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## **Special issue : Neuronal protein mobility**

### **KCC2 membrane diffusion tunes neuronal chloride homeostasis**

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## Abstract

Neuronal  $\text{Cl}^-$  homeostasis is regulated by the activity of two cation chloride co-transporters (CCCs), the  $\text{K}^+-\text{Cl}^-$  cotransporter KCC2 and the  $\text{Na}^+-\text{K}^+-\text{Cl}^-$  cotransporter NKCC1, which are primarily extruding and importing chloride in neurons, respectively. Several neurological and psychiatric disorders including epilepsy, neuropathic pain, schizophrenia and autism are associated with altered neuronal chloride ( $\text{Cl}^-$ ) homeostasis. A current view is that the accumulation of intracellular  $\text{Cl}^-$  in neurons as a result of KCC2 down-regulation and/or NKCC1 up-regulation may weaken inhibitory GABA signaling and thereby promote the development of pathological activities. CCC activity is determined mainly by their level of expression in the plasma membrane. Furthermore, CCCs undergo “diffusion-trapping” in the membrane, a mechanism that is rapidly adjusted by activity-dependent post-translational modifications i.e. phosphorylation / dephosphorylation of key serine and threonine residues. This represents probably the most rapid cellular mechanism for adapting CCC function to changes in neuronal activity. Therefore, interfering with these mechanisms may help restoring  $\text{Cl}^-$  homeostasis and inhibition under pathological conditions.

## Keywords

KCC2; NKCC1; GABAergic transmission; chloride homeostasis; epilepsy; lateral diffusion; clustering

## Introduction

The main inhibitory neurotransmitter receptors in the central nervous system (CNS) are type A  $\gamma$ -aminobutyric acid receptors (GABA<sub>A</sub>R) and glycine receptors (GlyR). Upon activation, GABA<sub>A</sub>R and GlyR selectively conduct Cl<sup>-</sup> - and, to a lesser extent, bicarbonate - through their pore. The direction of Cl<sup>-</sup> flux through the channels depends on the transmembrane electrochemical chloride gradient. Two members of the cation-chloride co-transporter (CCC) family (which is composed of nine members encoded by the genes *Slc12a1-9*) play a prominent role in controlling intra-neuronal chloride concentration ([Cl<sup>-</sup>]<sub>i</sub>): the Na<sup>+</sup>/K<sup>+</sup>/2Cl<sup>-</sup> co-transporter 1 (NKCC1) and the K<sup>+</sup>/Cl<sup>-</sup> co-transporter 2 (KCC2). NKCC1 and KCC2 are secondary active transporters, using the electrochemical gradients generated by the Na<sup>+</sup>/K<sup>+</sup> ATPase (Figure 1). Therefore, the net ion flux they carry primarily depends on transmembrane Na<sup>+</sup>, K<sup>+</sup> and Cl<sup>-</sup> gradients and should be expected to be reversible. However, whereas KCC2 functions near thermodynamic equilibrium and may extrude or import chloride depending on extracellular K<sup>+</sup> and intracellular Cl<sup>-</sup> concentration, NKCC1 mediates ion influx in most cell types and under most physiological conditions (Russell, 2000). Therefore, it is generally assumed that NKCC1 and KCC2 are primarily a chloride importer and extruder, respectively, at least under resting conditions.

The structural organization of KCC2 and NKCC1 is similar and comprises 12 transmembrane domains (TMs), six extracellular loops (ECL), as well as intracellular N- and C- termini (Hartmann and Nothwang, 2015) (**Figure 1**). TMs are required for ion translocation and are therefore highly conserved in KCC2 and NKCC1 (Gamba, 2005; Payne et al., 1995; 1996; Somasekharan et al., 2012). In contrast, KCC2 and NKCC1 differ by the position of a long extracellular loop (LEL) between TM5 and TM6 in KCC2, and between TM7 and TM8 in NKCC1 (Gamba, 2005; Payne et al., 1995; 1996). The intracellular N- and C-termini of KCC2

and NKCC1 carry regulatory sequences and phosphorylation sites. In addition, the C-terminus of KCC2 contains a motif responsible for the isotonic activity of the transporter (Acton et al., 2012; Bergeron et al., 2006; Mercado et al., 2006). KCC2 and NKCC1 N- and C- termini also participate in the regulation of membrane trafficking (Lee et al., 2010, 2007; Zhao et al., 2008), basolateral and apical sorting in polarized cells (Carmosino et al., 2008) and oligomerization (Casula et al., 2009, 2001; Parvin et al., 2007; Simard et al., 2004; Warmuth et al., 2009). Furthermore, two KCC2 and NKCC1 (a and b) isoforms have been identified, which differ by the presence of additional amino acid sequences in KCC2a and NKCC1a on their N- and C- termini respectively (**Figure 1**).

While other CCCs such as NKCC2 and NCC are mostly expressed in the kidney where they regulate salt re-absorption (Kahle et al., 2010; Russell, 2000), NKCC1 is ubiquitously expressed (Plotkin et al., 1997). In the CNS, NKCC1 is highly expressed in immature neurons and its expression level decreases during neuronal maturation in some but not all brain regions including the hippocampus (reviewed in Watanabe and Fukuda, 2015). In fact, the relatively high level of NKCC1 detected at adult stage is contentious with the global idea of a downregulation of NKCC1 during development (Yamada et al., 2004, reviewed by Kaila et al., 2014). In contrast to other KCCs, KCC2 is almost exclusively restricted to the CNS and is constitutively active under isotonic conditions (Acton et al., 2012; Mercado et al., 2006; Strange et al., 2000). The developmental upregulation of KCC2 expression is responsible for the early postnatal depolarizing shift in the polarity of GABAergic and glycinergic transmission in the CNS (Rivera et al., 1999). In addition to its function in inhibitory neurotransmission, KCC2 has been shown to play a role in dendritic spine formation and maturation (Li et al, 2007; Fiumelli et al 2015; Awad et al 2018) as well as in excitatory glutamatergic synaptic transmission and plasticity (Gauvain et al 2011; Chevy et al 2015).

In the adult CNS, pathological alterations of KCC2 and NKCC1 expression and/or function lead to a depolarizing shift in GABA signalling, which may underlie anomalous network activities. Several studies have reported a down-regulation of KCC2 and an up-regulation of NKCC1, resulting in increased  $[Cl^-]_i$  that promote depolarization/excitation in several neurological disorders such as neuropathic pain, spinal cord injury and epileptic disorders (reviewed in (Di Cristo et al., 2017)). Drugs aimed at reducing neuronal  $[Cl^-]_i$  therefore represent a promising therapeutic strategy. Although the expression of CCCs is regulated both at the transcriptional and posttranslational levels, mechanisms controlling their membrane stability also rapidly influence their net function and therefore offer novel opportunities for pharmacological intervention. Here we will review experimental evidence supporting the role of lateral diffusion and clustering in the functional regulation of the KCC2 transporter and discuss how similar mechanisms may apply to other CCCs such as NKCC1.

