal chloride/potassium KCC2 transporter at glutamatergic, excitatory synapses. The first section describes KCC2 expression and membrane dynamics in cortical neurons to show that KCC2 is enriched in dendritic spines that host excitatory synapses. Then, it reviews KCC2 protein binding partners, with a specific focus on those that may contribute to the specific confinement and role of KCC2 in dendritic spines. With this background, the next section describes how KCC2 contributes to both dendritic spine morphology and excitatory synaptic function and plasticity. The chapter ends with a discussion on the implications of the multiple functions of KCC2, in particular with respect to the pathology.

**Key Words:** Synaptic plasticity; LTP; Dendritic spines; Actin cytoskeleton; Cation chloride co-transporters; Neurological disorders

## 1. Introduction

Mature neurons maintain intracellular chloride concentrations way below those observed in other cell types. This ensures that activation of the chloride-permeable GABA and glycine receptors generates hyperpolarizing, inhibitory potentials. The cation-chloride co-transporters (CCC) KCC2 and NKCC1 are secondary active transporters that play a prominent role in regulating transmembrane chloride gradients, as discussed in previous chapters. Although their existence and function have been documented since the early 1970's, Payne and collaborators cloned the KCC2 gene (later termed *Slc12a5*) from the rat brain only in 1996 (Payne, Stevenson, & Donaldson, 1996). Three years later, KCC2 was identified as responsible for the early postnatal shift in the polarity of GABA transmission in the rat hippocampus (Rivera et al., 1999). Since then, more than 600 original publications and 80 reviews have explored KCC2 expression, function, regulation and involvement of the pathology. For obvious reasons, most of those have focused on KCC2 in the context of inhibitory neurotransmission. However, an ever-growing body of evidence indicates that KCC2 function extends beyond the mere control of transmembrane chloride gradients and GABA transmission. First, the pattern of KCC2 expression reveals specific clustering in dendritic spines, which harbor most glutamatergic synapses. Second, KCC2 genetic ablation or knockdown have revealed much more complex phenotypes than previously expected, including alterations in dendritic spine maturation and morphology as well as excitatory synaptic function. In 2015, Blaesse and Schmidt coined this heterogeneity of KCC2 functions with the term *moonlighting protein*, which appeared in the late 1990's to designate the existence of multifunctional proteins (Jeffery, 1999).

In this chapter, we review experimental data supporting the notion that KCC2 is indeed multifunctional and that, through interactions with a variety of molecular partners, it participates in several biological processes, which only partly involve its ion-transport function. We suggest that, considering the variety of pathological conditions involving down-regulation of KCC2 expression (Kahle et al., 2008; Kaila, Price, Payne, Puskarjov, & Voipio, 2014), fully understanding the various processes and molecular interactions KCC2 is engaged in will help predicting and designing most efficient and specific therapeutic strategies.

## **2. KCC2 expression in the vicinity of excitatory synapses**

The *Slc12a5* gene encodes two KCC2 protein isoforms, KCC2a and KCC2b. that are expressed under the control of distinct promoters (Markkanen et al., 2014; Uvarov et al., 2007; Uvarov et al., 2009). KCC2a differs from KCC2b by a unique 40-amino acid sequence in its N-terminal domain, containing a putative regulatory domain by the SPS1-related proline/alanine-rich kinase (SPAK)(Uvarov et al., 2007). Using antibodies raised against subtype specific epitopes, KCC2b was shown to be the most prominently expressed isoform in the adult forebrain, whereas KCC2a expression is higher in neonates and remains relatively constant during development (Markkanen et al., 2014). Remarkably, whereas full KCC2 KO mice die at birth due to severe motor and respiratory deficits (Hubner et al., 2001), isoform-selective KO show less severe phenotypes. Thus, KCC2b KO mice survive for 2-3 weeks and then die due to generalized seizures (Woo et al., 2002) whereas KCC2a KO show altered breathing behavior, most importantly at early postnatal stages (Dubois et al., 2018; Markkanen et al., 2014). Therefore, although both isoforms form functional transporters (Markkanen et al., 2017; Uvarov et al., 2007), whether they fulfill distinct or partially overlapping functions remains unclear. In the rest of this chapter, KCC2 implicitly refers to KCC2b only.

In heterologous systems, KCC2 forms both homo- and hetero-oligomeres with other cation-chloride co-transporters (Simard et al., 2007). Dimers of the two isoforms, KCC2a and KCC2b, are also formed both *in vivo* and in heterologous cells (Uvarov et al., 2009). Oligomerization appears to rely mainly on disulfide bonds, as treatment with reducing agents mostly yields monomeric forms (Agez et al., 2017; Blaesse et al., 2006) and may involve KCC2 carboxy-terminal domain (Agez et al., 2017), as also demonstrated for KCC1 (Casula et al., 2001). KCC2 oligomerization and function were suggested to be correlated, based in part on their parallel developmental profile in the rat lateral superior olive (Blaesse et al., 2006). In addition, lipid rafts may also influence KCC2 oligomerization and clustering, even though the impact of KCC2 accumulation in lipid rafts on its transport function remain debated (Hartmann et al., 2009; Watanabe, Wake, Moorhouse, & Nabekura, 2009).

