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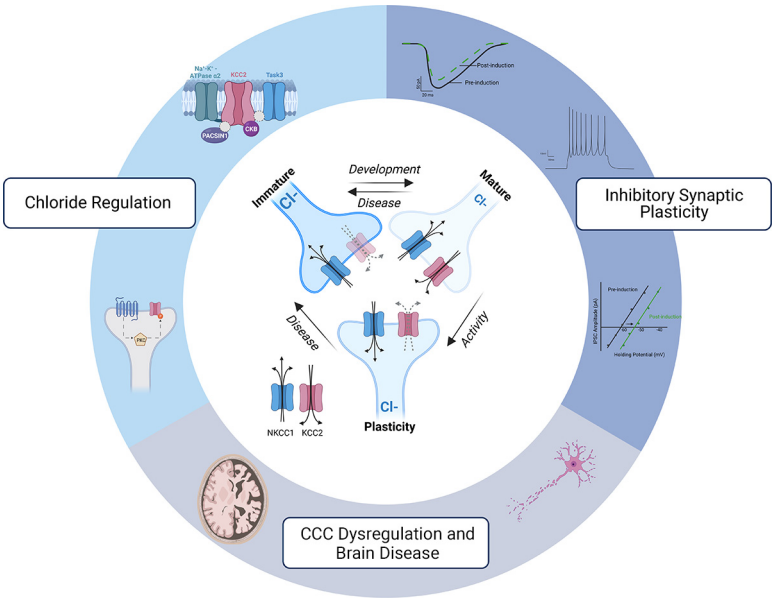
CHLORIDE TRANSPORTERS CONTROLLING NEURONAL EXCITABILITY



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KEY WORDS

chloride; inhibition; KCC2; neurophysiology; synaptic transmission

CLINICAL HIGHLIGHTS

- Numerous neurodevelopmental, neuropsychiatric, and neurological disorders result from the dysfunction of Cl^- transporters, which alters the strength of synaptic inhibition.
- A decrease in, or dysfunction of, the neuron-specific Cl^--extruding transporter KCC2 contributes to seizure generation, neuropathic pain, and autism spectrum disorders (ASDs).
- Inhibition of the Cl^- importing transporter NKCC1, to reduce the concentration of intracellular Cl^-, has been a successful strategy to rescue synaptic inhibition and improve neuronal circuit function and behavioral outcomes in animal models of neurological disorders resulting from KCC2 dysfunction.
- The development of *1*) next-generation Cl^- imaging tools, *2*) next-generation NKCC1 inhibitors, and *3*) a KCC2 enhancer/activator toolkit will facilitate a dramatic increase in both our understanding of CCC function and our ability to treat neurological disorders with underlying CCC dysfunction.

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CHLORIDE TRANSPORTERS CONTROLLING NEURONAL EXCITABILITY

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Abstract

Synaptic inhibition plays a crucial role in regulating neuronal excitability, which is the foundation of nervous system function. This inhibition is largely mediated by the neurotransmitters GABA and glycine that activate Cl^--permeable ion channels, which means that the strength of inhibition depends on the Cl^- gradient across the membrane. In neurons, the Cl^- gradient is primarily mediated by two secondarily active cation-chloride cotransporters (CCCs), NKCC1 and KCC2. CCC-mediated regulation of the neuronal Cl^- gradient is critical for healthy brain function, as dysregulation of CCCs has emerged as a key mechanism underlying neurological disorders including epilepsy, neuropathic pain, and autism spectrum disorder. This review begins with an overview of neuronal chloride transporters before explaining the dependent relationship between these CCCs, Cl^- regulation, and inhibitory synaptic transmission. We then discuss the evidence for how CCCs can be regulated, including by activity and their protein interactions, which underlie inhibitory synaptic plasticity. For readers who may be interested in conducting experiments on CCCs and neuronal excitability, we have included a section on techniques for estimating and recording intracellular Cl^-, including their advantages and limitations. Although the focus of this review is on neurons, we also examine how Cl^- is regulated in glial cells, which in turn regulate neuronal excitability through the tight relationship between this nonneuronal cell type and synapses. Finally, we discuss the relatively extensive and growing literature on how CCC-mediated neuronal excitability contributes to neurological disorders.

chloride; inhibition; KCC2; neurophysiology; synaptic transmission

CLINICAL HIGHLIGHTS

- Numerous neurodevelopmental, neuropsychiatric, and neurological disorders result from the dysfunction of Cl^- transporters, which alters the strength of synaptic inhibition.
- A decrease in, or dysfunction of, the neuron-specific Cl^--extruding transporter KCC2 contributes to seizure generation, neuropathic pain, and autism spectrum disorders (ASDs).
- Inhibition of the Cl^- importing transporter NKCC1, to reduce the concentration of intracellular Cl^-, has been a successful strategy to rescue synaptic inhibition and improve neuronal circuit function and behavioral outcomes in animal models of neurological disorders resulting from KCC2 dysfunction.
- The development of *1*) next-generation Cl^- imaging tools, *2*) next-generation NKCC1 inhibitors, and *3*) a KCC2 enhancer/activator toolkit will facilitate a dramatic increase in both our understanding of CCC function and our ability to treat neurological disorders with underlying CCC dysfunction.

1. INTRODUCTION

Excitability is a unique property of some cells, including neurons. Neuronal excitability, which is foundational to the functioning of the nervous system, is determined by both passive and active membrane properties and synaptic signaling. Passive and active membrane properties contribute to the intrinsic excitability of the neuron and are largely determined by the number and distribution of ion channels and receptors. More dynamic to the regulation of neuronal excitability is the contribution of excitatory and inhibitory synaptic signaling, with the inhibitory role being complex, in large part because of the relationship between neuronal chloride (Cl^-) regulation and inhibition.

Synaptic inhibition is mediated by the neurotransmitters γ-aminobutyric acid (GABA) and glycine, which both bind to Cl^--permeable ligand-gated ionotropic receptors ($GABA_A$Rs and GlyRs). Because of the Cl^--permeable nature of these receptors, the strength of this inhibitory

transmission depends upon the neuronal gradient for Cl^-, which is primarily determined by cation-chloride cotransporters (CCCs), and the membrane permeability for Cl^- (1, 2).

Pioneering experiments starting in the 1950s, which investigated the ionic permeability underlying inhibitory postsynaptic potentials (IPSPs), revealed Cl^- as the predominant charge carrier (1–3); however, as discussed below, $GABA_A$Rs and GlyRs are also permeable to bicarbonate (HCO_3^-), but with lower permeability than Cl^-. It was not until the mid-1980s that researchers determined that a dedicated transport mechanism was required to maintain the Cl^- gradient for neuronal inhibition (4–7). Collectively, these historic studies led to the realization that neuronal Cl^- must be regulated by two Cl^- transport processes (one that accumulates and the other that extrudes). However, it was not until more than a decade later that the neuron-specific K^+-Cl^- cotransporter (KCC2) was demonstrated to be the Cl^- extrusion transporter required for fast synaptic inhibition in the central nervous system (CNS) (8). Although it has now been several decades that we have known that CCCs are required for inhibition and thus regulate neuronal excitability, we continue to discover how even relatively small changes in expression and function can have big impacts on excitability (9). This review is written to be of interest to foundational neurophysiologists and clinical trainees and researchers interested in the impact of CCCs on neuronal excitability, with a particular focus on GABAergic inhibition in the healthy and diseased CNS.

2. AN OVERVIEW OF NEURONAL Cl^- TRANSPORTERS

The passive and active movement of Cl^- is central to neuronal Cl^- homeostasis and plays a critical role in regulating neuronal excitability. In this section we review the integral membrane proteins primarily responsible for Cl^- transport across the neuronal membrane, the cation-chloride cotransporters (CCCs), including the history of their discovery and cloning, their basic biophysical properties and molecular structures, and their developmental and brain region expression patterns.

2.1. *SLC12A Gene Family of Cation-Chloride Cotransporters*

Cl^- is primarily transported across the neuronal membrane by the CCCs, which are secondarily active transporters that do not consume ATP directly but rather derive energy from ionic gradients established by primary transporters. The sodium-potassium ATPase (Na^+-K^+-ATPase) is an integral membrane protein and primary active transporter fueled by ATP. The Na^+-K^+-ATPase transports three Na^+ out of the neuron in exchange for every two K^+ transported in, resulting in a net extracellular positive charge (10). The CCCs use the energy stored in the ionic gradients established by the Na^+-K^+-ATPase to transport other ions, including Cl^-, against their electrochemical gradients.

CCCs are members of the *SLC12A* nine-member gene family, which have a common evolutionary origin and are categorized into functional paralogs (11, 12) and include four K^+-Cl^- cotransporters (KCCs), two Na^+-K^+-$2Cl^-$ cotransporters (NKCCs), an Na^+-Cl^- cotransporter (NCC), and two orphan cotransporters, CCC9 and CIP (13, 14); they are all predicted to have similar tertiary structures (**FIGURE 1*A***). CCCs are large glycoproteins (~110–130 kDa) with 12 transmembrane segments and intracellular amino- and carboxy-terminal domains (12, 18, 19) (**TABLE 1**). Readers interested in learning more about the history, evolutionary origin, sequence homology, and structure of the entire gene family are directed to a comprehensive review by Hartmann and Nothwang (12). For the purposes of this review, we focus on those members of the SLC12A family with robust expression in the vertebrate brain and whose function is integral to neuronal excitability: NKCC1 and KCC2.

SLC12A2 (NKCC1) was cloned in the mid-1990s from shark rectal gland and rabbit and mouse kidney (20–22). This bumetanide-sensitive CCC is often referred to as the secretory cotransporter and is found in abundance in secretory epithelia (22, 23). It is also highly expressed by multiple cell types in the brain, including immature neurons, astrocytes, and oligodendrocytes (24–28). NKCC1 has alternatively spliced isoforms, which could alter the functionality of this transporter in different brain regions and at different developmental time points (29–31). Native NKCC1 normally exits as a dimer (~355 kDa) in the plasma membrane (32), and this dimerization requires the COOH terminus (33, 34). The Na^+-K^+-ATPase establishes an inwardly directed Na^+ gradient, and NKCC1 uses that gradient to uptake K^+ and Cl^- into the cell, with a stoichiometry ratio of $1Na^+$:$1K^+$:$2Cl^-$ (35). This electroneutral NKCC1-mediated inward transport of Cl^- is responsible for the relatively high neuronal Cl^- early in development, which underlies depolarizing and excitatory GABAergic transmission (36–38). The other NKCC in the *SLC12A* family is encoded by *SLC12A1* and is primarily expressed in the apical membrane of the thick ascending limb of Henle (19, 20, 39). NKCC1, like the other NKCCs and KCCs, is organized into a highly conserved 12-transmembrane domain at the NH_2 terminus and a conserved COOH-terminal domain (18, 40–42). The structure of NKCC1 was recently reported with cryo-electron microscopy (cryo-EM) (43), which helped to reveal the ion translocation pathway, ion-binding