## **1. KCC2 clustering**

KCC2 is clustered in the somato-dendritic plasma membrane of cortical neurons (Gauvain et al., 2001; Barthó et al., 2004; Chamma et al., 2013, 2012; Gulyás et al., 2001; Heubl et al., 2017; Hübner et al., 2001; Watanabe et al., 2009). In these cells, most clusters are found near both excitatory and inhibitory synapses, with no preferential enrichment at one vs. the other (Chamma et al., 2013). Ultrastructural studies reported transporter accumulation at the periphery of excitatory synapses within dendritic spines as well as along the dendritic shaft (Báldi et al., 2010; Gulyás et al., 2001). However, these studies lacked a quantitative analysis. The use of quantitative electron microscopy or super-resolution microscopy is thus required to more precisely evaluate the subcellular localization of KCC2 in the neuronal membrane.

## **1.1 Clustering mechanisms**

### **1.1.1. Oligomerization and actin-binding**

Several mechanisms may account for KCC2 aggregation. Oligomerization-induced trapping was proposed to be involved in - or result from - protein activation (for review see (Kusumi et al., 2005)). KCC2 is known to form homo- and hetero-oligomers with other CCC family members (Blaesse et al., 2006; Simard et al., 2007; Uvarov et al., 2009). The expression of KCC2 Tyrosine 1087 phosphorylation mutants induced a loss of transporter oligomerization and function without a change in its membrane expression (Watanabe et al., 2009), suggesting a link between oligomerization, clustering and function. Multimeric assembly of KCC2 with other membrane proteins via low-energy interactions could also regulate KCC2 function in neuronal plasma membrane (Goldman et al., 2004; Marguet et al., 2006). For instance, KCC2 assembly with the kainate-sensitive glutamate receptor GluK2 subunit or with Neuropilin and tolloid like-2 (Neto-2), the auxiliary subunit of the kainate receptor, increases its oligomerization and  $\text{Cl}^-$  transport function in hippocampal neurons (Ivakine et al., 2013; Mahadevan et al., 2014; Pressey et al., 2017). However, it is difficult to conclude that the formation of a multimeric complex increases KCC2 function since it is always associated with an increase of its surface expression.

KCC2 binds to the actin cytoskeleton in neurons through interaction of its carboxy-terminal domain (CTD) with protein 4.1N (H. Li et al., 2007). The relief of KCC2 diffusion constraints observed upon either 4.1N knockdown by RNA interference, overexpression of the KCC2-CTD, or actin depolymerisation with Latrunculin A suggests that a sub-population of transporters may be anchored near excitatory synapses via KCC2-4.1N-actin interaction (Chamma et al., 2013). However, cytoskeleton tethering to the membrane does not appear to be

responsible for KCC2 cluster formation *per se*, and might instead influence the specific localization of a sub-population of KCC2 clusters (Chamma et al., 2013).

### **1.1.2. Lipid rafts**

What molecular mechanism other than actin anchoring may be responsible for KCC2 clustering? KCC2 has been shown to associate with lipid rafts (Hartmann et al., 2009; Watanabe et al., 2009), as do numerous synaptic transmembrane proteins such as GABA<sub>A</sub>Rs (Li et al., 2007) or AMPARs (Hou et al., 2008). Lipid raft-dependent segregation is thought to provide mobile signaling platforms that influence clustering and function of plasma membrane molecules (Pike, 2009; Tillman and Cascio, 2003). A study on the modulation of the serotonin reuptake transporter (SERT) by lipid raft association and cytoskeleton tethering showed increased transport activity when SERT was localized into lipid rafts. Interestingly, actin filament disruption increased the lateral diffusion of the transporter that however remained localized in lipid rafts (Chang et al., 2012). The cytoskeleton anchoring of SERT-containing lipid rafts was shown to rely on SERT interaction with intracellular partners, which can be modulated by phosphorylation. Hence, a complex interplay between phosphorylation, cytoskeleton tethering and lipid raft association seems to regulate the localization, diffusion and function of the transporter. Regarding KCC2, two studies focused on the interplay between KCC2 clustering and lipid raft association (Watanabe et al., 2009; Hartmann et al., 2009). Both groups found the transporter within lipid rafts but reached opposing conclusions regarding its functional impact. Thus, Watanabe et al. showed that KCC2 distribution in neurons switched from punctate to diffuse upon lipid raft disruption while KCC2 activity decreased, suggesting that localization in lipid rafts increases KCC2 transport activity (Watanabe et al., 2009). Hartmann et al. on the other hand observed increased KCC2 transport and aggregation upon lipid raft disruption in HEK-293 cells (Hartmann et al., 2009). In these cells, however, lipid raft disruption also increased KCC2 surface expression, which complicates the interpretation of



these observations. Therefore, how KCC2 clustering in lipid rafts influences its transport function remains unclear.

Collectively, these data suggest that distinct KCC2 subpopulations may differentially associate with lipid rafts. In addition, interaction between KCC2 and actin cytoskeleton may tether KCC2-containing lipid-raft platforms to specific plasma membrane domains where local molecular partners form functional complexes. Phosphorylation of the transporter may then regulate KCC2 binding to intracellular partners and targeting to lipid rafts. It would be interesting to characterize in more detail how and under which conditions KCC2 accumulates in lipid rafts and whether KCC2-containing lipid rafts are associated with synaptic or extrasynaptic markers, as shown for GABA<sub>A</sub>Rs (X. Li et al., 2007).

## **1.2. Clustering function**

Clustering may help to localize and/or stabilize transporters in sub-membrane compartments e.g. near excitatory synapses or to form a barrier in dendritic spines surrounding glutamatergic PSDs. The enrichment of KCC2 near excitatory synapses raises the question of whether the transporter is active in dendritic spines? Addressing the function of an electroneutral transporter at the subcellular level is extremely challenging. However, we have obtained indirect evidence that KCC2 is functional in dendritic spines. In experiments where KCC2 function was blocked using a specific KCC2 antagonist, we observed spine head swelling with no change in spine length or density (Gauvain et al., 2011). This effect is consistent with local changes in osmotic pressure due to the loss of solute export in spine heads. Importantly, this effect was independent of the structural role of KCC2 on spine actin cytoskeleton, as this effect was not observed by preventing KCC2 interaction with actin. Whether and to which extent dendritic shaft chloride concentration is influenced by KCC2 function in spines remains unknown. However, it is remarkable that modeling data suggest the presence of dendritic spines impacts chloride

diffusion in dendritic compartments (Mohapatra et al., 2016). How KCC2 further contributes to this effect remains unknown.