At the subcellular level, KCC2 is present throughout the somato-dendritic membrane of most cortical neurons including GABAergic interneurons (Gulyas, Sik, Payne, Kaila, & Freund, 2001), but is virtually excluded from their axon, including their axon initial segment (Baldi, Varga, & Tamas, 2010; Szabadics et al., 2006). In the hippocampus, high-resolution pre-embedding KCC2 immunolocalization revealed cell-type specific differences in the somatic/dendritic expression levels (Baldi et al., 2010). While granule cells show more intense membrane expression in dendritic than somatic regions, KCC2 seems more evenly distributed in CA1 pyramidal cells. Most strikingly, as initially reported by Gulyas and collaborators (Gulyas et al., 2001), KCC2 expression is often observed in dendritic spines, and is more abundant near excitatory than inhibitory synapses (Baldi et al., 2010). Confocal imaging of hippocampal neurons immunostained for KCC2 also revealed a higher intensity of KCC2 cluster immunofluorescence in dendritic spines than on the adjacent shafts (Gauvain et al., 2011)(Fig. 1A). Although these clusters were mostly found near the postsynaptic densities, KCC2 and PSD95 immunostainings were mutually exclusive, suggesting KCC2

primarily aggregates perisynaptically. These intriguing observations raise several important questions, in particular regarding the specific targeting and aggregation in dendritic spines, as well as the possible role of a K/Cl co-transporter near excitatory synapses.

*(Figure 1 near here)*

**Figure 1. Molecular determinants of KCC2 aggregation near synapses.** *A, Confocal images of a hippocampal neuron immunostained for KCC2. Right, magnification of boxed area in the image shown on the left. Note high-intensity fluorescent KCC2 clusters in dendritic spines. Scale, 5 $\mu$ m. B, Reconstructed trajectories of recombinant KCC2 molecules tracked with quantum dots (white), overlaid with fluorescent micrographs showing Homer1c-GFP (green) and gephyrin-mRFP (red) identifying glutamatergic and GABAergic synapses, respectively. KCC2 molecules show less constrained trajectories in the extrasynaptic membrane. Scale, 1  $\mu$ m. C, Schematic representation of KCC2 diffusion in the neuronal plasma membrane. Near excitatory synapses, interaction with submembrane actin via 4.1N hinders KCC2 lateral diffusion. Similarly, KCC2 diffusion is constrained near inhibitory synapses, through so far unidentified molecular interactions, possibly with GABA receptors and/or scaffolding molecules such as gephyrin. Extrasynaptic KCC2 on the other hand are more mobile, as indicated by the larger arrow.*

*Credits. Adapted from (Gauvain et al., 2011) (A) and (Chamma et al., 2013) (B) with permission.*

KCC2 aggregation in dendritic spines is somewhat reminiscent of postsynaptic receptor accumulation, which is known to reflect interactions with submembrane scaffolding proteins

(Choquet & Triller, 2013). Single particle tracking experiments using photostable fluorescent nanocrystals called *quantum dots* (Bannai, Levi, Schweizer, Dahan, & Triller, 2006) have revealed that the somewhat static picture of the postsynaptic element with tightly anchored receptors was oversimplified. Thus, receptors shown near-Brownian diffusion within the plasma membrane and only get temporarily trapped at synapses by high-affinity interaction with scaffolding molecules, such that synaptic and extrasynaptic receptors undergo a continuous and dynamic exchange, a phenomenon termed *diffusion-trapping* (Choquet & Triller, 2003). Such phenomenon represents a general mechanism allowing molecular heterogeneity within the plasma membrane, based on reversible interactions of transmembrane molecules with membrane or sub-membrane molecules. Using similar approaches as those used to study postsynaptic receptors dynamics and synaptic trapping, Chamma et al. have explored KCC2 membrane dynamics in cultured hippocampal neurons (Chamma et al., 2013). Recombinant KCC2 showed diffusion properties comparable to postsynaptic receptors with greater diffusion coefficients and confinement domains in the extrasynaptic membrane than near excitatory and inhibitory synapses. Importantly, the dwell-time of KCC2 molecules near excitatory synapses was longer than near inhibitory synapses, suggestive of distinct molecular constraints over KCC2 diffusion at these synapses (Fig. 1B). This raised the question of the molecular identity of the scaffolding molecules that hinder KCC2 diffusion at excitatory vs. inhibitory synapses.

As we discuss below (Section 3, see also Chapter 8 and Chapter 12), KCC2 interacts with a variety of molecular partners that may accumulate near synapses. In particular, dendritic spines that harbor glutamatergic synapses are highly enriched in actin and actin-binding proteins (Cingolani & Goda, 2008) which act as scaffolding molecules for numerous ion transport proteins (ion channels, transporters, exchangers...)(Denker & Barber, 2002). Thus, the depolymerizing agent latrunculin A increases KCC2 lateral diffusion and reduces its

dwelling-time near excitatory synapses (Chamma et al., 2013). This effect likely involves KCC2 interaction with submembrane actin scaffold via interaction of its carboxy-terminal domain with the 4.1 Ezrin Radixin Moesin (FERM)-domain protein 4.1N, an actin/spectrin-binding protein also enriched in dendritic spines and that was shown to interact with KCC2 (Li et al., 2007). Thus, overexpression of KCC2 carboxy-terminal domain or 4.1N knockdown by RNA interference both mimicked the effect of latrunculin. Importantly, the dwelling-time of KCC2 near inhibitory synapses was unaffected by these manipulations. This suggests that distinct molecular interactions are at play to confine KCC2 near inhibitory vs. excitatory synapses (Fig. 1C). Interactors responsible for trapping KCC2 near GABAergic synapses remain to be identified.

### **3. KCC2 interacts with synaptic and perisynaptic proteins**

Molecular interactions are not just governing the subcellular distribution of transmembrane proteins but also modulate their function. Most of them are engaged in macro-molecular complexes with intricate physical and functional interactions. Full understanding of the function and regulation of individual transmembrane proteins therefore relies on a comprehensive identification of the molecular interactions they engage in. Thus, several recent studies have revealed a number of somewhat unexpected molecular interactions involving KCC2 (Table 1). Those shed new light on its functions and regulation in neurons.