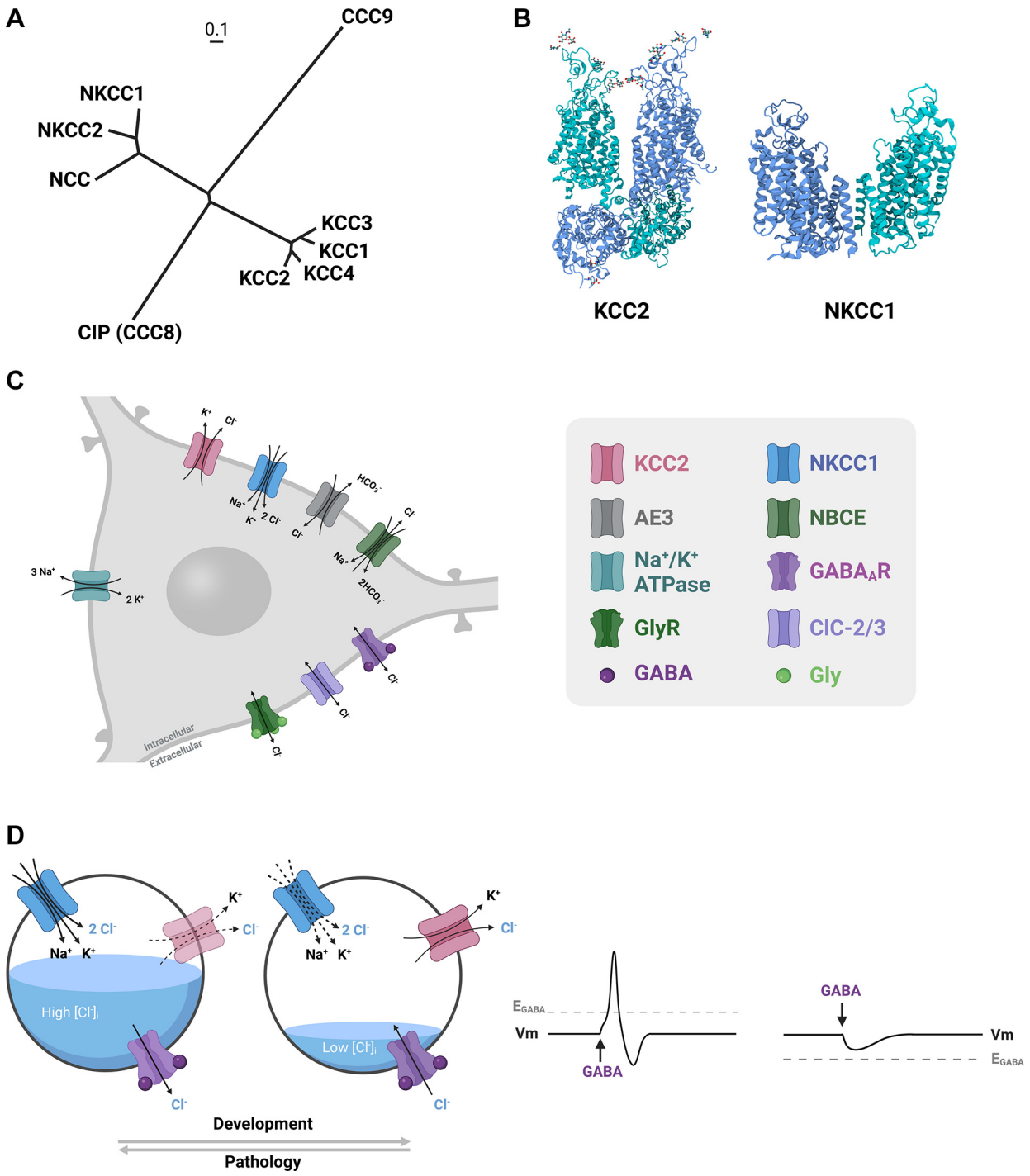

FIGURE 1. Cation-chloride cotransporters (CCCs) maintain intracellular chloride concentration in neurons. *A*: cluster dendrogram of human SLC12A cotransporters adapted from Ref. 15. Among CCCs in humans, the Na^+-independent cotransporters (KCCs) and the Na^+-dependent cotransporters (NKCCs and NCC) form the 2 major branches, and they separated early during evolution. The Na^+-independent cotransporters are more closely related to each other than to Na^+-dependent cotransporters. *B*: crystal structure of human KCC2 (adapted from Ref. 16, reproduced from Protein Data Bank 10.2210/pdb6M23/pdb) and NKCC1 (adapted from Ref. 17 and reproduced from Protein Data Bank 10.2210/pdb6PZT/pdb) proteins with associated glycans. *C*: CCCs including KCC2 and NKCC1, the Na^+-independent anion exchanger (AE3), and the Na^+-coupled bicarbonate transporter (NBCE) are active transport mechanisms that establish the chloride gradient in a mature neuron. This gradient allows for the movement of chloride across the membrane through passive mechanisms such as the $GABA_A$ receptor ($GABA_AR$), glycine receptor (GlyR), and ClC-2/3. *D*: increased NKCC1 expression during early development leads to relatively high intracellular Cl^- concentration in neurons, which underlies depolarizing and excitatory GABAergic transmission. Decreased NKCC1 activity and increased KCC2 expression during neuronal maturation maintain a low intracellular chloride concentration, leading to chloride influx and hyperpolarization following activation of the $GABA_AR$. E_{GABA}, GABA equilibrium potential; V_m, membrane potential. Figure created with BioRender.com, with permission.

Table 1. ***SLC12A gene family of cation-chloride cotransporters***

Human Gene Name	Protein Name	Neuronal Expression	Ions Transported and Stoichiometry
Slc12a1	NKCC2	No	$1Na^+:1K^+:2Cl^-$
Slc12a2	NKCC1	Yes	$1Na^+:1K^+:2Cl^-$
Slc12a3	NCC	No	$1Na^+:1Cl^-$
Slc12a4	KCC1	Yes	$1K^+:1Cl^-$
Slc12a5	KCC2	Yes	$1K^+:1Cl^-$
Slc12a6	KCC3	Yes	$1K^+:1Cl^-$
Slc12a7	KCC4	Yes	$1K^+:1Cl^-$
Slc12a8	CCC9	No	None
Slc12a9	CIP1	No	Unknown

sites, and key residues for transport activity, and thus our understanding of the structure-function relationship of CCCs has grown significantly, as recently reviewed by Jawhari (44).

Among the four KCC isoforms [KCC1 (*SLC12A4*), KCC2 (*SLC12A5*), KCC3 (*SLC12A6*), and KCC4 (*SLC12A7*)], KCC2 is predominantly expressed in CNS neurons (11), where it is necessary to extrude intracellular Cl^- (8, 45). KCC2 was cloned from rat brain shortly after the cloning of *SLC12A2* (40). KCC2 has two NH_2-terminally spliced neuron-specific isoforms (KCC2a and KCC2b), which are generated with alternative promoters and first exons (46–49); KCC2b protein represents ~90% of the total KCC2 protein in the mature cortex, whereas KCC2a expression remains low throughout life (46). Despite the fact that the KCCs show a relatively high sequence homology, there are numerous conserved domains within individual KCCs, including the ISO domain in the KCC2 COOH terminus, which is required for KCC2 to transport under isotonic conditions (50, 51).

The existence of K^+-Cl^- cotransport had already been characterized well before its cloning, first in human red blood cells (52–56) and then in nephrons (57), as an *N*-ethylmaleimide (NEM)-sensitive, hypotonically activated, Cl^--driven, K^+-efflux mechanism involved in regulatory volume decrease. The critical role of Cl^- transport in neurons was revealed in the 1970s, when Lux and Neher (3) discovered the existence of a dedicated neuronal Cl^- transport mechanism required for hyperpolarizing inhibition. But it was not until the following decade that the Cl^- gradient was shown to be reduced by the "loop diuretic" furosemide and to be dependent on extracellular K^+ (4–7), which together revealed a distinct neuronal K^+-Cl^- transport process.

KCC2 derives energy from the outward-directed K^+ gradient established by the Na^+-K^+-ATPase for the outward transport of Cl^- with a 1:1 stoichiometry (11). KCC2 is unique among its family members because of its exclusive neuronal expression in the CNS, high affinity for extracellular K^+, and constitutive activity (58); in addition, KCC2 is unique because it is the only CCC capable of, and required for, hyperpolarizing inhibition in pyramidal neurons (8). The importance of KCC2, however, is not exclusive to pyramidal neurons; KCC2 is also involved in regulating Cl^- homeostasis in other cell types, including cerebellar Purkinje and granule cells as well as retinal ganglion cells, though not exclusively required for hyperpolarizing inhibition in these cell types (59, 60).

KCC structures have recently been reported for KCC1 (17), KCC2 (16, 61), KCC3 (16, 61), and KCC4 (61). Collectively these structural reports reveal that all four KCCs have the following similar overall architecture, as was predicted from sequence homology: 12 transmembrane-spanning domains with an extracellular large loop between transmembrane helix (TM)5 and TM6 and intracellular COOH and NH_2 termini; the COOH-terminal domain contains numerous phosphorylation sites that mediate activity and regulate expression and trafficking (12, 39, 62–66); an NH_2-terminal peptide-mediated autoinhibitory mechanism (61); and an inward facing conformation (61) (**FIGURE 1*B***). KCC2 exists as a monomer, dimer, trimer, or tetramer in the mature brain (67). However, KCC2 monomers are more common in immature brain, which suggests that an age-dependent oligomerization of KCC2 activates its function.

SLC12A6 (KCC3), which was cloned from both mouse and human placenta in the late 1990s (68, 69), is also expressed (nonexclusively) in CNS cells, where it regulates intracellular Cl^- concentration and mediates volume regulation (70, 71). But, unlike KCC2, KCC3 does not appear to maintain Cl^- homeostasis under resting neurophysiological states (isosmotic conditions) (66, 72). In addition to this, microarray mRNA expression data by Sedmak et al. (73) showed that KCC3 is expressed at relatively lower levels than KCC2 in human brain after birth, and thus it will not be discussed further. Similarly, KCC4 is nonexclusively expressed in the CNS and is also involved in cell volume regulation (74, 75), although its specific role in the nervous system is not well understood, and thus it will not be discussed further.

In addition to the *SLC12A* Cl^- transporters, neurons also move Cl^- across the neuronal membrane via the *SLC4A* family of anion transporters, including the Na^+-independent anion exchanger AE3 (76–79) and the Na^+-coupled bicarbonate transporter NBCE (**FIGURE**

1C). AE3 is an electroneutral secondarily active neuronal Cl^-/HCO_3^- anion exchanger that countertransports 1 Cl^- for 1 HCO_3^- and thereby accumulates Cl^- in immature neurons (77, 80, 81). Interestingly, a genome-wide linkage analysis identified the *SLC4A3* locus, which encodes AE3, as a susceptibility locus for idiopathic generalized epilepsy (IGE) (82). Subsequent mutation analysis of the AE3 coding region revealed a significant increase of the Ala867Asp substitution polymorphism in IGE patients (83). A later study using an AE3-knockout (KO) mouse model found that these mice exhibited reduced seizure threshold in response to proconvulsant substances while not exhibiting an overt epileptic phenotype (84). These findings suggest that AE3 deficiency contributes to increased seizure susceptibility, thereby highlighting one of the many roles of Cl^- transport in neuronal excitation. NBCE (*SLC4A10*) is also expressed in the brain (85), where it serves as an acid-base transport mechanism that plays a role in controlling intracellular pH (pH_i). It is electroneutral and transports Cl^- out of the neuron, thereby promoting inhibition. During bouts of neuronal activity, which result in bicarbonate efflux via $GABA_A$Rs and consequent neuronal acidosis, activation of NBCE will decrease intracellular Cl^- and thereby help to maintain the inhibitory action of GABA (86). It is important to recognize that of all the Cl^- transporters and exchangers, only KCC2 and NBCE can reduce the intracellular Cl^- concentration below equilibrium under physiological conditions in mature neurons.

2.2. NKCC1 and KCC2 Expression in Brain

There are developmental, cell type-specific, and brain region-specific variations in NKCC1 expression. In general, it is well accepted that NKCC1 is highly expressed early in development throughout the neuroepithelium (87, 88). Although there are experimental discrepancies regarding the developmental expression of NKCC1, both postnatal declines (37, 89, 90) and increases (25, 30, 80, 88, 91, 92) in NKCC1 expression have been reported. A recent review from Virtanen et al. (Table 1, Ref. 93) has summarized the discrepancies in the postnatal expression of NKCC1, along with the details of differences in the methodology used, including the cell type and the brain region studied and the type of assay used to quantify RNA or protein.

KCC2 mRNA expression largely follows neurogenesis, with neurons born earlier showing KCC2 mRNA expression earlier (87, 88, 94). KCC2 mRNA is not expressed in the neuroepithelium or in glutamatergic neuronal precursors or migrating glutamatergic cells (87, 88, 94) but can be detected as early as embryonic day (E)10.5 from the brain stem. In addition, KCC2 expression has been observed with migrating forebrain GABAergic interneuron precursors at E13.5, and KCC2's increase coincides with their migration termination (95, 96). With regard to KCC2 protein, there is a gradient in onset of expression from caudal to rostral CNS (87, 88, 94). In rodents, KCC2 expression is already relatively high before birth in the spinal cord and brain stem (47, 67, 80, 94), whereas detectable expression in the cortex does not begin until birth or shortly thereafter, at which point it increases dramatically throughout the first month of life (8, 80, 87, 88, 94). In the mature nervous system, KCC2 is abundantly expressed in CNS neurons; either not expressed or with very low expression in PNS neurons; and not expressed in glia or other nonneuronal cells (8, 11, 40, 80, 81, 87, 88, 97–99), with the exception of its expression in the islet cells of the pancreas (100).