Could KCC2 clustering modulation account for local changes in intra-neuronal chloride concentrations? Chloride is not uniformly distributed along neurites (Doyon et al., 2011; Price and Trussell, 2006; Waseem et al., 2010). Using computational models and N-(ethoxycarbonylmethyl)-6-methoxy-quinolinium bromide (MQAE) - based fluorescence life time imaging microscopy (FLIM) in mature neurons, Doyon et al. showed that  $[Cl^-]_i$  increased proportionally to the distance from the soma. They suggested that the specific geometry of the dendrites may hinder chloride diffusion, while chloride concentration at the soma remains clamped. This somato-dendritic chloride gradient was abolished by inhibition of KCC2 or blockade of GABAergic neurotransmission (Doyon et al., 2011), suggesting that KCC2 function is necessary to establish the gradient.

Simulation of intra-neuronal chloride concentrations suggested that the activation of a single GABAergic synapse can lead to a local change in  $[Cl^-]_i$  of about 5 mM (Doyon et al., 2011). A single focal synapse activation at 50 Hz could therefore influence the response of neighbouring GABAergic synapses within a distance of 50  $\mu m$  (Doyon et al., 2011; Jedlicka et al., 2010). In these simulations however chloride diffusion was assumed to be similar to that measured in water (2.03  $\mu m^2/ms$ ). In addition, the influence of intracellular or extracellular matrix proteins on  $[Cl^-]_i$  via the Gibbs-Donan effect (Glykys et al., 2014) as well as local chloride attraction by positive charges such as polyamines were ignored. Estimated diffusion may then be overestimated in these models, suggesting that even stronger local chloride accumulation may occur near activated inhibitory synapses. Therefore, increasing or decreasing CCC clustering near synapses may rapidly and efficiently modulate  $[Cl^-]_i$ .

## **2. Lateral diffusion regulates membrane protein trafficking**

Protein diffusion in the neuronal plasma membrane was first evidenced for neurotransmitter receptors and this mechanism was shown to be of particular interest in the context of synaptic transmission and plasticity (Choquet and Triller, 2013; Triller and Choquet, 2008). In brief, receptors are randomly exocytosed in the plasma membrane where they diffuse until they get trapped by the postsynaptic scaffold that anchors them to the sub-membrane cytoskeleton. Pools of synaptic and extrasynaptic receptors undergo a continuous and reciprocal exchange that can be rapidly regulated by neuronal activity, thereby modulating the density of synaptic receptors. Here, we will discuss the relevance of lateral diffusion as a novel mechanism to regulate KCC2 activity, and the importance of cluster formation and maintenance in this context.

## **2.1. Brownian-type motion and confinement**

KCC2 membrane diffusion was studied using quantum-dot-based single particle tracking (QD-SPT) (Chamma et al., 2013). SPT is an immunocytochemistry-based method which allows following the movements of individual molecules at the surface of living cells, and to detect heterogeneities in the diffusion behaviors of independent molecules. This technique therefore provides accurate information about the influence of microstructures that restrict diffusion, and of local interactions that retain proteins and confines them to a given space (Triller and Choquet, 2008). SPT consists in following fluorescent markers that are bound to the protein of interest in a specific manner through a linker, usually an antibody recognizing an extracellular epitope of the endogenous protein or a tag (e.g. HA, Flag, GFP) inserted in the extracellular region of the recombinant protein. Several criteria are required for efficient protein-linker coupling: nanomolar affinity between the protein, the linker, and the marker to ensure the stability of the protein-linker-marker complex, and a stoichiometry that enables one-to-one interaction between the protein of interest and the marker (Triller and Choquet, 2008; Pinaud et al., 2010). Moreover, the photostability of the fluorescent marker determines the maximal time of acquisition (due to photobleaching). The high photostability and signal-to-noise-ratio of QDs

allow to follow single molecules for long duration with a QD localization accuracy of 5-10 nm (Dahan et al., 2003). Following QD labeling, real time movies are acquired and QD tracking and trajectory reconstructions are performed with homemade software (MATLAB; MathWorks) as described previously (Bonneau et al., 2005). Then, the center of the fluorescence spots is determined with a spatial accuracy of 5-10 nm by cross-correlating the image with a Gaussian fit of the point spread function (for details, see Triller and Choquet, 2008). Trajectories are classified as synaptic when they overlapped with the synaptic mask of gephyrin or homer1c fluorescent clusters to identify excitatory and inhibitory synapses, respectively, or extrasynaptic for spots two pixels (~380 nm) away. Analysis of reconstructed trajectories gives access to diffusion speed, confinement index and dwell time of tracked molecules in a given sub-cellular compartment.

Analysis of hundreds of KCC2 trajectories at the surface of hippocampal cultured neurons revealed a reduced diffusion coefficient and an increased confinement of the transporter near both excitatory glutamatergic and inhibitory GABAergic synapses as compared to extrasynaptic areas where transporters display Brownian-type motion (Chamma et al., 2013, 2012; Heubl et al., 2017) (**Figure 2**). Transporters are temporarily immobilized near synapses and can escape the perisynaptic zone by lateral diffusion to explore neighboring membrane areas. This is reminiscent of the neurotransmitter receptors diffusive behaviour (Choquet and Triller, 2013; Triller and Choquet, 2008). KCC2, however, diffused faster at excitatory and inhibitory synapses (~1.9 fold and 1.6 fold, respectively) as compared to AMPARs and GABA<sub>A</sub>Rs (**Table 1**).

Increased confinement near excitatory and inhibitory synapses may lead to local KCC2 accumulation. Interestingly, the vast majority of KCC2 clusters are detected near synapses, where KCC2 confinement is also the highest (Chamma et al., 2013). Furthermore, most experimental paradigms (see below) known to increase KCC2 diffusion also induce cluster

dispersion and loss. Therefore, KCC2 clustering requires a constant exchange between pools of mobile transporters in the extrasynaptic membrane and pools of less mobile transporters near synapses. A dynamic equilibrium between these pools of transporters may be rapidly modified to locally increase or decrease transporter clustering and thereby influence local, intra-neuronal chloride levels.

## **2.2. Molecular crowding and anchoring to cytoskeleton**

Several factors are hindering the diffusion of trans-membrane proteins in biological membranes. These include transient interactions with proteins present in the membrane (Marguet et al., 2006), direct immobilization through stable molecular interactions (Gerrow and Triller, 2010), and/or corralling of membrane proteins by matrix-bound or aggregating proteins (Lippincott-Schwartz et al., 2001). Lateral diffusion of a protein also depends on its size (Ramadurai et al., 2009). Thus, the diffusion of a protein within an oligomeric complex will be reduced compared to that of a freely-moving molecule. Furthermore, lateral diffusion is limited by the concentration of obstacles in the plasma membrane (Saxton and Jacobson, 1997). In a very crowded environment, such as the postsynaptic membrane, most particles are slowed down and confined (Renner et al., 2012; M. L. Renner et al., 2009). Some proteins may also undergo specific interaction with scaffolding molecules that anchor them to the sub-membrane cytoskeleton. In this case, diffusion speed is also reduced, and confinement increased (Choquet and Triller, 2013).