Yeast two-hybrid screening was first used to identify KCC2 binding partners. This approach revealed KCC2 interactions that are likely important for local regulation of KCC2 function. For instance, KCC2 was shown to interact with the brain-type creatine kinase (CKB) (Inoue, Ueno, & Fukuda, 2004), a neuronal ATP-generating enzyme acting to increase KCC2 function through mechanisms that remain to be fully explored (Inoue et al., 2004; Inoue,



Yamada, Ueno, & Fukuda, 2006). Interestingly, the alpha2 subunit of the Na<sup>+</sup>/K<sup>+</sup> ATPase was also found to interact with KCC2 (Ikeda et al., 2004). The Na<sup>+</sup>/K<sup>+</sup> ATPase establishes ion gradients across the plasma membrane used for KCC2-mediated ion transport. Therefore, a functional complex comprising KCC2, CKB and the Na<sup>+</sup>/K<sup>+</sup> ATPase may constitute an ion-transport metabolon (Kaila et al., 2014), in which ATPase-dependent potassium import would be promoted by local ATP production by CKB which would in turn support secondary active chloride export by KCC2, via a locally generated potassium gradient.

Other interactions identified by immunoprecipitation assays suggested new mechanisms for KCC2 regulation, further extending an already vast repertoire of regulatory posttranslational modifications ((Chamma, Chevy, Poncer, & Levi, 2012; Kaila et al., 2014; Medina et al., 2014) for review; **See also Chapter 11**). For instance, the clathrin-binding adaptor protein-2 (AP-2), Ras-associated binding protein 11b (Rab11b) and the actin-associated protein 4.1N were shown to be critical for KCC2 endocytosis, recycling and clustering, respectively (Chamma et al., 2013; Li et al., 2007; Roussa et al., 2016; Zhao et al., 2008). Perhaps more intriguing is the discovery of KCC2 interaction with the glutamatergic, kainate receptor subunit 2 (GluK2; (Mahadevan et al., 2014; Pressey et al., 2017)) and its auxiliary subunit neuropilin and tolloid-like 2 (Neto2; (Ivakine et al., 2013)). These interactions were shown to promote KCC2 function primarily by enhancing its recycling to the plasma membrane, leading to enhanced membrane expression and function (Pressey et al., 2017). Interestingly, kainate receptors also interact with 4.1N (Copits & Swanson, 2013) that in turn interacts with the AMPA receptor subunit GluA1 (Lin et al., 2009). Collectively, these data support the view that KCC2 may be part of a macromolecular complex comprising postsynaptic glutamate receptors and elements of the submembrane cytoskeleton. This complex may both stabilize KCC2 in specific subcellular membrane compartments as well as locally regulate glutamate receptor confinement (Table 1; see section 4).

(Table 1 near here)

**Table 1.**

Protein name	Alt. name	Gene	Interaction detection	References
<b>Proteins enriched at excitatory synapses</b>				
Band 4.1-like protein 1	4.1N	Epb4111	IP	(Chamma et al., 2013; Li et al., 2007)
Rho guanine nucleotide exchange factor 7	Beta-Pix	Arhgef7	IP	(Chevy et al., 2015; Llano et al., 2015)
Cofilin-1	p18	Cfl1	LC-MS	(Mahadevan et al., 2017)
Metabotropic glutamate receptor 1	mGluR1	Grm1	LC-MS	(Kato et al., 2012)
Metabotropic glutamate receptor 5	mGluR5	Grm5	LC-MS	(Farr et al., 2004)
Glutamate receptor ionotropic, kainate 2	GluK2	Grik2	LC-MS, IP	(Mahadevan et al., 2017; Pressey et al., 2017)
Neuropilin and tolloid-like protein 2	Neto2	Neto2	GST/LC-MS, IP	(Ivakine et al., 2013; Mahadevan et al., 2017)
Voltage-dependent R-type calcium channel subunit alpha-1E	Cav2.3	Cacna1e	LC-MS	(Muller et al., 2010)
Amyloid-beta A4 protein	APP	App	IP	(M. Chen et al., 2017)
Neuroigin-1	Neuroigin-1	Nlgn1	IP	(Loh et al., 2016)
<b>Proteins enriched at inhibitory synapses</b>				
Gamma-aminobutyric acid receptor subunit alpha-1	GABAAR1	Gabra1	IP	(Y. Huang et al., 2012)
Gamma-aminobutyric acid receptor subunit beta-1	GABABR1	Gabrb1	LC-MS, IP	(Wright et al., 2017)
Neuroigin-2	Neuroigin-2	Nlgn2	IP	(Sun, Zhang, & Chen, 2013)
<b>Proteins enriched at synapses</b>				
Protein kinase C and casein kinase substrate in neurons 1	SdpI	Pacsin1	LC-MS, IP	(Mahadevan et al., 2017)
Serine/threonine-protein phosphatase PP1-alpha catalytic subunit	PP-1A	Ppp1ca	LC-MS	(Mahadevan et al., 2017)
Adaptor protein complex AP-2	Alpha2-adaptin	Ap2	LC-MS, IP	(Mahadevan et al., 2017; Zhao et al., 2008)
<b>Non-synaptic proteins</b>				
STE20/SPS1-related proline-alanine-rich protein kinase	PSTK1	Stk39	IP	(Friedel et al., 2015)
Sodium/potassium-transporting ATPase subunit alpha-2	Na(+)/K(+) ATPase alpha-2 subunit	Atp1a2	LC-MS, IP	(Ikeda et al., 2004; Mahadevan et al., 2017)
Creatine kinase B-type	CPK-B	Ckb	LC-MS, YTH, IP	(Inoue et al., 2004; Inoue et al., 2006; Mahadevan et al., 2017)