This description of the timeline for KCC2 expression is very generalized, as there is significant heterogeneity in expression profiles across brain regions and cell types (27, 87, 101, 102). In fact, some mature neuron types lack KCC2 expression, including dopaminergic neurons of the substantia nigra (103) and neurons of the thalamic reticular nucleus (104, 105). Moreover, KCC2 expression can vary across neuronal compartments, with the most extreme variation being found in the axon initial segment, where KCC2 expression does not increase developmentally, and as a result this compartment has high intracellular Cl^- concentration ($[Cl^-]_i$) in the mature brain (106) (**FIGURE 1*D***).

Generally, the ontogeny of NKCC1 and KCC2 expression in mouse brain is similar to that in rat brain, but with the rat being delayed ~2 days; the references above and throughout this review to "early development" and "immature" are defined as embryonic in the rodent brain. In the human brain, KCC2 mRNA is reported to increase dramatically in the second half of gestation (92), with KCC2 protein being detected in the cortex in the 25th postconceptual week (107) and continuing to increase in abundance throughout the first year of life (37, 73).

2.3. CCC Posttranslational Modifications

NKCC1 and KCC2 are regulated by posttranslational modifications, including glycosylation, (de)phosphorylation, and ubiquitination, which have been recently described in several comprehensive reviews (11, 12, 27, 108). CCCs have glycosylation sites in the extracellular loop, between TM7 and TM8 in NKCCs and between TM5 and TM6 in KCCs (18, 22, 109). Glycosylation at these sites is required for the accurate folding and membrane localization of CCCs and can prevent their internalization and degradation. In the case of NKCC1, inhibiting this *N*-linked glycosylation in hypothalamic

paraventricular nucleus neurons reduced NKCC1 expression, thereby decreasing intracellular Cl^- and strengthening synaptic inhibition (110). Moreover, inhibition of *N*-glycan biosynthesis decreased total and plasma membrane NKCC1, which significantly reduced cotransport function in COS7 cells (111). KCC2 has six *N*-glycosylation sites on the extracellular loop between TM5 and TM6 (112), and KCC2 has been found to be glycosylated in both immature and mature neurons (67). In a study of KCC2 loss of function in patients with severe epilepsy, *SLC12A5* mutations were found to negatively impact KCC2 protein expression and glycosylation, which in turn reduced synaptic inhibition (113).

Phosphorylation of key residues in the NH_2- and COOH-terminal domains is also critical for the regulation of CCC expression, oligomerization, and function. An extensive list of major phosphorylation sites in CCCs and their regulatory roles was recently published in a review by Portioli et. al. (114), and so we only highlight here the two most well-documented types of phosphorylation. Protein kinase C (PKC) phosphorylates the serine 940 (S940) residue on the KCC2 COOH terminus, which increases surface stability and transporter efficacy (63). S940 can be dephosphorylated by protein phosphatase 1 (PP1), which promotes the rate of KCC2 internalization at the plasma membrane (115). CCCs are also phosphorylated by the With no lysine kinase (WNK)-regulated Ste20-related proline/alanine-rich kinase (SPAK)/Oxidative stress response 1 (OSR1) kinases, which decrease KCC2 activity while increasing NKCC1 activity, resulting in a combined increase in intracellular Cl^- (15, 116, 117).

Ubiquitination is an important posttranslational modification for many proteins; however, it has not been widely studied for CCCs, even though multiple NCC ubiquitination sites have been characterized and can directly affect NCC endocytosis, degradation, and transporter function. Ubiquitination can also cause the degradation of kinases involved in phosphorylation of NCC and indirectly affect the membrane expression (118). However, the precise mechanisms behind this regulation need further investigation.

3. THE RELATIONSHIP BETWEEN CCCS, Cl^- REGULATION, AND INHIBITION

CCC-mediated transport of Cl^- is critical for regulating the intracellular concentration of Cl^- ($[Cl^-]_i$), which in turn is responsible for the direction and strength of Cl^- currents. As we explain below, although $GABA_AR$ currents can be either hyperpolarizing or depolarizing, there is an important distinction between "depolarizing" and "excitatory." However, to understand the relationship between CCCs, $GABA_ARs$ and Cl^- currents, and to understand whether depolarizing currents are inhibitory or excitatory, we first review some fundamental concepts and nomenclature.

3.1. The Driving Force for Cl^- and Cl^- Current Conventions

In the absence of active Cl^- transport, the intracellular chloride concentration would be set by the membrane potential (V_m) and would equal the equilibrium potential for Cl^- (E_{Cl}). The equilibrium potential for a particular ion can be calculated from the Nernst equation and is the membrane potential at which there is no net flow of that ion. Thus, in the absence of NKCC1- and KCC2-mediated Cl^- transport, $V_m = E_{Cl}$, which is the case for most non-neuronal cells at isotonic conditions. However, in the presence of Cl^- transport, $V_m \neq E_{Cl}$, and the difference between these potentials represents the driving force for Cl^- ($DF_{Cl} = V_m - E_{Cl}$). However, the DF_{Cl} only results in a Cl^- current when there is membrane permeability for Cl^-, allowing for passive diffusion down the electrochemical gradient for Cl^-, thereby pulling the V_m toward E_{Cl}. Opening of a neurotransmitter-gated Cl^- channel increases membrane permeability to Cl^-, which will result in a Cl^- current (if there is a DF_{Cl}). Ionic current is the flow of ions and is often referred to by electrophysiologists as "inward" or "outward." These conventions depend on whether the ions flowing in the current are positively or negatively charged (cations or anions); for the anion Cl^- an outward current occurs if Cl^- flows inward, and an inward current occurs if Cl^- flows out.

3.2. GABA Receptors

GABA is a neurotransmitter in the CNS that primarily exerts its actions by activating ionotropic $GABA_ARs$ and metabotropic G protein-coupled $GABA_B$ receptors ($GABA_BRs$). Because $GABA_BRs$ are metabotropic receptors that activate an inwardly rectifying K^+ channel and/or inhibit high voltage-activated Ca^{2+} channels, and do not depend on Cl^-, we will not discuss their involvement in inhibitory neurotransmission further, but readers interested in learning more about $GABA_BRs$ are directed to a comprehensive review (119).

$GABA_ARs$ are Cl^--permeable ligand-gated ion channels composed of five subunits, with the individual receptor's agonist affinity, conductance, and other properties emerging from the combination of 19 different $GABA_AR$ subunit isoforms (in humans, identified based on sequence homology): α (1–6), β (1–3), γ (1–3), δ, ρ (1–3), ε, θ, and π (119). $GABA_AR$ function is determined primarily by subunit composition, localization, pharmacology, and

kinetic properties (120). $GABA_A$Rs have been heavily studied not only for their role in regulating synaptic transmission in the healthy brain but also as the site of pharmacological treatments for neurological disorders, which has revealed them to be the site of action of benzodiazepines, barbiturates, neuroactive steroids, anesthetics, and convulsants (121). Although GABA is often referred to as "inhibitory," the postsynaptic effect (excitatory vs. inhibitory) does not in fact depend on the transmitter itself but rather on the Cl^- flow through the $GABA_A$R. As we explain below, GABA can be excitatory or inhibitory depending on the direction of the DF_{Cl}, which in turn depends on the expression of NKCC1 and KCC2.

3.3. The "GABA Switch" from Excitation to Inhibition

In the immature nervous system, when the embryonic brain is still developing, NKCC1 is highly expressed and $[Cl^-]_i$ is relatively high (~30 mM). Thus, E_{Cl} sits relatively depolarized (approximately −35 to −40 mV) (36–38). As a result, activation of $GABA_A$Rs results in Cl^- efflux down its electrochemical gradient, and this negative charge flowing out of the neuron (inward current) depolarizes V_m. The V_m will continue to depolarize as it moves toward E_{Cl}, and because in this stage of development E_{Cl} is depolarized with respect to the action potential (AP) threshold, an action potential(s) will be generated. Thus, in early development, because of the high expression of NKCC1, GABAergic transmission is both depolarizing and excitatory (11, 18, 122, 123). However, the excitatory effects of GABA are not sustained in the healthy mature brain, as the dramatic increase in KCC2 expression around the time of birth in rodents rapidly decreases $[Cl^-]_i$.

Depolarizing GABAergic transmission in the developing brain contributes to the recurrent, synaptically evoked suprathreshold depolarizations called giant depolarizing potentials (GDPs) (123–126), which are a type of spontaneous activity transient that is the hallmark of developing neuronal networks (127, 128). However, the causality of GDPs remains unclear. Ben-Ari and colleagues (123, 124) hypothesize and present evidence for GABAergic interneurons pacing GDPs in a phasic manner. However, other groups demonstrate that depolarizing GABAergic transmission is faciliatory to GDPs but does not drive them, and rather they are paced by glutamatergic pyramidal neurons in the CA3 region of the hippocampus (129). Strikingly, it has been shown that GDP-like network events are generated in NKCC1 knockout ($NKCC1^{-/-}$) slices (130). Moreover, a recent study demonstrates that loss of NKCC1 in glutamatergic neurons in vivo has little effect on synaptic development, network dynamics, or hippocampus-dependent behaviors largely unaffected (131).

In mature neurons, when KCC2 is the dominant Cl^- transporter, $[Cl^-]_i$ = 5–10 mM, which results in a hyperpolarized E_{Cl} (approximately −65 to −75 mV). Now, activation of $GABA_A$Rs results in Cl^- influx down its electrochemical gradient (an outward current) toward E_{Cl}, which sits hyperpolarized to the resting membrane potential. In this case, the outward current is hyperpolarizing and prevents neuronal excitation (8, 45, 132). In fact, this KCC2-mediated developmental switch from GABAergic excitation to inhibition can be induced early by overexpression of KCC2 (133–135).

Shortly after the discovery that KCC2 is responsible for the shift from GABAergic excitation to inhibition, there was significant investigation to determine whether neuronal activity regulated this process. Although evidence emerged that GABA itself promotes its own developmental switch from GABAergic excitation to inhibition (136), this was not corroborated by studies demonstrating that inhibiting glutamatergic and GABAergic transmission (137, 138), or spiking itself, failed to alter the developmental expression of KCC2 and GABAergic switch. But although blocking transmission and activity does not appear to alter the upregulation of KCC2, neonatal seizures do trigger a fast and profound enhancement of KCC2-mediated Cl^- extraction, likely via an increase in KCC2 membrane insertion, which accelerates the "switch" (139).

This dramatic change in neuronal Cl^- regulation (from high in embryonic development to low in mature neurons) is unique among the physiologically relevant ions. And this developmental change in ionic regulation exemplifies that what makes a neurotransmitter excitatory or inhibitory is not a property of the chemical transmitter itself but, rather, depends on the postsynaptic response.

Because of the relatively rapid upregulation in KCC2, the shift from GABAergic excitation to inhibition is often called the "switch," although this is somewhat of an overstatement, especially given the brain region and cell type variability in the upregulation of KCC2. In addition, the term "switch" suggests that there are only two states, which is a further oversimplification because depolarizing currents are not necessarily excitatory. When E_{Cl} sits between the AP threshold and resting membrane potential (V_{rest}), GABA is depolarizing but usually not excitatory. $GABA_A$R activation may still be excitatory by virtue of activating low-threshold calcium currents (140, 141), removing the Mg^{2+} block of the NMDA receptor (NMDAR) (142, 143), or inactivating hyperpolarization-activated current (I_h) or K^+ currents (144). To further add to the complexity, this inward depolarizing current can be inhibitory by virtue of inactivating Na^+

conductance or by shunting more strongly depolarizing synaptic or voltage-gated membrane conductances.

Shunting inhibition occurs when the opening of ligand-gated Cl^- channels reduces the input resistance and thereby shunts other synaptic currents (145, 146). Think of the following scenario: A GABAergic synapse sits beside a glutamatergic synapse, and both overlap temporally and spatially in their activity: the Cl^- conductance will counteract the cation influx, resulting in smaller excitatory synaptic potentials, thereby exerting an inhibitory action. This form of inhibition is not exclusive to the scenario where E_{Cl} sits between the AP threshold and V_{rest} but also occurs when E_{Cl} is hyperpolarized with respect to V_{rest}; the difference is that when E_{Cl} sits between the threshold and V_{rest}, shunting is the only form of inhibition, versus when E_{Cl} is hyperpolarized and inhibition occurs because the membrane is pulled away from the AP threshold and because of shunting. Predicting the impact of a depolarizing potential on excitability is challenging because the magnitude of the shunting inhibition will depend on the total Cl^- conductance and the magnitude of neighboring excitatory postsynaptic potentials (EPSPs). For a more fulsome explanation regarding the factors that will determine whether a depolarizing GABA current is sufficient to trigger an action potential, readers are directed to Kilb (147).