To gain insight into the influence of obstacles and interactions with scaffolding molecules on KCC2 diffusion, the diffusion properties of KCC2 were compared to that of NCAM120, a glycosylphosphatidyl inositol (GPI)- anchored neural cell adhesion protein, that lacks intracellular domain (Rønn et al., 1998) and displays no preferential synaptic accumulation. Compared to KCC2, NCAM120 diffusion coefficient was increased by ~2 fold near

glutamatergic and GABAergic synapses, but NCAM120 still showed reduced diffusion and increased confinement at inhibitory and excitatory synapses as compared to extrasynaptic zone, indicative of molecular crowding (Renner et al. 2009; Renner et al. 2012) (**Table 1**). The synaptic dwell time which informs on protein-scaffold tethering (Renner et al., 2012) revealed that KCC2 spends more time near excitatory synapses than near inhibitory synapses, suggesting higher tethering near glutamatergic synapses. Compared to NCAM120, which is not specifically enriched at excitatory synapses, KCC2 dwell time was drastically increased (~5 fold), indicating KCC2 tethering near glutamatergic synapses. Although the diffusion coefficient of KCC2 was significantly (~2 fold) higher than that of AMPARs, KCC2 dwell time was only slightly lower (~1.6 fold) than that of AMPARs. There is no correlation between the diffusion coefficient and the dwell time (Renner et al., 2012). The lateral diffusion of a protein is hindered by the presence of diffusion barriers such as pickets and fences. The explored area and dwell time, but not diffusion coefficient, are correlated with the synaptic trapping and concentration of molecules. This means that although KCC2 moves rapidly in the membrane (faster than AMPARs), transporters are captured by the scaffold enriched at/near excitatory synapses (almost as efficiently as AMPARs which are known to interact with actin scaffold). In contrast, the dwell times of KCC2 and of NCAM120 did not significantly differ near inhibitory synapses (**Table 1**). Collectively, these results show that KCC2 specifically interacts with scaffolding molecules at excitatory glutamatergic synapses. Therefore, KCC2 undergoes a diffusion-trapping mechanism at/near excitatory synapses but not at/near inhibitory synapses. This is consistent with the recent characterization of the KCC2 interactome showing that KCC2 interacts with more proteins located at excitatory synapses than at inhibitory synapses (Mahadevan et al., 2017).

Excitatory and inhibitory synapses differ in the composition of their associated cytoskeleton, with filamentous actin highly enriched in dendritic spines, which harbour most excitatory

synapses (Korobova and Svitkina, 2010). Interactions with sub-membrane actin cytoskeleton likely contributes to hinder KCC2 diffusion near excitatory synapses. An initial study by Li et al. showed that the CTD of KCC2 interacts with actin via protein 4.1N and that this interaction is involved in spine morphogenesis (H. Li et al., 2007). Overexpression of KCC2-CTD as a dominant negative inhibitor of KCC2-4.1N interaction, knockdown of 4.1N by RNA interference or inhibition of actin polymerization using Latrunculin A increased KCC2 diffusion and decreased its dwell time near excitatory (~2 to 5 fold) but not inhibitory synapses (Chamma et al., 2013). This indicates that KCC2 is tethered near excitatory synapses and in dendritic spines, but not near inhibitory synapses, via interaction of its CTD with 4.1N and the actin cytoskeleton. Since the discovery of the KCC2-4.1N interaction, several actin-related proteins have been shown to interact with KCC2 (Chevy et al., 2015; Llano et al., 2015; Mahadevan et al., 2017), suggesting that additional KCC2 interactors may participate in KCC2 recruitment at excitatory synapses at rest and upon synaptic activity.

What is the implication of a dynamic regulation of KCC2 tethering at the periphery of glutamatergic synapses? This may contribute to both dendritic spine morphology and excitatory synaptic function and plasticity. Direct KCC2 interaction with actin-associated proteins (such as 4.1N) contributes to form a molecular barrier hindering the lateral diffusion of transmembrane proteins within dendritic spines (Gauvain et al., 2011). Disrupting this barrier by knocking down KCC2 expression or preventing its interaction with intracellular partners promotes AMPA receptor lateral diffusion and disperses the perisynaptic reserve pool of receptors and, subsequently, the synaptic pool, leading to reduced efficacy of glutamatergic synapses. On the other hand, KCC2 binding to proteins controlling actin polymerization (such as the guanine exchange factor  $\beta$ PIX) controls spine actin cytoskeleton (Chevy et al., 2015; Llano et al., 2015). Changes in KCC2 diffusion and clustering near excitatory synapses are then likely to further influence synaptic plasticity at active synapses (Chevy et al., 2015). Therefore,

the dynamic regulation of KCC2 may be particularly relevant for the physiological regulation of excitatory glutamatergic transmission.

### **3. Activity-dependent regulation of KCC2**

KCC2 expression and function are regulated by both excitatory and inhibitory synaptic activity. In most cases, enhanced physiological (Lee et al., 2011; Kitamura et al., 2008; Wang et al., 2006a; 2006b; Fiumelli et al., 2005; Woodin et al., 2003) or pathological (Shimizu-Okabe et al., 2011; Li et al., 2008; Pathak et al., 2007; Rivera et al., 2004; Reid et al., 2001) neuronal activity have been shown to down-regulate KCC2 expression and activity, further altering the excitation/inhibition balance and promoting pathological activities. Recently, KCC2 down-regulation was also observed in conditions of reduced GABAergic inhibition in mature neurons (Heubl et al., 2017). Several cellular and molecular mechanisms underlying activity-dependent KCC2 down-regulation have been identified and lateral diffusion was shown to represent a rapid mechanism for adapting cell surface KCC2 function to changes in activity (Chamma et al., 2013; Heubl et al., 2017).