Voltage-dependent P/Q-type calcium channel subunit alpha-1A	Cav2.1	Cacna1a	LC-MS	(Muller et al., 2010)
Huntingtin	HD protein homolog	Htt	LC-MS, IP	(Dargaei et al., 2018; Shirasaki et al., 2012)
Ras-related protein Rab-11B	Rab11B	Rab11b	IP	(Roussa et al., 2016)
Serine/threonine-protein kinase WNK1	Protein kinase with no lysine 1	Wnk1	IP	(Friedel et al., 2015)
Melatonin-related receptor	H9	Gpr50	YTH	(Grunewald, Kinnell, Porteous, & Thomson, 2009)
Solute carrier family 12 member 9 (CIP1)	CCC6	Slc12a9	IP	(Wenz, Hartmann, Friauf, & Nothwang, 2009)
Solute carrier family 12 member 6	KCC3	Slc12a6	IP	(Ding, Ponce-Coria, & Delpire, 2013)
Protein Associated with Myc	PAM	Pam	YTH, IP	(Garbarini & Delpire, 2008)

**Table 1. KCC2 molecular interactions in cortical neurons.** The table shows putative KCC2 interactors, as identified in yeast two-hybrid (YTH), GST pulldown (GST), immunoprecipitation (IP) or liquid chromatography and mass spectrometry (LC-MS). Note that a large proportion of putative interactors are enriched at or near excitatory synapses. The impact of putative or validated KCC2 interactors on KCC2 function, membrane expression or traffic has not always been assessed and is therefore not indicated.

Functional proteomic analysis of KCC2 recently further extended the list of putative KCC2 interactors (Mahadevan et al., 2017). It is remarkable that most identified or putative KCC2 partners are expressed at or near excitatory and, to a lesser extent, inhibitory synapses (Table 1), where KCC2 clusters are most abundant as discussed previously (Baldi et al., 2010; Chamma et al., 2013). Thus, KCC2 candidate interactors predominantly include ion-transport proteins (including ion channels, transporters and postsynaptic receptors), cytoskeleton-

related proteins and proteins involved in receptor trafficking (Mahadevan et al., 2017). This study also identified the protein kinase C and casein kinase II substrate in neurons (PACSIN1) as a new interactor that acts as a negative regulator of KCC2 expression and function, through mechanisms that remain to be determined. Interestingly, PACSIN1 is known to interact with the PKC-interacting proteins PICK1 and regulate activity-dependent trafficking of the AMPA receptor (Anggono et al., 2013), further supporting the tight interaction of KCC2 with glutamatergic signaling.

In conclusion, KCC2 appears to interact with a wealth of synaptic and perisynaptic proteins and notably proteins related to glutamatergic rather than GABAergic signaling (Table 1). These interactions have most often been explored from the point of view of KCC2 function, expression and regulation. However, KCC2 expression undergoes rapid and multifactorial up- and down-regulation both under physiological and pathological conditions (Chamma et al., 2012; Kaila et al., 2014; Medina et al., 2014). It seems unlikely that changes in KCC2 expression at the plasma membrane may not reciprocally affect the expression and/or function of its molecular partners. In particular, KCC2 clustering in dendritic spines and interaction with several synaptic and perisynaptic proteins raise the question of its role in dendritic spine physiology and excitatory synaptic function.

#### **4. KCC2 activity and the regulation of dendritic spine volume**

As described above, although KCC2 is expressed throughout the somato-dendritic membrane of most CNS neurons, it specifically aggregates in dendritic spines, near glutamatergic synapses through specific interactions with scaffolding molecules (Chamma et al., 2013; Gauvain et al., 2011). Why should a K/Cl co-transporter accumulate in dendritic spines?

K/Cl co-transport is one of the major mechanisms of cell volume regulation in mammalian cells (Hoffmann, Lambert, & Pedersen, 2009; Zeuthen, 2010). Since CNS neurons are mostly devoid of dedicated water channels such as aquaporins (Amiry-Moghaddam & Ottersen, 2003; Badaut, Lasbennes, Magistretti, & Regli, 2002; Kahle et al., 2015), the reported ability of CCCs to transport water alongside the ion fluxes (Hamann, Herrera-Perez, Bundgaard, Alvarez-Leefmans, & Zeuthen, 2005; Zeuthen, 1991, 2010) may be particularly important to deal with osmotic challenges. In neurons, such osmotic challenges may prominently result from intracellular influx of Na<sup>+</sup> and Cl<sup>-</sup> ions associated with synaptic activity. Thus, whereas synaptic activation of postsynaptic AMPARs may yield Na<sup>+</sup> transients that could lead to local concentration up to 10 mM that rapidly get cleared through spine neck diffusion, NMDAR activation leads to way larger and longer-lasting transients (Miyazaki & Ross, 2017). Therefore, repetitive activation of postsynaptic receptors is likely to generate significant, local osmotic challenges that could be partly compensated by KCC2 activity (Gulyas et al., 2001).