While $GABA_A$Rs are permeable to Cl^-, they are also permeable to HCO_3^- (bicarbonate), with a reported permeability ratio of 0.2–0.4 (HCO_3^-/Cl^-) (1, 146, 148–150). The equilibrium potential for HCO_3^- (E_{HCO3}) is relatively depolarized (approximately −10 to −20 mV), and the HCO_3^- gradient is robustly reestablished by cytosolic carbonic anhydrase-mediated conversion of CO_2 to HCO_3^- under constant intracellular pH (151). Therefore, $GABA_A$ conductance is a combination of the outward Cl^- current and the inward HCO_3^- current in mature neurons. Consequently, the reversal potential for $GABA_A$Rs is not equivalent to E_{Cl} but is rather a combination of E_{Cl} and E_{HCO3}. This results in E_{GABA} being slightly depolarized with respect to E_{Cl}. When experiments seek to record E_{Cl}, they most often do so by buffering extracellular solutions with HEPES instead of bicarbonate and CO_2 (152–156), and thus E_{GABA} is often recorded and reported as $\approx E_{Cl}$. However, during high-frequency activation of $GABA_A$Rs, the Cl^- influx can overwhelm KCC2-mediated Cl^--extrusion, resulting in a collapsed Cl^- gradient. In this situation, the HCO_3^- current predominates and drives V_m toward the AP threshold, resulting in GABAergic depolarization and even excitation (157, 158).

In addition to their classic role in mediating rapid phasic inhibitory synaptic transmission, $GABA_A$Rs also mediate tonic inhibition by producing currents extrasynaptically and perisynaptically (159). Because tonic activation of $GABA_A$Rs increases the cell's input resistance, the magnitude of any coincident excitatory postsynaptic potential is reduced, which reduces the probability that an action potential will be generated. Thus, although the focus of this review is on the effect of changes in Cl^- homeostasis on neuronal excitability, the reader should keep in mind that changes in Cl^- conductance will also affect the magnitude of tonic inhibition.

Although Cl^- flow through $GABA_A$Rs is the primary way that Cl^- passively diffuses across the neuronal membrane in the brain, there are other integral membrane proteins that are also permeable to Cl^-, including a Ca^{2+}-activated Cl^- channel called anoctamin-2, which is involved in spike adaptation (160), and ClC-2 and -3, which contribute to input resistance and vesicular neurotransmitter filling, respectively (161, 162).

Regardless of how Cl^- passively diffuses across the membrane, because *1*) DF_{Cl} is dynamic during development and *2*) E_{Cl} sits so close to V_{rest} in mature neurons, even relatively small changes in Cl^- transport function and expression can have big impacts on excitability, as has been revealed through various types of inhibitory synaptic plasticity.

4. INHIBITORY SYNAPTIC PLASTICITY

Similar to glutamatergic synapses, synaptic plasticity at inhibitory synapses can occur through mechanisms located either pre- or postsynaptically (157, 163–168). However, unlike glutamatergic synapses, where the mechanism of action is most commonly a change in postsynaptic receptor conductance or abundance, inhibitory synaptic plasticity can also result from a postsynaptic change in the driving force for Cl^-. Changes in postsynaptic Cl^- gradients were first revealed as a mechanism underlying inhibitory synaptic plasticity in the hippocampus when spike timing-dependent plasticity (STDP) protocols were found to reduce the strength of inhibition through a depolarization of E_{Cl} (169). This correlated, activity-induced depolarization of E_{Cl} resulted from a Ca^{2+}-dependent decrease in KCC2-mediated Cl^- extrusion (**FIGURE 2, *A–C***). Shortly thereafter, prolonged postsynaptic spiking of mature hippocampal neurons was also shown to depolarize E_{Cl} in a Ca^+-dependent downregulation of KCC2 (170). Moreover, the activity-induced regulation of the postsynaptic gradient was found to also occur at developing synapses when E_{Cl} was still depolarized (153, 155). However, there are two reported mechanisms for this activity-induced regulation of NKCC1. One mechanism requires a Ca^{2+}-dependent upregulation of NKCC1 (155), whereas the other mechanism

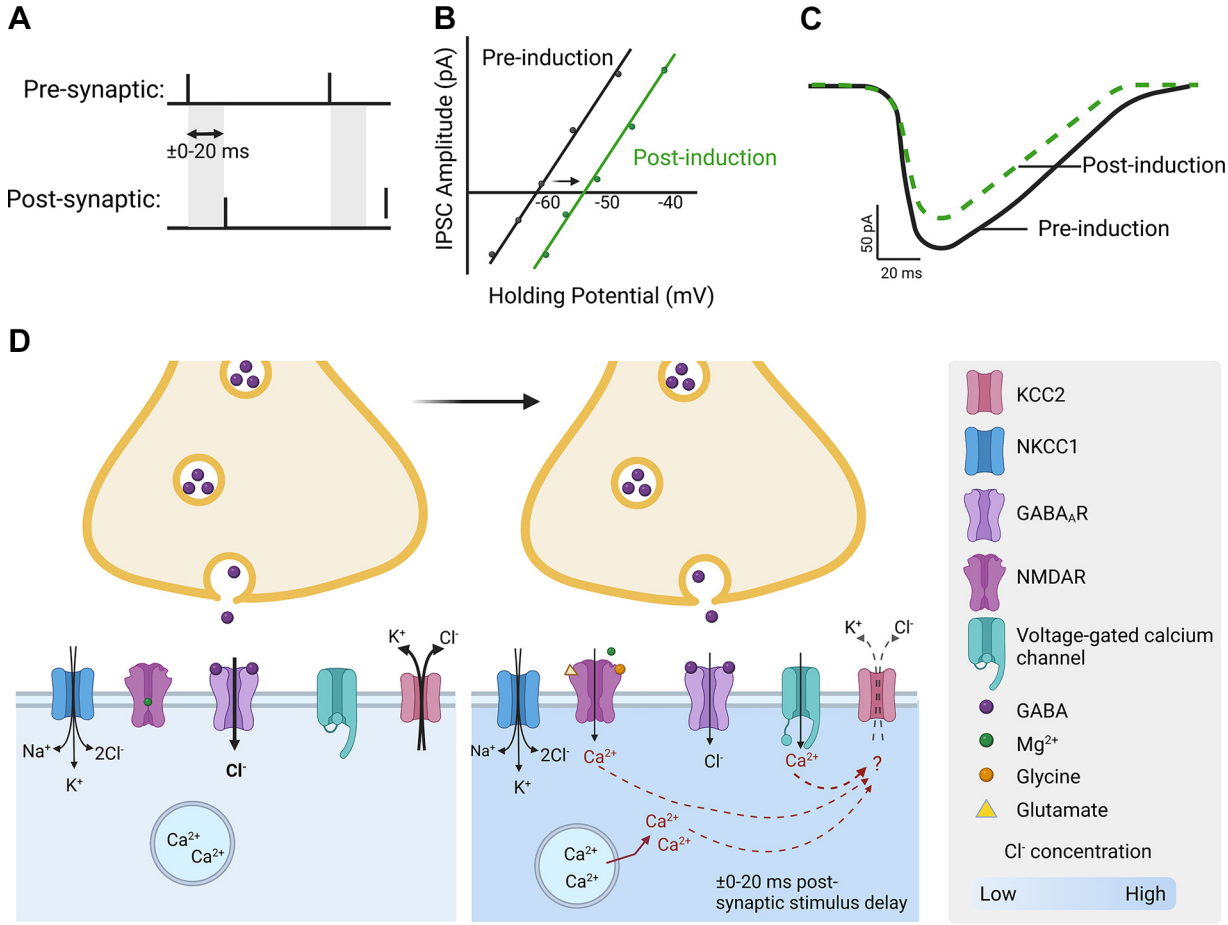

FIGURE 2. Inhibitory synaptic plasticity. *A*: inhibitory spike timing-dependent plasticity protocol. From Ref. 169, with permission from *Neuron*. Postsynaptic spiking within ±20 ms of GABAergic synapse activation results in persistent modifications to GABAergic synaptic strength. *B* and *C*: induction of inhibitory spike timing-dependent plasticity results in a depolarizing shift in Cl^- equilibrium potential (E_{Cl}) (green line) compared with preinduction (black line) (*B*) and a reduction in the inhibitory postsynaptic current (IPSC) amplitude (green dashed line) (*C*), thus reducing the strength of inhibition. *D*: inhibitory synaptic plasticity induction promotes Ca^{2+}-dependent downregulation of KCC2 through unknown mechanisms. This Ca^{2+}-dependent regulation is attained by Ca^{2+} release from intracellular stores and influx through NMDA receptors (NMDARs) and/or voltage-gated calcium channels (*right*). $GABA_AR$, $GABA_A$ receptor. Figure created with BioRender.com, with permission.

requires a thermodynamic regulation of NKCC1 itself, whereby the rate of NKCC1-mediated Cl^- transport depends on both the conductance of, and the driving force for, Cl^- (153). Collectively, this work revealed that NKCC1 and KCC2 can be regulated in a Ca^{2+}-dependent manner by correlated activity in both the juvenile and mature (171, 172) hippocampus, resulting in changes in synaptic strength, which led to coining of the phrase "ionic shift plasticity" (164). The Ca^{2+}-dependent regulation of Cl^- transporters underlying ionic shift plasticity can be achieved through Ca^{2+} influx from L-type and T-type voltage-gated channels (VGCCs) (155, 169, 173), VGCCs together with NMDARs (171, 172), or release from intracellular stores (170), which appears to result in a posttranslational modification of the transporter itself (172) (**FIGURE 2*D***).

Whether ionic shift plasticity is synapse specific likely depends on a multitude of factors, including developmental stage and synapse proximity. At hippocampal synapses that have recently matured as defined by the emergence of hyperpolarizing inhibition, plasticity was found to spread to some, but not all, neighboring synapses. In these cases in which plasticity spread from the synapses where plasticity was induced to noninduced synapses, the spread was likely due to the highly diffusible nature of Cl^- within the soma (169). However, at other synapses plasticity did not spread to neighboring synapses, which is likely due to the ability of neuronal compartments to maintain domain-specific intracellular Cl^- gradients (106, 174). Indeed, although Cl^- is highly diffusible in the cytoplasm, immobile intracellular anions that repel Cl^- from their vicinity are unevenly distributed throughout the cell and thereby maintain domain-specific intracellular Cl^- gradients (175). Therefore, at maturing hippocampal synapses, ionic shift plasticity demonstrates a range of input specificity, due to the level of Cl^- regulation within the neuronal compartment where the plasticity was induced. However, in the adult rodent brain plasticity

was found to be synapse (input) specific, through a heterosynaptic increase in GABAergic conductance at neighboring synapses, which countered local depolarization of E_{Cl} (171).

Ionic shift plasticity helped reveal how even relatively modest changes in Cl^- transport can have big changes in excitability. Take for example the STDP of inhibitory GABAergic synapses, where this activity-induced plasticity shifts E_{Cl} from being hyperpolarized to V_{rest} to being depolarized to V_{rest}, and thus flips the "polarity" of GABAergic transmission from hyperpolarizing to depolarizing (169). Computational modeling combined with experimental validation has revealed that even a modest increase in $[Cl^-]_i$ of 2.5 mM in a pyramidal neuron, which reflects the calculated increase in Cl^- that results from ionic shift plasticity, can increase the firing rate by 40% (176).

What is the impact of ionic shift plasticity on neuronal output? The answer involves both synaptic integration and neural circuits. Take for example the feedforward circuitry in the CA1 region of the hippocampus, where activity of the Schaffer collaterals (axons from CA3 pyramidal neurons) produces both directly an excitatory postsynaptic potential (EPSP) and indirectly an overlapping inhibitory postsynaptic potential (IPSP) in the same postsynaptic pyramidal neuron. This IPSP is slightly delayed with respect to the EPSP because it is generated disynaptically by Schaffer collateral-driven activation of inhibitory basket cells (177, 178). The EPSP and IPSP are integrated, and because of both the IPSP-induced hyperpolarization of V_m and shunting inhibition, the EPSP is attenuated. With this scenario in mind, it is straightforward to conceptualize the impact of ionic shift plasticity on that integration; the depolarization of E_{Cl} reduces inhibition, which in turn reduces the attenuation of the EPSP. With this inhibitory "brake" being eased, the EPSP is more likely to generate an action potential, and it is this phenomenon that has been termed "disinhibition-mediated long-term potentiation (LTP)" (172). This form of inhibitory plasticity was later found to be synapse specific (171) and to increase pyramidal neuron spiking as predicted (176). The significant impact of ionic shift plasticity on synaptic integration and neuronal output is well supported in the literature by the elegant demonstrations of the critical role that GABAergic inhibition itself plays in direction of synaptic integration and neural network activity in multiple brain regions (178–181).