#### **3.1. Regulation by glutamatergic activity**

KCC2 is rapidly down-regulated by enhanced neuronal activity and glutamatergic neurotransmission in mature hippocampal neurons (Chamma et al., 2013; Lee et al., 2011; Wang et al., 2006). NMDAR-induced  $\text{Ca}^{2+}$ -influx leads to PP1-dependent KCC2 Serine 940 (S940) dephosphorylation and KCC2-CTD cleavage by  $\text{Ca}^{2+}$ -activated protease calpain (Lee et al., 2011; Puskarjov et al., 2012; Zhou et al., 2012). This in turn increases endocytosis and degradation of KCC2 and intra-neuronal chloride concentration. In this context, lateral diffusion contributes to the regulation of KCC2 membrane stability and neuronal  $\text{Cl}^-$  homeostasis (Chamma et al., 2013) (**Figure 3**). Increasing glutamatergic synaptic activity



enhanced KCC2 mobility within minutes while reducing its confinement. This effect was blocked by preventing S940 dephosphorylation or calpain-dependent cleavage of the transporter, suggesting S940 dephosphorylation and dispersion of KCC2 clusters may be a prerequisite for  $\text{Ca}^{2+}$ -activated calpain cleavage. Interestingly, even in the presence of a membrane-permeable dynamin inhibitory peptide which blocked KCC2 endocytosis, KCC2 diffusion still increased upon enhanced glutamatergic synaptic activity, indicating that altered transporter diffusion precedes its removal from the membrane.

### 3.2. Regulation by GABAergic inhibition

A rapid regulation of KCC2 membrane trafficking and function by GABA<sub>A</sub>R-dependent inhibition was also recently demonstrated in hippocampal neurons (Heubl et al., 2017). GABA<sub>A</sub>R activation decreases KCC2 diffusion coefficient thereby increasing its membrane confinement and stability. Conversely, GABA<sub>A</sub>R blockade increases KCC2 mobility while reducing its surface clustering, stability and activity (Heubl et al., 2017). This mechanism may serve for “self-tuning” GABAergic signaling via rapid regulation of KCC2-mediated  $\text{Cl}^-$  export (**Figure 3**). The underlying molecular mechanism was shown to be independent of  $\text{Ca}^{2+}$  signaling and KCC2 S940 phosphorylation but instead involved  $\text{Cl}^-$  itself acting as a second intracellular messenger in this regulation. Thus, increasing  $[\text{Cl}]_i$  using the selective KCC2 inhibitor VU0463271 or local photo-stimulation of the chloride pump halorhodopsin confined KCC2 in the neuronal membrane. Reciprocally, lowering  $[\text{Cl}]_i$  by substituting extracellular  $\text{Cl}^-$  with methanesulfonate relieved KCC2 diffusion constraints. Since KCC2 confinement increased within tens of seconds after halorhodopsin photo-stimulation (Heubl et al., 2017), lateral diffusion is probably among the first cellular mechanisms modulating chloride homeostasis. GABA<sub>A</sub>R-dependent modulation of KCC2 diffusion involved the  $\text{Cl}^-$ -sensing serine/threonine kinase WNK1 (With No lysine (K) serine-threonine kinase 1) and its effectors, the kinases SPAK (STE20/SPS1-related, proline alanine-rich kinase) and OSR1 (Oxydative

stress response 1). The GABA<sub>A</sub>R-mediated activation of the WNK1/SPAK/OSR1 signaling pathway in turn promoted the phosphorylation of KCC2 at positions T906 and T1007 (Heubl et al., 2017), which is known to result in KCC2 inhibition (Rinehart et al 2009, Inoue et. al. 2012, de Los Heros et al 2014).

Therefore, enhanced NMDAR-mediated excitation or reduced GABA<sub>A</sub>R-mediated inhibition both act to increase KCC2 lateral diffusion through altered phosphorylation of specific residues (S940 dephosphorylation upon increased excitation; T906/1007 phosphorylation upon GABA<sub>A</sub>R blockade). KCC2 phosphorylation/dephosphorylation may then induce conformational changes (Groban et al., 2006), thereby altering transporter-scaffold interactions and promoting cluster dispersion. This may in turn facilitate KCC2 interaction with the endocytic machinery, a process that occurs at distance from the postsynaptic zone (Blanpied et al., 2002). Endocytosed transporters may then be recycled back to the membrane or sent to lysosomes for degradation. Inversely, KCC2 confinement in the plasma membrane may protect transporters from endocytosis. Interfering with transporter diffusion/endocytosis may then help to restore KCC2 membrane expression and function under pathological conditions (**Figure 3**).

### **3.3. Putative contribution of NKCC1 lateral diffusion to chloride homeostasis**

The increase in  $[Cl^-]_i$  and subsequent depolarizing shift in the reversal potential of GABA<sub>A</sub>R-mediated currents ( $E_{GABA}$ ) observed in the epileptic brain is most often attributed to a reduction in KCC2-dependent  $Cl^-$  export (reviewed in (Di Cristo et al., 2017)). However, an up-regulation of NKCC1 may also contribute to increased  $[Cl^-]_i$  and depolarized  $E_{GABA}$  (Kourdougli et al., 2017; Wang et al., 2017). Although the mechanisms regulating KCC2 membrane expression and function have been extensively studied, the cellular and molecular mechanisms controlling NKCC1 membrane turnover are less well understood. Phosphorylation-dependent alteration of NKCC1 diffusion may regulate its membrane turnover and function. QD-SPT experiments in

385 mature hippocampal neurons support a contribution of lateral diffusion to NKCC1 regulation  
386 at the plasma membrane (unpublished work from our laboratory). SPT experiments require  
387 labeling live neurons with an antibody targeting an extracellular epitope of NKCC1. In the  
388 absence of such antibody, we expressed a recombinant human NKCC1a transporter bearing a  
389 2xHA tag at position Histidine 398 in its second extracellular loop (i.e. the NT931 construct in  
390 Somasekharan et al., 2013). The recombinant transporter also carries a 3xFlag tag and a  
391 monomeric Venus tag inserted at the N-terminus after the initiation codon (Somasekharan et  
392 al., 2013). The insertion of these tags did not alter the membrane expression and transport  
393 function of the NKCC1 protein as checked by HA surface labeling and  $^{86}\text{Rb}$  influx studies in  
394 HEK 293 cells (Somasekharan et al., 2013). In agreement with these results, we reported using  
395 surface labeling that the recombinant transporter is well expressed in mature hippocampal  
396 cultured neurons and is targeted to both the axonal and somato-dendritic plasma membrane  
397 (**Figure 4**), in a similar way than the endogenous protein (not shown). Confocal microscopy  
398 further indicated that the recombinant NKCC1a transporter formed clusters near both excitatory  
399 and inhibitory synapses (**Figure 4**). The cellular and sub-cellular distributions of NKCC1b were  
400 indistinguishable from that of NKCC1a (data not shown). We thus focused on NKCC1a. We  
401 explored the membrane dynamics of NKCC1a by QD-SPT. As shown in Figure 4, NKCC1a  
402 transporters explored large areas of the axonal and somato-dendritic extrasynaptic plasma  
403 membrane while others were confined near excitatory and inhibitory synapses. The transporter  
404 mobility was significantly higher at the surface of the axon as compared to the dendrites,  
405 suggestive of lower diffusion constraints in the axon. Transitions between significant NKCC1  
406 confinement at/near synapses and less constrained diffusion in extrasynaptic areas is  
407 reminiscent of KCC2 diffusion behavior (Chamma et al., 2013, 2012; Heubl et al., 2017).  
408 Therefore, we concluded that NKCC1 also responds to the diffusion-trapping mechanism.  
409 Unlike KCC2, we found that NKCC1 diffused along the axon (**Figure 4**) suggesting functional

differences between the two transporters. This is in agreement with the fact that  $E_{\text{GABA}}$  is depolarized at the axon initial segment (AIS) due to NKCC1 expression (Khirug et al., 2008) and KCC2 exclusion from CNS axons (Hübner et al. 2001; Williams et al. 1999). Clearly, more work is needed to examine whether NKCC1a and NKCC1b membrane dynamics and clustering are modulated by normal and pathological activities in the axonal and somato-dendritic compartments. An activity-dependent regulation of NKCC1 diffusion/clustering, similar to that described for KCC2, may then locally affect its function, and in turn chloride homeostasis and GABA signaling.