The first demonstration for a role of KCC2 in water fluxes and neuronal volume regulation was provided in a study using digital holographic microscopy, a noninvasive optical imaging technique to monitor transmembrane water fluxes as detected by the phase signal (Jourdain et al., 2011). Blocking KCC2 with furosemide reduced phase shifts induced by glutamate application onto cultured cortical neurons. Since then, several experimental observations supported a specific role of KCC2 in spine volume regulation. For example, chronic KCC2 suppression in mature hippocampal neurons using RNA interference leads to a prominent increase in spine head volume (Chevy et al., 2015; Gauvain et al., 2011). This effect likely reflects the loss of KCC2 transport function as it is mimicked by chronic application of an antagonist of the transporter (Gauvain et al., 2011) but not by overexpression of its carboxy-terminal tail, acting to prevent KCC2 interaction with molecular partners involved in actin remodeling (see sections 5-6). In addition, activity-dependent KCC2 down-regulation was

also recently associated with increased spine volume (Heubl et al., 2017). In these experiments, phosphorylation-dependent dispersion of KCC2 resulted in increased spine head volume in just 30 minutes. Even though the precise timing of this effect should be further characterized, these data suggest that loss of KCC2 activity could rapidly control spine volume. Remarkably, these effects were not associated with changes in spine length or density or in the proportion of filopodia-like structures (Gauvain et al., 2011). This contrasts with the genetic ablation of KCC2, which compromises the formation and maturation of dendritic spines in immature hippocampal neurons, through a mechanism involving its interaction with actin cytoskeleton (Li et al., 2007) (See Chapter XX). Therefore, whereas KCC2 interaction with actin is required for spinogenesis during development, KCC2 expression and function in mature neurons appear to be predominantly required to control spine head volume, not spine maintenance or structure.

Increased spine volume is associated with long term potentiation (LTP) at a variety of glutamatergic synapses in the CNS (Bosch & Hayashi, 2012). Such increase is extremely rapid and usually reaches its maximum during or immediately after the conditioning stimulus and then decays over minutes to stabilize at above-control values, involving actin cytoskeleton rearrangements (Bosch et al., 2014; Kopec, Real, Kessels, & Malinow, 2007; Murakoshi, Wang, & Yasuda, 2011). Although the initial increase in spine head volume may also reflect massive protein translocation into the spine head, as suggested using FRET-based probes for several scaffolding molecules (Bosch et al., 2014), the bi-phasic time-course of these structural changes is hard to explain solely by cytoskeleton remodeling. Alternatively, a tempting hypothesis would be that intense synaptic activity and postsynaptic ion influx during LTP induction may lead to near-instantaneous spine head swelling, and that KCC2 may then

serve to restore osmotic pressure through ion and water export. This hypothesis however currently lacks experimental validation.

### **5. KCC2-actin interaction hinders protein diffusion in dendritic spines**

Many integral membrane proteins serve as cytoskeleton anchors to the plasma membrane. These include adhesion molecules as well as ion channels, pumps, cotransporters and exchangers (Denker & Barber, 2002). Such interactions are critical for the stability and maintenance of the shape of subcellular compartments. The role of ion transport proteins in cytoskeleton anchoring to plasma membrane was first illustrated in erythrocytes by the anion exchanger 1 (AE1), which binds to spectrin/actin via ankyrin and the FERM domain containing adaptor protein 4.1R, thereby contributing to the typical shape and viscoelastic properties of these cells (Bennett & Baines, 2001; Jons & Drenckhahn, 1992).

As discussed above, KCC2 interacts through its carboxy-terminal domain with 4.1N, a member of the 4.1 family (Li et al., 2007). This interaction is at least partly responsible for confining KCC2 near glutamatergic synapses onto dendritic spines (Chamma et al., 2013). Conversely, through this interaction, KCC2 is expected to anchor spine actin cytoskeleton to the plasma membrane. Given the prominent role of actin dynamics in spine morphogenesis (Tada & Sheng, 2006), it is therefore not totally surprising that cortical KCC2<sup>-/-</sup> neurons fail to develop mature, functional spines when cultured *in vitro* (Li et al., 2007)(but see (Seja et al., 2012)) whereas KCC2 precocious expression increases dendritic spinogenesis (Fiumelli et al., 2013).

However, actin dynamics are not only involved in spinogenesis but also influence synaptic function and plasticity, through modulation of AMPA receptor lateral diffusion. As demonstrated using single molecule tracking techniques (Triller & Choquet, 2008), synaptic

AMPA receptors are confined within the postsynaptic density via interactions with scaffolding molecules and cytoskeleton (Choquet & Triller, 2003; Sheng & Hoogenraad, 2007). They also continuously exchange between synaptic, perisynaptic, and extrasynaptic pools by lateral diffusion, which acts to maintain a steady state level of functional receptors at synapses (Opazo & Choquet, 2011). Altering spine actin cytoskeleton therefore influences AMPAR diffusion and anchoring, and thereby affects the synaptic pool of receptors and synaptic efficacy (Kerr & Blanpied, 2012; Rust et al., 2010). Actin membrane-anchoring proteins located near glutamatergic synapses are therefore predicted to influence AMPAR diffusion within dendritic spines.

*(Figure 2 near here)*

**Figure 2. KCC2 hinders protein diffusion in dendritic spines.** *A, Left, Immunostaining of GFP and GluA1 in dendritic sections of hippocampal neurons expressing non-target (shNT) or KCC2-directed shRNA. KCC2 knockdown leads to reduced GluA1 immunofluorescence in dendritic spines (arrowheads). Scale, 1  $\mu$ m. Right, quantification of the normalized cluster intensity of GluA1 immunofluorescence in dendritic spines. B, image sequences of quantum dot-labeled, mobile GluA1 (arrowheads) in dendritic spines of neurons expressing non target vs. KCC2-directed shRNA as in A. Quantum dot images are shown in green while spine membrane is outlined in red. Explored area over 24s is shown on maximum intensity projections on the right panels. Note the larger explored area of GluA1 and its escape from the spine head in the absence of KCC2. Scale, 1  $\mu$ m. C, Schematic representation of structural changes in dendritic spines upon KCC2 suppression. Under control conditions, KCC2 interaction with submembrane actin scaffold hinders the diffusion of the mobile (persisynaptic) fraction of AMPA receptors and other actin-interacting proteins such as*