However, as described above, the situation becomes increasingly complex when GABA is depolarizing but not excitatory ($V_{rest} < E_{Cl} <$ AP threshold). If we think about this scenario in the CA1 feedforward example provided above, the impact of the GABAergic transmission on the EPSP would be significantly different. While the GABAergic transmission would still shunt the cationic influx and therefore limit the EPSP, the GABA-induced depolarization of V_m would facilitate the EPSP and result in bidirectional control of neuronal firing rates (122, 182).

What happens when the GABAergic transmission is already inhibitory and then E_{Cl} hyperpolarizes even further, thereby increasing DF_{Cl}? Increasing the driving force will increase the magnitude of the hyperpolarizing current, which in turn will increase the probability of activating a hyperpolarization-sensitive cation channel, such as in the auditory brain stem, where hyperpolarizing inhibition activates I_h and T-type calcium currents that then induce rebound spiking (183).

4.1. CCCs Regulate Glutamatergic Synaptic Transmission and Plasticity

Early in neurodevelopment, depolarizing GABA plays a critical signaling role for migrating neurons. Although migrating neurons express both α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)- and *N*-methyl-D-aspartic acid (or *N*-methyl-D-aspartate; NMDA)-type glutamate receptors (AMPARs and NMDARs, respectively), these neurons do not display the requisite AMPAR-mediated currents required to remove the depolarization block from NMDARs (77, 180, 184–187). However, at these "silent" synapses, the NKCC1-dependent GABA-mediated depolarization can be sufficient to remove the depolarization block from NMDARs, leading to Ca^{2+} influx and, in turn, AMPAR insertion (186) (**FIGURE 3**). Evidence for the critical role of depolarizing GABA in vivo can be found in the visual system of the tadpole, where depolarizing GABA converted nonspiking tectal neurons into spiking tectal neurons with a visual conditioning paradigm (186). When NKCC1 was inhibited with the antagonist bumetanide, or when NMDARs were pharmacologically inhibited in vivo, nonspiking tectal neurons could no longer be converted into spiking neurons.

Depolarizing GABA also plays a critical role in the maturation of newborn granule cells of the hippocampus. These newborn cells have a high $[Cl^-]_i$ for the first 2–3 wk of postmitotic development because of a relatively high expression of NKCC1 (188), which results in depolarizing GABA. During this same time period, glutamatergic neurotransmission is insufficient to reach action potential threshold, and a recent publication of adult-born granule cells demonstrated that combined GABAergic depolarization and NMDAR activation rapidly drives AMPAR insertion and glutamatergic unsilencing (189, 190).

During neuronal development, KCC2 expression plays a significant role in dendritic spine formation (191, 192) and confines postsynaptic glutamate receptors

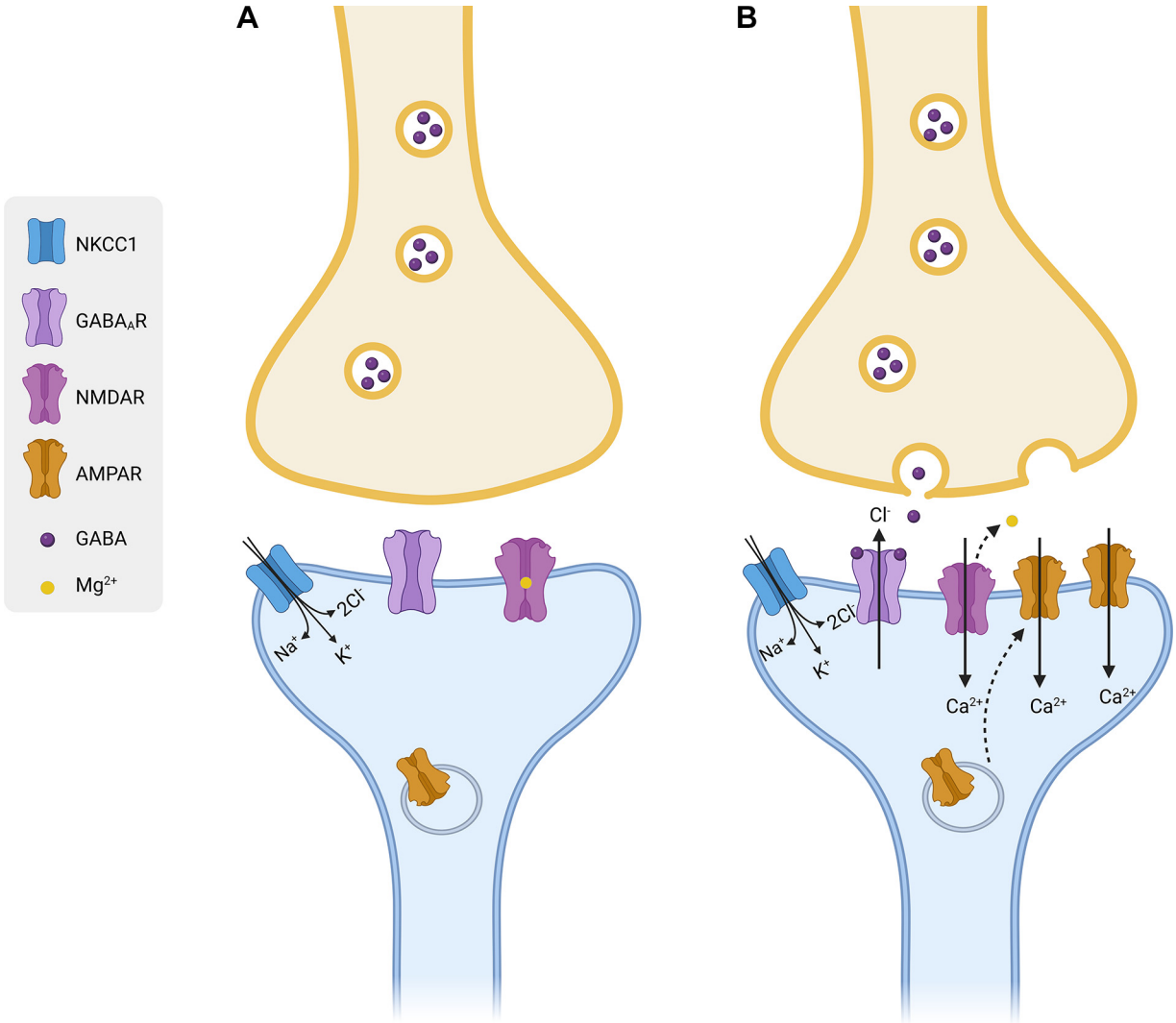

FIGURE 3. NKCC1-dependent GABA-mediated depolarization. *A*: early in development, migrating neurons exhibit high intracellular chloride concentrations due to predominant NKCC1-mediated chloride import. These migrating neurons form silent synapses that do not display AMPA receptor (AMPAR) currents. *B*: at these silent synapses, binding of GABA to postsynaptic $GABA_A$ receptors ($GABA_A$Rs) results in NKCC1-dependent GABA-mediated depolarization. This depolarization can be sufficient to remove the depolarization block from NMDA receptors (NMDARs), resulting in Ca^{2+} influx and consequent AMPAR insertion into the membrane. Figure created with BioRender.com, with permission.

within those spines (193). Moreover, KCC2 also gates long-term potentiation at glutamatergic synapses (194). All of these interactions in spines and at glutamatergic synapses require KCC2's large carboxy-terminal domain with actin-related proteins, such as the FERM domain and 4.1N (191), and β-pix (194, 195) (**FIGURE 4*A***). Although these requirements for KCC2 in spines and at glutamatergic synapses do not require ion extrusion, it is not known whether ion extrusion occurs at these sites. Even though KCC2 has a morphogenic role in synapse development, KCC2 disruption in granule cells or Purkinje cells changed neither synapse density nor spine morphology (59).

Amidst the published literature delineating KCC2's potential ion extrusion-independent function, KCC2 itself is reported to be regulated by proteins associated with glutamatergic synaptic transmission (196–199). The first demonstration of the regulation of KCC2 by glutamate receptors was via tonic activation of group I metabotropic glutamate receptors (mGluRs) at hippocampal CA3 synapses (196) (**FIGURE 4*C***). More recently, a robust bidirectional regulation of KCC2 expression was determined in cerebellar Purkinje synapses, where genetic ablation and pharmacological inactivation of mGluR5 decreased KCC2 expression and pharmacological activation increased KCC2 expression (200). In addition, the GluK2 subunit of kainate receptors mediates the surface trafficking and abundance of KCC2 at the neuronal membrane, in a process that requires phosphorylation of GluK2 that stabilizes the channel in the membrane and mediates synaptic excitation (201) (**FIGURE 4*B***). Finally, GluK2-mediated excitatory

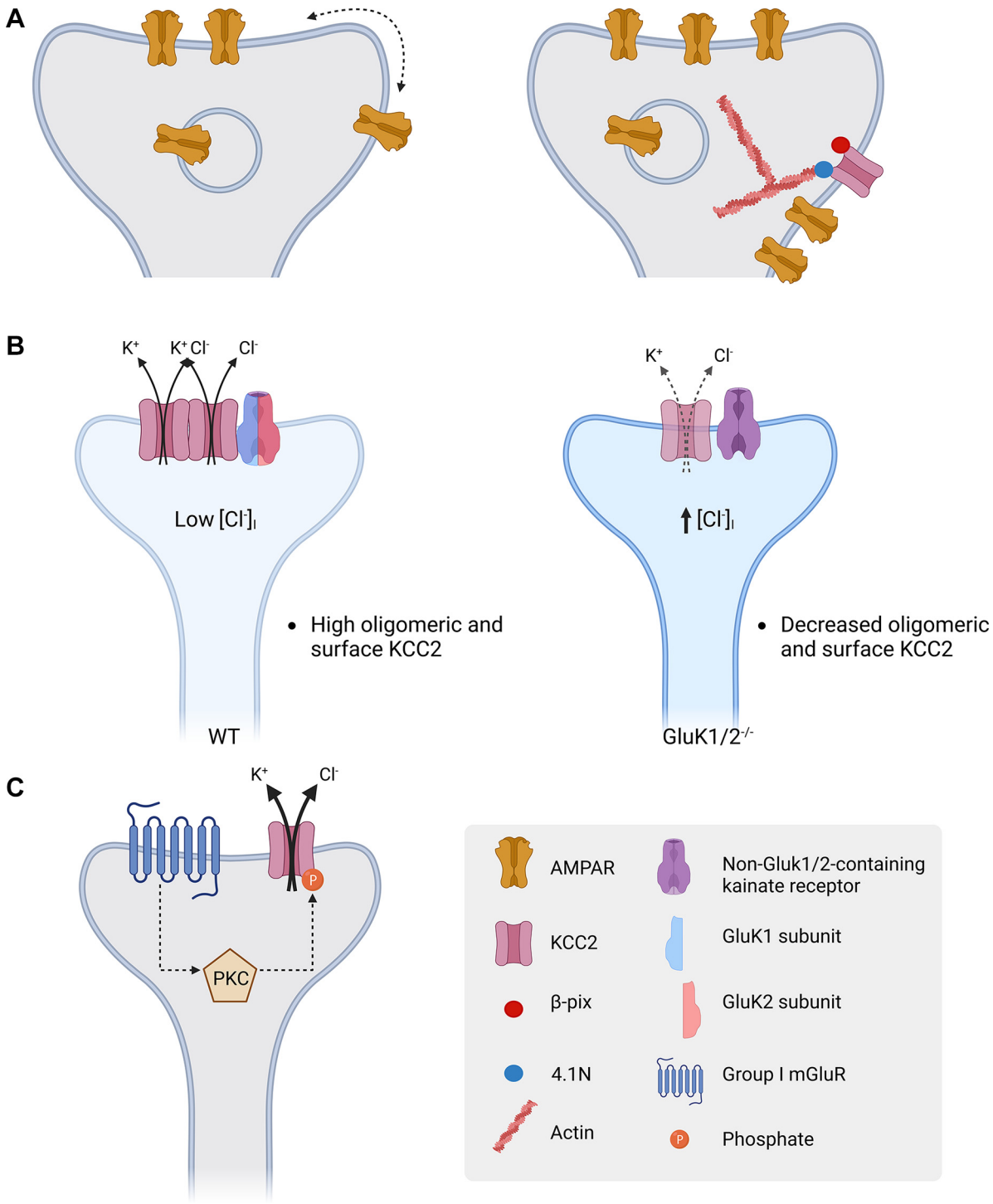

FIGURE 4. KCC2 at excitatory synapses. *A*: KCC2 expression restricts AMPA receptors (AMPARs) to dendritic spines, preventing AMPAR lateral diffusion. This is likely accomplished through KCC2's interaction with actin mediated by 4.1N. *B*: the GluK1/2 subunits of kainate receptors mediate the membrane surface abundance of KCC2. In the absence of GluK1/2, oligomerization and surface expression of KCC2 decreases, resulting in an increase in intracellular chloride concentration ($[Cl^-]_i$). *C*: activity of group I metabotropic glutamate receptors (mGluRs) results in the activation of PKC, which phosphorylates KCC2. Phosphorylation of KCC2 potentiates its chloride extrusion capacity. WT, wild type. Figure created with BioRender.com, with permission.

transmission itself can potentiate KCC2-dependent Cl^- extrusion in CA3 pyramidal neurons (202). Is this arrangement between synaptic excitation and KCC2 function restricted to only glutamatergic synapses? It appears that spontaneous cholinergic excitation mediated by α3- and α7-subunits of nicotinic acetylcholine receptors (α3/α7-nAChRs) can also regulate KCC2 expression. Together, this begs the questions *1*) Does KCC2-dependent Cl^-

extrusion occur at excitatory synapses? and *2*) Why does a biological mechanism mediating Cl^- transport at excitatory synapses occur during glutamatergic neurotransmission? Indeed, the role for KCC2 at excitatory synapses is only getting more "exciting," paving way for a more nuanced, and reciprocal, regulation of excitation and KCC2 function.