In conclusion, different subpopulations of CCCs exist in the plasma membrane: freely moving transporters outside of clusters and confined transporters in clusters near synapses. KCC2 and NKCC1 clustering probably result from accumulation in lipid-rafts, interaction with the cytoskeleton via scaffolding proteins and oligomerization. Freely moving transporters are probably more susceptible to interaction with molecules involved in clathrin-dependent endocytosis. The balance between “freely moving” and “clustered” pools of KCC2 and NKCC1 might be rapidly changed by activity through phosphoregulation and impact the overall density of transporters at the plasma membrane. Since activity-induced changes in KCC2 mobility occurs within seconds - i.e. well before transporter internalization, which occurs within tens of minutes - lateral diffusion is probably among the first cellular mechanism modulating chloride transporter membrane stability.

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## Figure legends

### Figure 1. CCC activity influences the direction of chloride flux through GABA<sub>A</sub>Rs.

**A**, KCC2 extrudes chloride out of the cell using the electrochemical potassium gradient generated by the Na<sup>+</sup>/K<sup>+</sup> ATPase. NKCC1, in contrast, transports chloride into neurons using the electrochemical gradient of sodium ions. In mature neurons, KCC2 transport dominates over NKCC1 function, leading to low intra-neuronal chloride concentration. Hence, under resting conditions in most neurons, GABA<sub>A</sub>R activation usually induces a hyperpolarizing influx of chloride ions. **B**, KCC2 (left) and NKCC1 (right) structure, key phosphorylation residues and domains. KCC2 and NKCC1 a and b isoforms differ by the presence of additional amino acid sequences in KCC2a and NKCC1a (exon 21). Note that most regulatory domains and phosphorylation sites of KCC2 are present in the large intracellular C-terminal domain while those of NKCC1 are located in the N-terminus. The ISO-domain, calpain and AP-2 binding domains in KCC2 are highlighted in orange, pink and salmon respectively. Key KCC2 and NKCC1 Serine (S), Tyrosine (Y) and Threonine (T) phosphorylation residues are shown. They are targeted by PKC and PP1 (green), Src family kinase (dark blue) and WNK-SPAK-OSR1 kinases (yellow). SPAK-binding domains in KCC2 and NKCC1 are also highlighted (blue).

### Figure 2. KCC2 membrane dynamics studied with QD-based SPT.

**A**, Representative trajectory (red) of QD-bound Flag-tagged recombinant KCC2 overlaid with fluorescent clusters of recombinant gephyrin–mRFP (green) to identify inhibitory (IS) synapses. The white spot indicates the starting point of the trajectory. Scale bar, 0.5  $\mu$ m. **B**, Median QD diffusion coefficients  $D$  values  $\pm$  25-75% IQR in the extrasynaptic membrane (black) or near excitatory (green) or inhibitory (red) synapses identified by homer1c–GFP and gephyrin–mRFP clusters, respectively. Note the reduced diffusion near synapses ( $**p = 2 \times 10^{-3}$ ,  $***p < 10^{-3}$ ). **C**, Decreased size of the confinement domain  $L$  for synaptic versus extrasynaptic QDs ( $***p < 10^{-3}$ ). **D**, Mean KCC2 dwell times near excitatory synapses (green) and inhibitory synapses (red) showing increased time spent near excitatory synapses ( $*p < 5 \times 10^{-2}$ ). Adapted from (Chamma et al., 2013).

### **Figure 3. Regulation of KCC2 membrane trafficking.**

**A**, KCC2 is mobile outside of clusters and confined within clusters. KCC2 clustering probably involves a multiple step process including transporter oligomerization, accumulation in lipid-rafts, and cytoskeleton interaction. Freely moving transporters are more susceptible to interact with molecules implicated in endocytosis. **B**, Activity-dependent tuning of KCC2 membrane trafficking: the balance between “freely moving” and “clustered” KCC2 pools can be rapidly modified by neuronal activity. Changes in the phosphorylation state of the transporter then influence its clustering and internalization rate, and in turn affect intracellular chloride concentration. C, clustered; D, diffusing; E, endocytosed transporters.

### **Figure 4. NKCC1 also undergoes diffusion-trapping at the plasma membrane.**

**A**, Total (Venus) and surface (HA tag) labeling of recombinant HA-Venus-NKCC1 transporter in 21 DIV hippocampal neurons showing that a large amount of the recombinant transporter traffics to the somato-dendritic and axonal plasma membrane. Scale bar, 10  $\mu$ m. **B**, NKCC1-HA surface staining (red) in neurons cotransfected with gephyrin-FingerGFP (green) and

816 homer1c-mRFP (blue), two markers of inhibitory (IS) and excitatory (ES) synapses. Scale bar,  
 817 2  $\mu$ m. Note that some NKCC1 clusters are surrounding inhibitory (open arrows) and excitatory  
 818 (closed arrows) synapses, whereas others are associated with the extrasynaptic membrane  
 819 (crossed arrows). **C**, Representative trajectories (white) of QD-bound HA-tagged recombinant  
 820 NKCC1 overlaid with either cytoplasmic eGFP (red) to identify axons, or with recombinant  
 821 homer1c-GFP (green) and gephyrin-mRFP (red) to identify ES and IS, respectively. Scale bars,  
 822 0.5  $\mu$ m. **D**, Instantaneous diffusion coefficients of the trajectories shown in (C). **E**, Median  
 823 diffusion coefficient D values  $\pm$ 25–75% IQR in axons (white), dendrites (grey), extrasynaptic  
 824 (extra, black), at/near excitatory synapses (red), and at/near inhibitory synapses (green). Axon,  
 825 n = 94 QDs, dendrite, n = 234 QDs, extra, n = 153 QDs, ES, n = 47 QDs, IS, n = 36 QDs, 2  
 826 cultures. axon vs. dendrite KS test p < 0.001, extra vs. ES KS test p = 0.05, extra vs. IS KS test  
 827 p = 0.4. **F**, Time-averaged MSD functions of axonal QDs (dashed), extrasynaptic QDs (black),  
 828 QDs at ES (red), and QDs at IS (green). The MSD versus time relationships show a steeper  
 829 initial slope for axonal vs extrasynaptic trajectories and for extrasynaptic vs synaptic  
 830 trajectories, suggesting that trajectories are less confined. **G**, Median explored area EA  $\pm$ 25–  
 831 75% IQR in axons (white), dendrites (grey), extrasynaptic (black), at/near ES (red), and at/near  
 832 IS (green). Axon, n = 276 QDs, dendrite, n = 408 QDs, extra, n = 488 QDs, ES, n = 89 QDs,  
 833 IS, n = 95 QDs, 2 cultures. axon vs. dendrite KS test p < 0.001, extra vs. ES KS test p = 0.001,  
 834 extra vs. IS KS test p = 0.001. Note that NKCC1 moved faster and explored a larger surface of  
 835 the axonal membrane compared to the extrasynaptic dendritic membrane. NKCC1 is also  
 836 slower and more confined near synapses as compared to extrasynaptic sites.