*NCAM180. KCC2 knockdown reduces this diffusion constraint, leading to enhanced diffusion of perisynaptic AMPA receptors. Continuous exchange between synaptic and perisynaptic pools then leads to the progressive partial depletion of the former. Note that diffusion of the less mobile (synaptic) fraction of AMPA receptors and of membrane-bound NCAM is unaffected. KCC2 suppression also leads to an increase in spine head volume as discussed in section 4.*

*Credits. Adapted from (Gauvain et al., 2011) with permission.*

This hypothesis was tested in experiments where KCC2 expression was suppressed in hippocampal neurons with RNA interference (Gauvain et al., 2011). Single particle tracking of AMPA receptors containing the GluA1 subunit revealed enhanced lateral diffusion of the mobile (likely non-synaptic) pool of receptors, with no detectable effect on the immobile (likely synaptic) fraction (Tardin, Cognet, Bats, Lounis, & Choquet, 2003)(Fig. 2B). Notably, this effect was observed in dendritic spines but not on dendritic shafts. It was not specific to AMPA receptors, as lateral diffusion of the cell adhesion molecule NCAM was also increased in absence of KCC2. More specifically, this effect was observed for the transmembrane isoform NCAM 180, bearing a short intracellular domain that interacts with actin cytoskeleton through  $\beta$ -spectrin, but not the membrane-anchored NCAM 120 isoform that lacks an intracellular domain (Buttner & Horstkorte, 2010). These results support a model in which KCC2 is an element of a molecular complex acting as a barrier for the lateral diffusion of non-synaptic, transmembrane proteins within dendritic spines (Fig. 2). Functionally, enhanced lateral diffusion of perisynaptic AMPA receptors was associated with a reduced immunostaining of the receptors in spines and reduced synaptic efficacy at glutamatergic synapses (Fig. 2A). Thus, mEPSCs were reduced in amplitude but not frequency in neurons

with reduced KCC2 expression. Importantly, these effects were mimicked by preventing KCC2 interaction with intracellular partners using its carboxy-terminal domain as dominant negative, but not by a KCC2 antagonist. This suggests KCC2, like other transmembrane ion-transport proteins (Denker & Barber, 2002), acts as an anchor to submembrane actin cytoskeleton that contributes to confine perisynaptic AMPA receptors within dendritic spines, independent of its ion transport function.

In conclusion, KCC2 interacts with submembrane actin cytoskeleton and thereby contributes to a molecular barrier hindering the lateral diffusion of transmembrane proteins within dendritic spines. Disrupting this barrier promotes AMPA receptor lateral diffusion and likely depletes a perisynaptic reserve pool of receptors and, subsequently, the synaptic pool, leading to reduced efficacy of glutamatergic synapses (Fig. 2C). This phenomenon may be particularly relevant in the pathology but also for the physiological regulation of excitatory transmission. KCC2 clusters are rapidly dispersed upon sustained NMDA receptor activation (Chamma et al., 2013; Lee, Deeb, Walker, Davies, & Moss, 2011). Subsequent enhancement of lateral diffusion and depletion of the synaptic and perisynaptic pools may then contribute to NMDA receptor-induced plasticity of glutamatergic synapses.

## **6. KCC2-dependent control of actin dynamics and long term potentiation at glutamatergic synapses**

The relation between the KCC2 cotransporter and actin extends beyond the mere organization of a molecular barrier for the lateral diffusion of transmembrane proteins. KCC2 knockdown or precocious expression was shown to influence the dynamics of actin polymerization in several cell types. Although KCC2 is largely considered a neuron-specific transporter, it appears to be expressed in several human cancer cell lines (Wei et al., 2011). In some of these, manipulating KCC2 expression resulted in remarkable changes in cell spreading and

stress fiber organization. Thus, suppressing KCC2 resulted in increased filamentous actin (f-actin) content, suggestive of enhanced actin polymerization while overexpressing KCC2 led to the opposite phenotype. Conversely, neuron-specific overexpression of KCC2 in mouse embryos resulted in aberrant f-actin distribution with reduced density at the *adherens* junctions lining the neural tube (Horn, Ringstedt, Blaesse, Kaila, & Herlenius, 2010). In both studies, the observed phenotype was independent of the transporter function of KCC2 as it was mimicked by recombinant, Cl<sup>-</sup> transport-deficient mutant KCC2. These results suggested that KCC2 may not just interact with actin cytoskeleton but also somehow control actin polymerization.

Dynamic actin remodeling in neurons is controlled by small GTPases of the Rho family that includes Rho, Rac1, and Cdc42 (Cingolani & Goda, 2008). In dendritic spines, which are enriched in filamentous actin (f-actin), local Rac1 activation involves the synaptic anchoring by the G-protein-coupled receptor kinase-interacting protein 1 (GIT1) of its partner βPIX (H. Zhang, Webb, Asmussen, & Horwitz, 2003), a guanine nucleotide exchange factor (GEF) which facilitates the exchange of GDP for GTP. Activated Rac1 may then bind to several effector proteins including the p21 activated kinase (PAK), which in turn activates LIM kinase. This ultimately leads to inhibition of the actin-severing protein cofilin through phosphorylation of its Ser3 residue (Yang et al., 1998)(Fig. 3).