4.2. CCC-Protein Interactions Regulate Neuronal Excitability

Regulation of protein function is essential for maintaining neuronal homeostasis, and in addition to protein regulation by posttranslational modifications, protein function is also regulated by their interaction with other proteins (203). The CCC family is no exception, and in this section we review the literature of known CCC-protein interactions and how those interactions regulate CCC function and thus how they regulate neuronal excitability.

CCC-protein interactions were first identified with traditional biochemical assays such as yeast 2-hybrid screens (204). Early protein interaction studies revealed that KCC2 (36, 204), but not NKCC1 (205), functionally interacts with the Na^+-K^+-ATPase α2-isoform (206). KCC2 also interacts with brain-type creatine kinase (CKB), which is a kinase that transfers high-energy phosphate from phosphocreatine to ADP to rapidly generate ATP. It is hypothesized that increased KCC2 function depends on a concomitant increase in CKB and Na^+-K^+-ATPase. Coincidentally, there is a developmental regulation of the CKB and the α2-isoform expression (205), which further supports the hypothesis that CKB is critically required for KCC2 function. The identification of these protein interactions revealed that KCC2 interacts with proteins regulating synaptic homeostasis at the site of excitation and thus is not solely an "inhibitory protein," as it was initially characterized.

KCC2 also regulates neuronal excitability through its interaction with the tandem-pore leak K^+ channel Task-3 and regulates the traffic and expression of excitatory proteins (207). Goutierre and colleagues (207) determined that KCC2 interacts with Task-3 channels, which ultimately regulate membrane excitability. When total KCC2 expression was knocked down with an RNA interference (RNAi) strategy, the result was an overall increase in spiking and network activity in the dentate gyrus (59). This study reveals that not only does KCC2 interact with traditional inhibitory proteins such as GABA receptors but KCC2 also impacts the membrane targeting of interacting partners and thus has a profound impact on the intrinsic excitability of neurons. In a similar fashion, an earlier study from the Jentsch laboratory (59) reported that a cerebellar granule cell-specific genetic knockout of KCC2 also exhibited a depolarized resting membrane potential and resting E_{GABA} without altering the driving force of GABAergic neurotransmission. Although this study did not directly examine the role of KCC2 protein interactions with potassium channels, these two studies establish that KCC2 is capable of directly regulating membrane excitability in different cell types and brain regions.

More recently, proteomics has been used as a holistic strategy to identify the proteins that interact with KCC2, which is referred to as the KCC2 interactome. Mahadevan et al. (208) used unbiased affinity purification coupled with high-resolution mass spectrometry (AP-MS) to identify native protein interactions. Because of the hydrophobicity of KCC2, this protein had been exceptionally difficult to isolate for further biochemical and structural analysis (209), so before the application of proteomic analysis, the authors first had to empirically determine the optimal detergent and protocol for isolating stable and active membrane-embedded native KCC2 multiprotein complexes (MPCs) (202, 209). Mahadevan et al. determined that nonaethylene glycol monododecyl ether ($C_{12}E_9$) and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS) were the most effective detergents to extract native KCC2 samples for AP-MS. This approach revealed a KCC2 interactome comprised of 181 diverse proteins, with the predominant interactors playing important roles in postsynaptic receptor recycling.

Large-scale proteomic studies performed with high-throughput experimental methods such as AP-MS do not provide an exhaustive list of protein interactors, and each putative interacting protein needs to be thoroughly validated to be confirmed as a true interactor (to identify false positives). Validation can be performed with other in vitro or in vivo assays, including coimmunoprecipitation, yeast 2-hybrid, label transfer protein interaction analysis, and proximity ligation assay. The most abundant KCC2-protein interactor is the neuronal endocytic regulatory protein termed PACSIN1 (SYNDAPIN1). Biochemical validation of this protein interaction, combined with functional characterization studies, revealed that PACSIN1 is a negative regulator of KCC2 expression and function (201). When PACSIN1 expression was reduced with shRNA, KCC2 protein abundance increased and E_{GABA} hyperpolarized. This study is one of the first examples illuminating how protein interactions with KCC2 can bidirectionally regulate KCC2 function in neurons (**FIGURE 5**).

Since the publication of the first KCC2 proteome, an additional proteome was published by Smalley et al. in 2020 (210) combining mouse brain plasma membrane fractionation and a classical native PAGE protocol using the Triton X-100 extraction buffer. Although there is

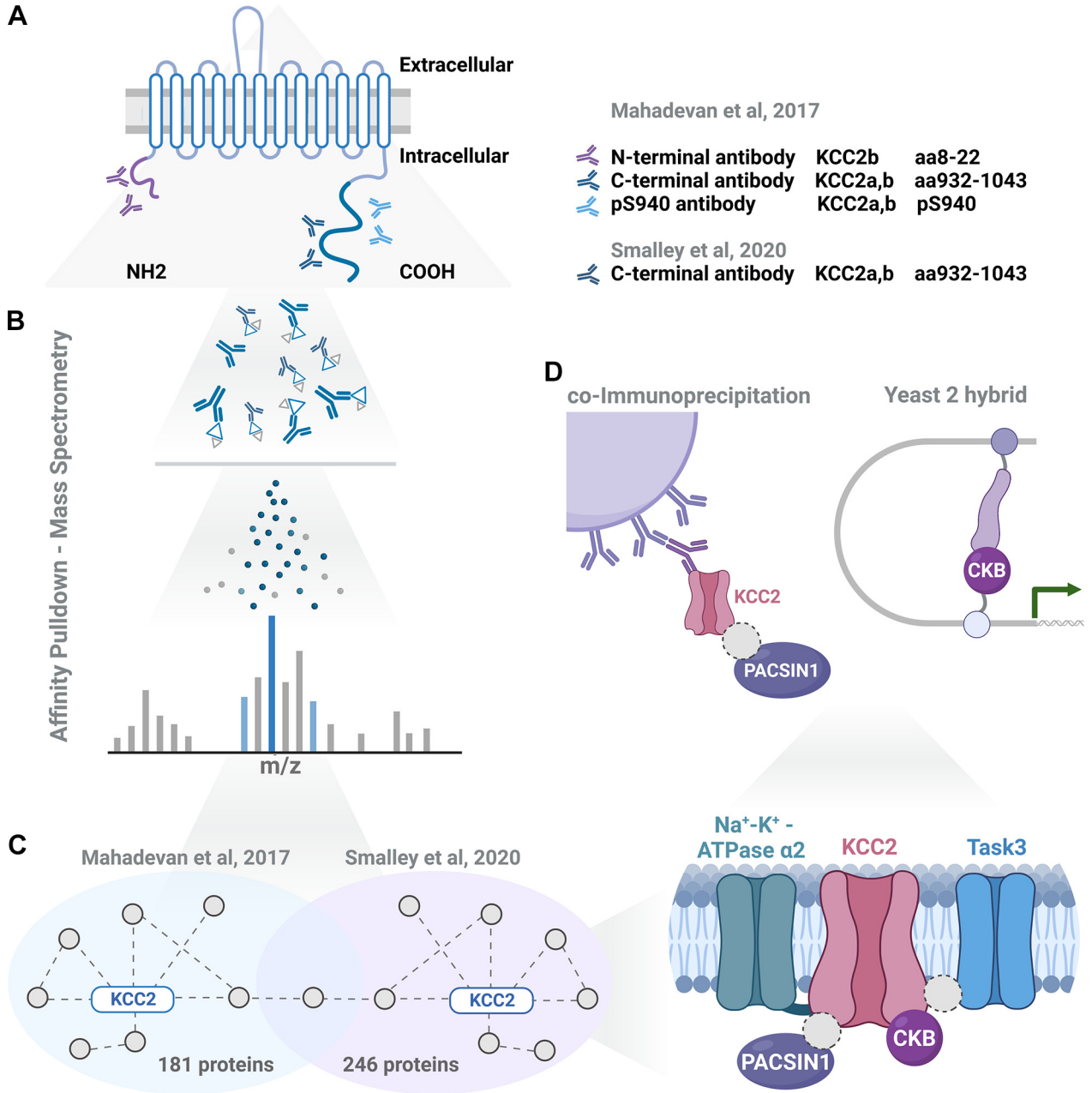

FIGURE 5. Strategies to identify and validate KCC2 interactome. *A–C*: schematics showing the epitope locations of anti-KCC2 antibodies (*A*) and the technique (affinity pulldown-mass spectrometry) (*B*) used to identify the 2 published interactomes of KCC2 (*C*). The interactomes by Mahadevan et al. and Smalley et al. found a total of 181 and 246 KCC2 interacting proteins, respectively, with common proteins identified in both interactomes. *D*: these interacting partners of KCC2 can be further validated by additional methods such as coimmunoprecipitation and yeast 2-hybrid assay. Figure created with BioRender.com, with permission.

some overlap between the proteins identified in the KCC2 interactomes, not all proteins were found in both proteomes. This variability is not uncommon in proteomics and likely results from the extraction methods used to isolate KCC2 and the criteria used to analyze the mass spectrometry data. Other technical considerations, including the affinity and the location of epitope of the antibody used for the pulldown, can also influence the interactome. For example, in the publication by Mahadevan et al. (208), the interacting proteins varied depending on whether the antibody used for the pulldown targeted the NH_2 or COOH terminus. However, despite the differences, both KCC2 proteomes identified interacting proteins found at both inhibitory and excitatory synapses that have roles in receptor trafficking, ion homeostasis, and the dendritic cytoskeleton (208).

These proteomes highlight the potential role of KCC2 as a "hub" protein. Whereas most proteins interact with only a few others during their life span, some proteins interact with a very large number of other proteins and

are referred to as hub proteins (211–213). We recently argued for the role of KCC2 as a hub protein in the CNS, as it meets the following criteria (214): *1*) KCC2 is highly conserved (215, 216); *2*) KCC2 has an extensive interactome (208, 210); and *3*) KCC2 is essential for survival, as $KCC2^{-/-}$ are postnatal lethal (24).

To date no specific proteome has been completed for NKCC1; however, in 2019 He et al. (217) performed a proteomics analysis of the somatosensory cortex of FMR1-knockout mice, which recapitulate symptoms of fragile X syndrome. This mouse line exhibits delayed GABAergic inhibition, upregulated NKCC1 expression, and altered excitatory synaptic transmission during the first 10 days of postnatal development in the somatosensory cortex (217). The authors found that not only did chronic treatment with the NKCC1 inhibitor bumetanide during this critical period rescue the synaptic transmission deficits but bumetanide treatment also altered a subset of proteins identified in the proteome, including inhibitory markers parvalbumin (PV) and TrkB (217). Although this is the first publication investigating how the regulation of NKCC1 during a critical period may help normalize developmental pathologies, the primary focus remains on elucidating novel strategies for targeting KCC2 function and expression.