**Table 1. KCC2 diffusion properties compared to that of AMPAR, GABA<sub>A</sub>R and NCAM120.**

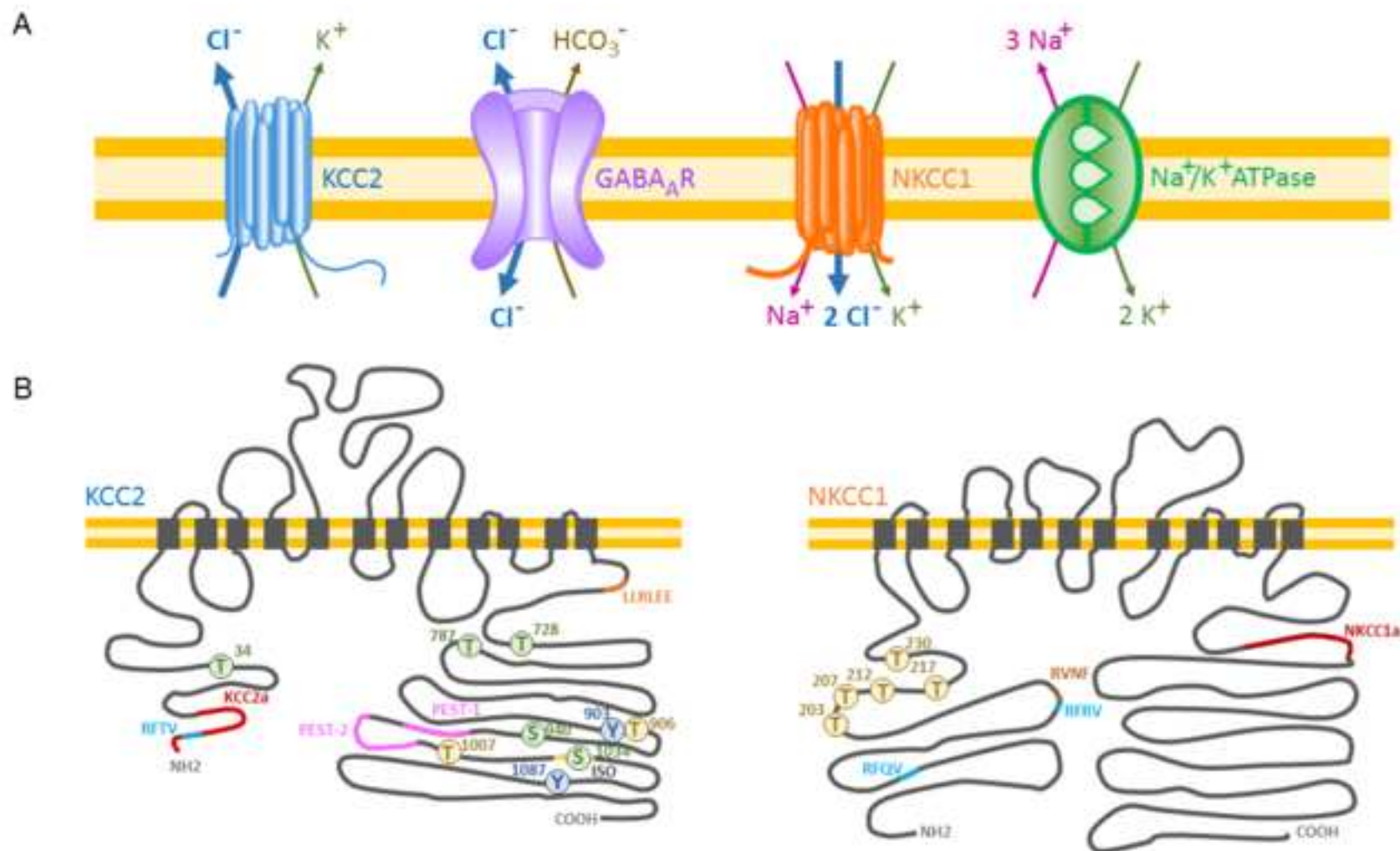
Molecules	Location	Median D ( $10^{-2} \mu\text{m}^2\text{s}^{-1}$ )	Mean DT (s)
<b>KCC2</b>	ES.	2.7 (281, 5)	$11.9 \pm 0.8$
	IS.	2.5 (202, 5)	$9.4 \pm 0.8$
	Extra.	3.3 (367, 5)	N.A.
<b>AMPA GluA1</b>	ES.	1.4 (40, 2)	$19.5 \pm 2.6$
	IS.	1.6 (39, 2)	$14.5 \pm 2.6$
	Extra.	2.1 (126, 2)	N.A.
<b>GABA<sub>A</sub>R <math>\gamma 2</math></b>	ES.	N.D.	
	IS.	1.6 (171, 4)	$15.5 \pm 0.9$
	Extra.	3.1 (546, 4)	N.A.
<b>NCAM120</b>	ES.	5.9 (76, 2)	$2.8 \pm 0.6$ (380)
	IS.	5.8 (68, 2)	$6.2 \pm 1.2$ (288)
	Extra.	8.1 (183, 2)	N.A.

Quantifications from 2-5 independent experiments. Numbers between brackets indicate the numbers of QDs and experiments analyzed. Data for KCC2 and GABA<sub>A</sub>R are from (Chamma et al., 2013; Lévi et al., 2015); those for NCAM120 and AMPAR (unpublished data from our laboratory). N.D. not determined; N.A. not applicable.