Increased dendritic spine actin polymerization upon KCC2 knockdown was observed in hippocampal neurons (Llano et al. 2015, Chevy et al. 2015). This effect likely involves a direct interaction between KCC2 and βPIX, as demonstrated in immunoprecipitation assays, even though this interaction was not identified in recent functional proteomic analysis (Mahadevan et al., 2017). Thus, KCC2 genetic ablation or knockdown by RNA interference leads to reduced actin turnover and accumulation of f-actin in dendritic spines. Stimulated-emission-depletion (STED) super-resolution microscopy showed enhanced f-actin content in

spine necks but did not reveal specific patterns of f-actin accumulation within spine heads (Chevy et al., 2015). This effect was associated with mobilization of  $\beta$ PIX in dendritic spines, a specific increase in Rac1 but not Rho-A activity as well as enhanced cofilin phosphorylation (Chevy et al., 2015; Llano et al., 2015). These observations are consistent with activation of the Rac1-PAK1-LIMK pathway and inhibition of cofilin. Importantly, again, these effects were independent of ion transport by KCC2 as they were mimicked by a dominant negative peptide that inhibits KCC2 interaction with intracellular partners (Chevy et al., 2015) and rescued by the expression of a transport-deficient recombinant KCC2 (Llano et al., 2015).

*(Figure 3 near here)*

***Figure 3. KCC2 interaction with  $\beta$ PIX controls spine actin dynamics and gates LTP at excitatory synapses.*** A, *Viral-based chronic KCC2 suppression by RNA interference. Confocal micrograph showing viral expression (as detected by GFP fluorescence, green) in a rat hippocampal slice with DAPI staining (blue). Stimulation and recording electrodes are shown. Scale, 200  $\mu$ m.* B, *Summary graph from experiment as in A, showing LTP of the fEPSP recorded in str. moleculare upon high frequency stimulation (HFS) of perforant path afferents. KCC2 knockdown (shKCC2) precluded LTP expression at this synapse.* C, *schematic representation of the molecular cascade involved in this effect. Upon KCC2 suppression,  $\beta$ PIX relocates at the postsynaptic density and interacts with GIT1 to specifically activate Rac1, leading to enhanced PAK1 and LIMK activity. This in turn inhibits cofilin leading to enhanced f-actin content in dendritic spines. f-actin is non-permissive for activity-driven AMPA receptor delivery, thereby precluding LTP expression. Preventing cofilin inhibition is sufficient to restore AMPA receptor exocytosis in KCC2 knockdown neurons.*

*Credits. Adapted from (Chevy et al., 2015) with permission.*

How KCC2 precisely regulates Rac1 activity through  $\beta$ PIX remains to be fully elucidated but may involve molecular trapping of  $\beta$ PIX by KCC2. As discussed above (section 2), KCC2 appears to be excluded from the glutamatergic postsynaptic density. KCC2 might therefore contribute to sequester  $\beta$ PIX away from the postsynaptic density, where GIT1 acts as a postsynaptic scaffold for multiprotein signaling complex with Rac1 (H. Zhang et al., 2003). Overexpressed KCC2 carboxy-terminal domain may then compete with KCC2- $\beta$ PIX interaction and thereby favor  $\beta$ PIX binding to GIT1 within the postsynaptic density, similar to KCC2 knockdown. Although more evidence is needed to support this hypothesis, including PSD purification assays and super-resolution imaging, it is remarkable that KCC2 knockdown increases the clustering of  $\beta$ PIX but not GIT1 in dendritic spines (Chevy et al., 2015).

Tight regulation of actin dynamics is critical to the structural and functional changes involved in long term plasticity of glutamatergic synapses (Bosch & Hayashi, 2012). Long term potentiation is known to rely on a concomitant increase of both the number of postsynaptic AMPA receptors (Hayashi et al., 2000; Herring & Nicoll, 2016; Malinow, Mainen, & Hayashi, 2000; Poncer, 2003; Rumpel, LeDoux, Zador, & Malinow, 2005) and the volume of dendritic spines that host glutamatergic synapses (Bosch et al., 2014; L. Y. Chen, Rex, Casale, Gall, & Lynch, 2007; Fortin et al., 2010; Gu et al., 2010; Kopec et al., 2007; Okamoto, Bosch, & Hayashi, 2009; Park et al., 2006; Sala & Segal, 2014). The latter is associated with a persistent increase in actin polymerization within the potentiated dendritic spine (Okamoto, Nagai, Miyawaki, & Hayashi, 2004). However, an initial and transient actin depolymerization is required for both activity-driven AMPA receptor exocytosis and increase in spine volume (Gu et al., 2010; Ouyang et al., 2005). This reflects the transient activation (i.e., dephosphorylation) of the actin-severing enzyme cofilin at the time of LTP induction (Gu et al., 2010). Transient actin depolymerization may be required for activity-driven AMPA

receptor membrane insertion by promoting vesicle exocytosis that may otherwise be hindered by cortical actin, as described in adrenal chromaffin cells (Gasman et al., 1999). Alternatively, partial cytoskeleton disassembly may be required to release secretory vesicles and promote their traffic to the plasma membrane.