Frantzi et al. (218) aptly described diseases being the result of changes in the proteome, whether protein abundance, structure, or function, and the resulting pathology is an outcome of these changes. Proteomics research is a burgeoning field, with several review articles highlighting this technique as the next logical path forward in targeting protein interactions for drug discovery (218–220). By characterizing pertinent KCC2 protein interactors that negatively impact KCC2 function in pathological conditions, new therapeutic strategies can be developed to specifically target these adverse events and rescue KCC2-related neurological conditions.

5. CHLORIDE AS AN INTRACELLULAR SIGNALING MOLECULE

Cl^- has recently been hypothesized to act as a signaling molecule, analogous to known effector molecules such as Ca^{2+} (for comprehensive reviews see Refs. 221–224). Although studies have predominantly framed the regulation of intracellular Cl^- as a secondary effect of the activity of membrane proteins themselves, as we review below evidence exists that Cl^- itself can act as a second messenger effector. In particular, we discuss how Cl^- signaling via specific kinases regulates cellular excitability via NKCC and KCC cotransporters.

5.1. Cl^- Regulates Signaling Kinases

Although Cl^- has been implicated in the regulation of multiple kinases, the most highly studied Cl^- regulated signaling pathways are the With No Lysine (WNK) kinases (**FIGURE 6**). There are four members of the WNK kinase family, WNK1–4 (18); WNK1, 3, and 4 are expressed by any tissue that regulates Cl^- (18), whereas WNK2 is expressed exclusively in brain tissue (225). The WNKs are regulated by changes in $[Cl^-]_i$, osmolarity, and/or cell volume. When the $[Cl^-]_i$ is reduced, WNKs autophosphorylate their T-loop residue and transition into an active state; however, when Cl^- is present it physically binds to this residue and prevents activation (226–228). Additionally, when cells become hyperosmotic or cell volume is reduced, WNKs can also become activated through autophosphorylation (227).

The downstream effectors of the WNKs are the serine/threonine kinase 39 (STK39)/Ste20-related proline alanine-rich kinase (SPAK) and oxidative stress-responsive kinase 1 (OSR1). The WNK-SPAK/OSR1 signaling cascade was first linked to the regulation of CCCs in the kidney, where mutating the function of WNK1 or WNK4 resulted in hypoaldosteronism type II (Gordon syndrome) and hyperkalemia (229). Since then, WNKs have been shown to also regulate CCCs in the nervous system.

WNK1 and WNK3 appear to be the dominant regulators of NKCCs and KCCs in brain tissue either directly or indirectly via SPAK/OSR1. Phosphorylation by WNK-SPAK/OSR1 activates NKCCs, whereas phosphorylation of the KCCs inhibits them (230, 231). Various phosphorylation sites have been well characterized and are discussed in detail in other reviews (232–234). Because of the ability of the WNK-SPAK/OSR1 pathway to bidirectionally regulate the activity of NKCCs and KCCs, this signaling pathway has garnered considerable experimental attention in the past few years.

As members of the WNK family are highly sensitive to small changes in Cl^-, this signaling cascade provides a unique opportunity where inhibitory synaptic currents, and thus Cl^- flux across the membrane, can regulate the activity of NKCC1 and KCC2. This mechanism for "fine-tuning" inhibition was demonstrated by Heubl et al. (235), who directly linked $GABA_AR$ currents to the phosphorylation of KCC2 at T906 and T1007. Driving GABAergic synaptic activity was sufficient to increase $[Cl^-]_i$ and activate WNK1, which was shown to act on KCC2 by limiting the lateral diffusion of this protein in the neuronal membrane and promoting KCC2-mediated Cl^- extrusion (235). Studies such as this have revealed this signaling pathway to be a potent regulator of neuronal Cl^-, with targeted genetic disruption of WNK3 (resulting from partial deletion of the X chromosome that

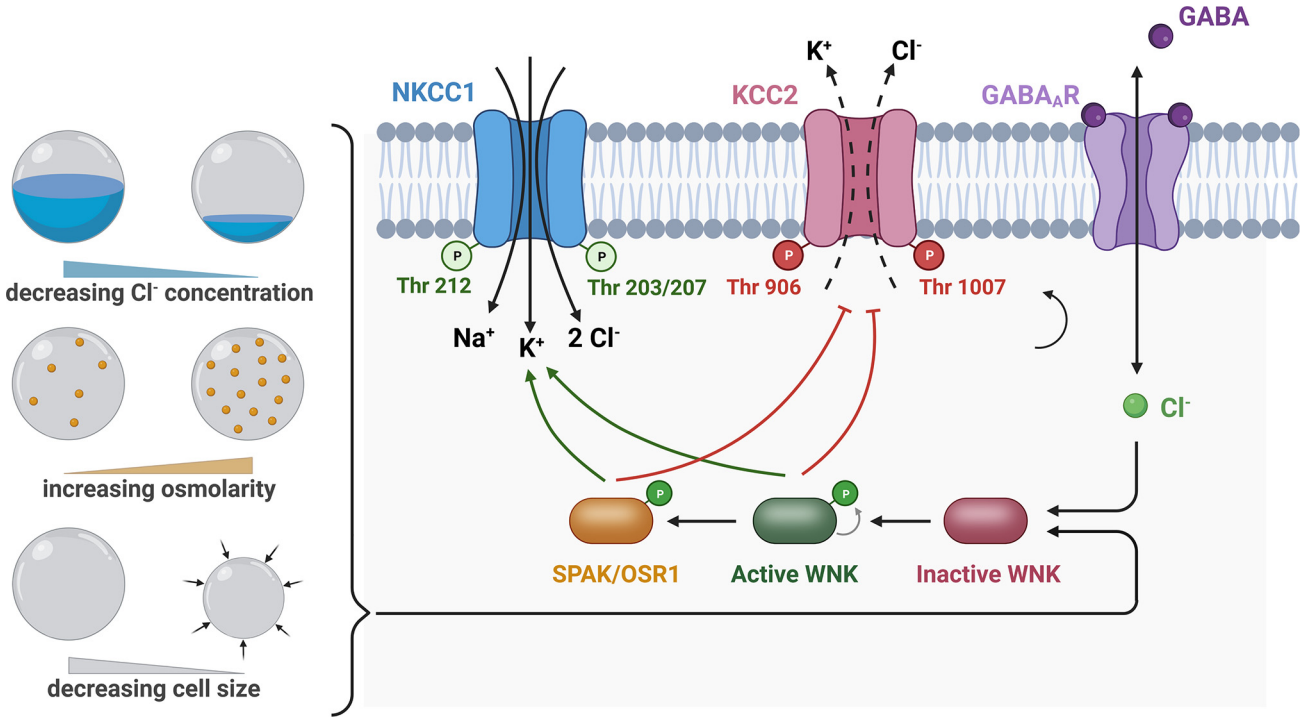

FIGURE 6. WNK kinases regulate KCC2/NKCC1 expression and function. Changes in intracellular properties and cell phenotype including decreased chloride concentration, increased osmolarity, and decreased cell size activate WNKs. Active WNKS, which can perform autophosphorylation, can directly or indirectly (via SPAK/OSR1) activate NKCC1 and inhibit KCC2 via phosphorylation of key residues to reestablish homeostasis. $GABA_AR$, $GABA_A$ receptor. Figure created with BioRender.com, with permission.

disrupts an additional 2 genes) implicated in some instances of autism spectrum disorder (ASD) (236), and a specific mutation in WNK1 contributes to neuropathic pain (237). Accordingly, this signaling pathway has garnered much attention, and considerable efforts are underway to develop novel therapeutic targets for treating WNK-SPAK/OSR1-related neurological disorders (232). It is currently unclear whether the scope of WNK-dependent Cl^- signaling during the regulation of neuronal excitability extends beyond their currently explored regulation of KCC family members. However, few members of the excitatory receptors (238–240) and transporters (241, 242) are known to be directly regulated by Cl^- binding in their extracellular or intracellular pockets, and it is currently not known how this property affects cellular excitability.

6. CHLORIDE REGULATION IN GLIA CELLS AND THE INTERPLAY WITH NEURONAL EXCITABILITY

All cells regulate Cl^- across their plasma membrane and within intracellular organelles (243), including the glial cells of the CNS (astrocytes, oligodendrocytes, and microglia). Reports on the $[Cl^-]_i$ in glia vary considerably, and it is reported to be in the range of 5–50 mM (244, 245), with the Cl^- gradient being regulated by NKCC1, $GABA_AR$s, GlyRs, voltage-gated Cl^- channels, calcium-dependent Cl^- channels, BEST family proteins, cystic fibrosis transmembrane conductance regulator (CFTR), and volume-regulated anion channels (VRACs) (246–248). Glial cells are essential for maintaining a low extracellular K^+ concentration, and in doing so astrocytes also regulate Cl^-, as this ion is often used to counterbalance the movement of cations to maintain cellular electroneutrality (243). Thus, Cl^- regulation in glia is often studied in the context of cell volume regulation in response to swelling and the maintenance of ionic equilibrium (221). As we explain below, evidence is accumulating to suggest that the regulation of Cl^- in glia contributes to neuronal excitability in a myriad of ways.

6.1. Glial NKCC1

With the advancement of RNA sequencing techniques, glial researchers have examined the relative expression profiles of NKCC1 in all cell types of the CNS including astrocytes (249, 250), oligodendrocytes and oligodendrocyte precursor cells (26, 251), microglia (252), and the choroid plexus (89, 253). Interestingly, NKCC1 mRNA expression is higher in glia than in neurons (254), and although mRNA expression does not always directly correlate with protein expression, the mRNA profile

suggests that NKCC1 is a significant regulator of Cl^- in glia. With the realization that NKCC1 plays a functional role in glia, a pressing preclinical question is whether the therapeutic benefits of the NKCC1 inhibitor bumetanide on ameliorating neuronal excitation/inhibition imbalances is due in part to the role of NKCC1 in glia (255).

6.2. The Tight Coupling of Astrocytic GABA/Glycine Receptors to Inhibitory Synaptic Transmission and Neuronal Excitability

Astrocytes are a key component of the "tripartite synapse," which was first coined in the early 1990s to describe the tight physical and functional coupling of pre- and postsynaptic terminals with astrocytes (256). Astrocytes not only encapsulate inhibitory synapses but also express proteins critically involved in inhibition, including $GABA_A$Rs, $GABA_B$Rs (257), GlyRs (257), and the GABA transporters GAT-1 and GAT-3 (258), which allow them to participate in inhibitory synaptic transmission (**FIGURE 7*A***). Astrocytes use their NKCC1-mediated Cl^- gradients to regulate the extracellular $[Cl^-]$ during periods of intense GABAergic signaling, when Cl^- is rapidly entering into neurons via GABA/glycine receptors (259, 260) (**FIGURE 7*B***). Activation of astrocytic $GABA_A$ and glycine receptors results in Cl^- efflux and depolarization of the astrocytic plasma membrane. The first study to record glycine currents in astrocytes was performed in the spinal cord of the developing rat and determined that the glycinergic current was much smaller than the GABAergic current (261). The authors predicted that glycine-induced currents were thus not a major signal to regulate astrocytic function. More recently, however, a study examined the astrocytic glycine and GABA currents in the inferior colliculus and the hippocampus and found similar results indicating that, although the GABA current often elicits a larger membrane depolarization than glycine, the ratio of the two currents is brain region specific (262).

6.3. Bestrophin Proteins

Finally, we briefly review the activity of the Bestrophin (BEST) family of proteins, which are Ca^{2+}-activated Cl^- channels (263). There are four BEST proteins, BEST1–4, with the BEST1 channel being highly expressed in astrocytes and the most well studied in the context of the CNS (248). BEST1 is specifically expressed in astrocytic endfeet surrounding inhibitory synapses of the cerebellum, hippocampus, and striatum, where BEST1 channels have been found to regulate tonic neuronal inhibition (244, 264). Astrocytes were discovered to synthesize the neurotransmitter GABA using the enzyme monoamine oxidase B (MAOB) instead of the neuronal enzyme glutamate decarboxylase (244). This intra-astrocytic GABA was classically thought to be complemented by GABA uptake through GABA transporters on glial cells. Remarkably, several studies have shown that glial cells also actively release GABA. Indeed, once produced in astrocytes, GABA can be released via the BEST1 channels, where it is free to bind to GABA receptors expressed on nearby neurons and contribute to tonic inhibition (244, 264). The significance of this signaling pathway was recently demonstrated in the cerebellum, where tonic inhibition is mediated by extrasynaptic $GABA_A$Rs (265). The impact of BEST1-mediated tonic inhibition was demonstrated with a global BEST1 KO mouse model and by examining the impact of tonic inhibition in cerebellar granule cells (**FIGURE 8, *A* and *B***). In the absence of BEST1 expression, tonic inhibition was significantly reduced, but phasic $GABA_A$ receptor currents remained unaffected (**FIGURE 8, *C* and *D***). Increasing GABA production by driving the expression of MAOB in Bergmann glial cells reduced overall granule cell excitability (265). Consequentially, mice with less astrocytic GABAergic signaling learned motor coordination tasks much better than mice that had their tonic inhibition intact (265) (**FIGURE 8*E***). VRACs have also been recently identified as GABA and glutamate transporters (266), and with more information being published on the role of astrocytes in the regulation of excitation-inhibition balance in various brain regions, the importance of this support cell is gaining traction.