#### References:

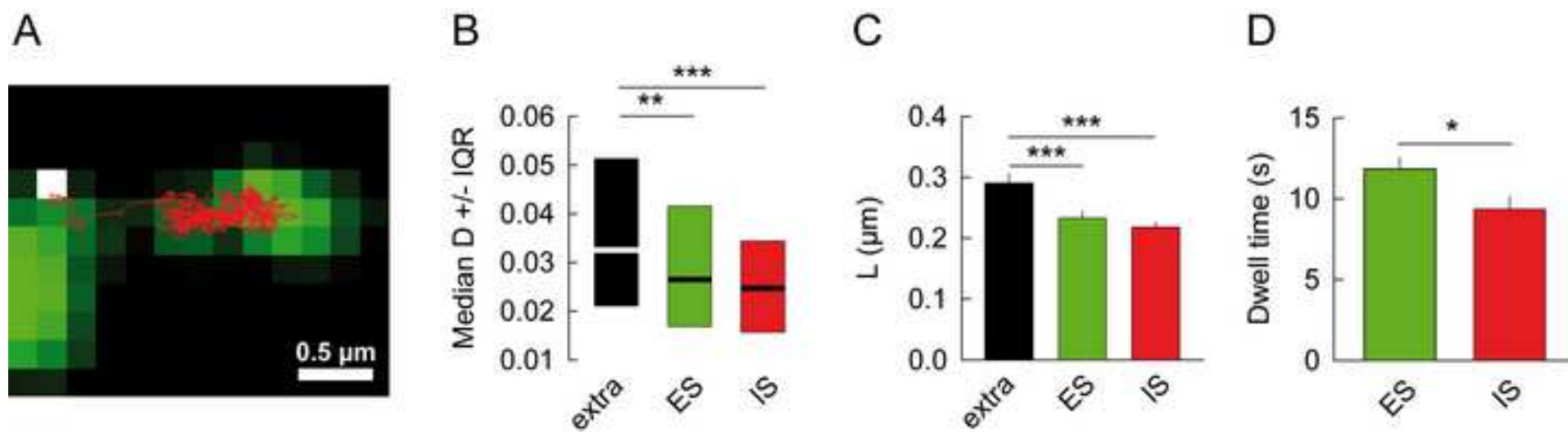
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**Figure 2**  
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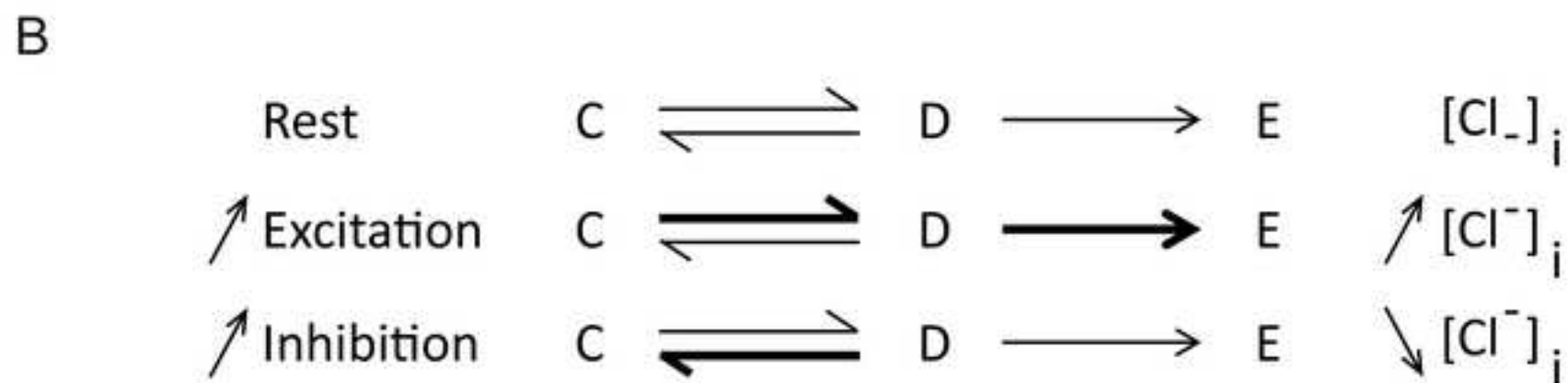
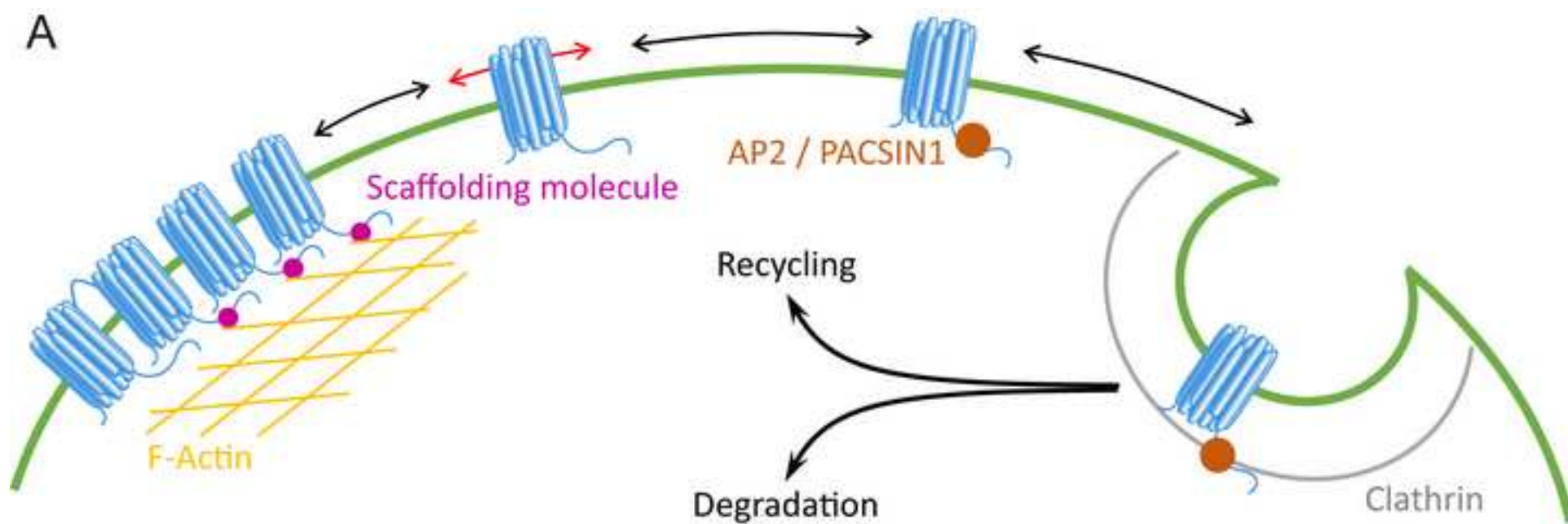


Figure 4  
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