KCC2 interaction with the Rac-PAK-LIMK pathway regulating cofilin activity is then expected to impact functional and structural plasticity at excitatory synapses. This hypothesis was first tested by knocking-down KCC2 in dentate gyrus granule cells where LTP of entorhinal afferents is mediated through postsynaptic AMPA receptor traffic (Poncer & Malinow, 2001). LTP at these synapses was almost fully abolished upon KCC2 knockdown (Chevy et al., 2015) (Fig. 3A-B). Suppression of KCC2 expression by RNA interference in primary hippocampal culture similarly precluded chemically-induced LTP (cLTP). Thus, both structural (i.e. change in dendritic spine volume) and functional (i.e. addition of glutamatergic receptors) cLTP was abolished in KCC2 knockdown neurons. This effect was acting downstream of CaMKII activation, since overexpression of a constitutively active enzyme also failed to induce LTP in neurons with suppressed KCC2 expression. In fact, LTP hindrance upon KCC2 suppression directly and specifically relied on dysregulation of actin dynamics, as inhibitors of either PAK1 or LIMK fully rescued structural and functional LTP expression (Chevy et al., 2015). Importantly, all these experiments were performed in the presence of the GABA<sub>A</sub> receptor antagonist bicuculline. Therefore, LTP hindrance was independent of any change in GABA signaling induced upon KCC2 suppression in hippocampal neurons (Pellegrino et al., 2011). A recent study, however, suggested that altered GABA signaling upon KCC2 downregulation may compromise synapse specificity of LTP in the ageing hippocampus, through mechanisms that remain to be clarified (Ferando, Faas, & Mody, 2016).

In conclusion, direct interaction of the KCC2 co-transporter with both actin-associated proteins (such as 4.1N) and proteins controlling actin dynamics (such as  $\beta$ PIX) turns out to strongly impact the structure, function and plasticity of glutamatergic synapses (Fig. 3C). This may be important in physiological settings. As discussed above, KCC2 clusters are rapidly dispersed upon NMDA receptor activation and subsequent  $\text{Ca}^{2+}$  influx, partly through Ser940 dephosphorylation and calpain-induced cleavage (Chamma et al., 2013; Lee et al., 2011; Puskarjov, Ahmad, Kaila, & Blaesse, 2012).  $\text{Ca}^{2+}$ -dependent KCC2 down-regulation has been shown to underlie some forms of activity-dependent plasticity of GABA signaling in cortical neurons (Fiumelli, Cancedda, & Poo, 2005; Woodin, Ganguly, & Poo, 2003). However, as discussed above, activity-induced dispersion of KCC2 clusters within dendritic spines may also rapidly affect spine actin cytoskeleton and polymerization. Such changes are likely to influence further induction of synaptic plasticity at recently active synapses. It is then tempting to suggest that KCC2 may then act as a metaplastic switch (Abraham, 2008) for glutamatergic synapses, acting to adjust synaptic plasticity to prior activity due to changes in actin polymerization. Thus, NMDA receptor activation has been shown to inhibit subsequent LTP induction in CA1 hippocampal neurons (Y. Y. Huang, Colino, Selig, & Malenka, 1992). The mechanisms underlying synaptic metaplasticity remain elusive (Abraham, 2008; Hulme, Jones, & Abraham, 2013), but PP1- and calpain-mediated KCC2 clearance and subsequent actin remodeling may represent a molecular substrate deserving further experimental investigation.

## **7. Conclusions**

As discussed in this and the preceding chapters, KCC2 clearly appears to fulfill more than just one function in neurons, with roles ranging from maintenance of neuronal transmembrane

chloride gradients to regulation of dendritic spine volume, spine actin membrane anchoring and dynamics.

KCC2 belongs to the CCC family, which itself is part of the family of the solute carriers with its 52 distinct subgroups (Hediger et al., 2004). Phylogenetic analysis of the CCC family shows that KCC proteins have emerged from a common ancestor gene through three main gene duplication events (Hartmann, Tesch, Nothwang, & Bininda-Emonds, 2014). As discussed by Blaesse and Schmidt (Blaesse & Schmidt, 2015), multi-functional proteins such as KCC2 may represent an intermediate stage of evolution, in which several functions have not yet led to gene duplication and specialization that may be required to optimize each individual function. The term *function*, however, should be considered with caution, as it is perhaps questionable whether the relationship between KCC2 and actin cytoskeleton and its functional impact represents an actual *function* of KCC2 or whether they primarily represent the *consequence* of molecular interactions and subcellular membrane anchoring required for fulfilling its original, ion- (and water-) transport function. In this context, it is worth noting that a variety of ion transport proteins are also *used* to anchor and regulate cytoskeleton in many different cells types (Denker & Barber, 2002).

Finally, in addition to the aforementioned activity-induced changes at the posttranslational level, KCC2 expression is down-regulated in a variety of pathological conditions ranging from epilepsy, neuropathic pain, spasticity and stroke to schizophrenia, autism-spectrum disorders and Alzheimer's disease (reviewed in (Kahle et al., 2008; Kaila et al., 2014; Moore, Kelley, Brandon, Deeb, & Moss, 2017); see also Chapter 14-23). Reduced KCC2 expression and activity are often assumed to contribute to pathological network activities underlying these conditions by reducing the efficacy of GABAergic transmission. Drugs acting to compensate for KCC2 suppression therefore hold great therapeutic potential. For instance, NKCC1 antagonists such as bumetanide may help rescuing intraneuronal chloride transport in



KCC2-lacking neurons and thereby normalize neuronal activity (Ben-Ari, 2017). However, this approach would only compensate ion-transport related KCC2 functions. In this context, it is interesting to note that the first KCC2 polymorphism associated with idiopathic epilepsy compromised both ion transport and dendritic spine morphogenesis (Puskarjov et al., 2014). How ion-transport independent functions actually contribute to the pathology remains to be fully explored. We suggest however that pharmacological strategies aiming to enhance KCC2 expression or promote its membrane stability (Gagnon et al., 2013; J. Zhang, Karimy, Delpire, & Kahle, 2017) rather than solely rescue neuronal chloride homeostasis may more fully compensate synaptic and neuronal deficits induced by KCC2 down-regulation in neurological and psychiatric disorders.

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