7. TECHNIQUES FOR MEASURING INTRACELLULAR CHLORIDE AND TRANSPORTER ACTIVITY

Neuroscientists use various forms of imaging and/or electrophysiology to estimate $[Cl^-]_i$ in neurons (**FIGURE 9**). Each of these techniques has its advantages and limitations for determining $[Cl^-]_i$ and CCC function, as summarized in **TABLE 2**.

7.1. Tools for Imaging Chloride

Cl^- imaging is of significant interest to neuroscientists, but it has been challenging to optimize Cl^- imaging tools to achieve the following technical requirements: high sensitivity to Cl^-, concentration quantitation, and low background fluorescence (269). The first Cl^- imaging tools included a set of three synthetic Cl^- dyes [SPQ (270), MEQ (271), and MQAE (272)], which emit reduced fluorescence intensity as $[Cl^-]_i$ increases (273–275). Despite their multiple limitations, these indicators were

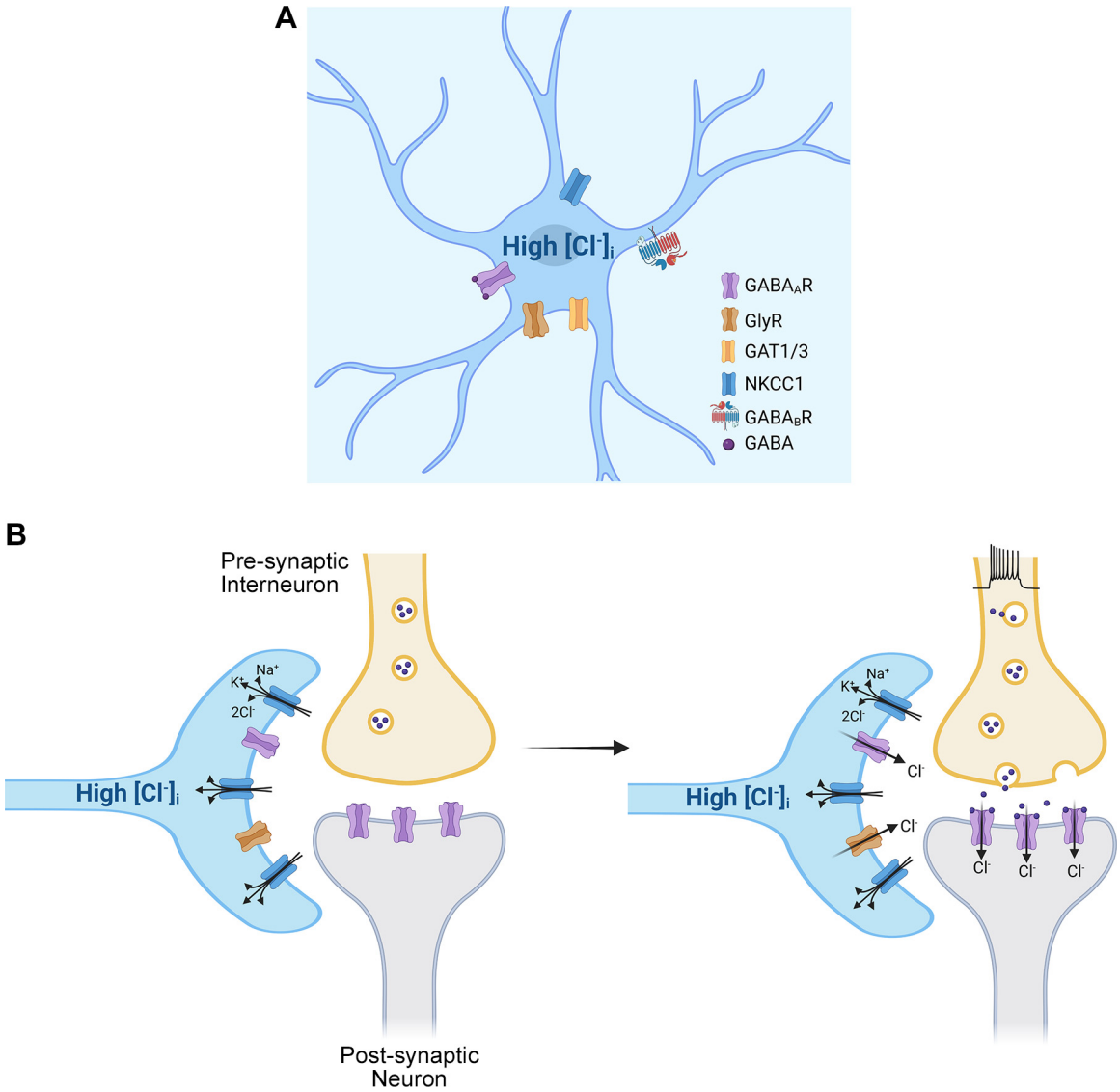

FIGURE 7. Astrocytes maintain high intracellular chloride Cl^- concentration ($[Cl^-]_i$) via NKCC1 and use this gradient to buffer extracellular Cl^- concentration ($[Cl^-]_o$) during periods of intense interneuron firing via $GABA_A$ receptors ($GABA_ARs$) and glycine receptors (GlyRs). *A*: glia express a variety of channels and transporters that regulate the Cl^- gradient, including $GABA_ARs$, GlyRs, GAT1/3, NKCC1, and $GABA_B$ receptors ($GABA_BRs$). *B*: NKCC1 expression in astrocytes maintains high $[Cl^-]_i$ in astrocytes during basal levels of activity. During periods of intense GABA-mediated inhibition (*right*), Cl^- in the extracellular space enters the postsynaptic neuron and mediates neuronal inhibition. During this time, the $[Cl^-]_o$ is maintained by the release of Cl^- from astrocytes via $GABA_ARs$ and GlyRs. Figure created with BioRender.com, with permission.

indispensable for our early understanding of neuronal Cl^- regulation and CCC function.

A significant advancement in Cl^- imaging tools came in 2000 with the development of Clomeleon, which was the first genetically encoded ratiometric Cl^- indicator and offered numerous advantages over Cl^- dyes (**TABLE 2**). Advantages of Clomeleon and its further generations, Cl-sensor and SuperClomeleon, include the ability to target them to specific subcellular compartments and/or cell types; the ability to repeatedly measure them in vivo; their photostability compared with chemical dyes; the ability to measure them ratiometrically with or without fluorescence resonance energy transfer (FRET); and the ability to calibrate them. Clomeleon has been used successfully in vitro (267, 276) and in vivo (277) and is sufficient for fluorescence lifetime imaging microscopy (FLIM), which facilitates quantitation of $[Cl^-]_i$ with good background separation (278). However, this Clomeleon is not sensitive to $[Cl^-]_i$ changes at lower physiologically relevant levels (K_d = ~100–119 mM Cl^-) (276, 279) and has several additional limitations including relatively low Cl^- affinity in the physiological range and a relatively high signal-to-noise ratio. Although both of these limitations were improved in the next-generation indicators, Cl-sensor and SuperClomeleon (280, 281), sensitivity to changes in pH remained (282). In an attempt to address the limitation of pH dependence, ClopHensor was designed to permit

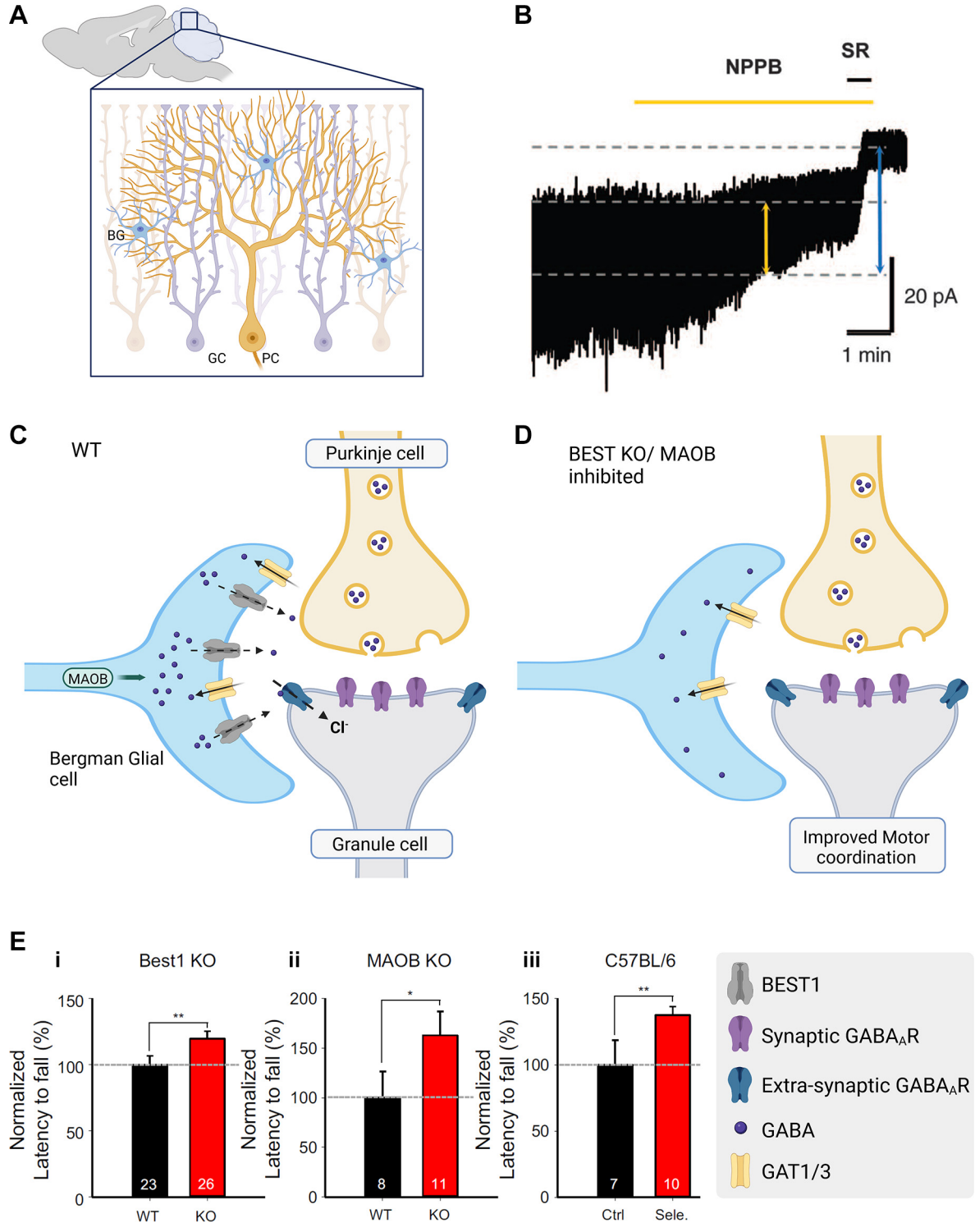

FIGURE 8. Astrocytic BEST1 channels mediate tonic inhibition at the cerebellar. *A*: Bergmann glia (BG) encapsulate Purkinje cells (PC) and interact with granule cells (GC) in the cerebellum. *B*: tonic inhibition mediated by $GABA_A$ receptors expressed by granule cells (GCs) is abolished by the GABA inhibitors 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB; depolarizing shift indicated with yellow line) and gabazine (SR95531; depolarizing shift indicated with blue line). From Ref. 264. Printed with permission from AAAS. *C* and *D*: BGs express both the enzyme monoamine oxidase B (MAOB), which produces GABA, and BEST1 channels, which allow for the release of GABA into the synaptic cleft to activate extrasynaptic $GABA_A$ receptors ($GABA_A$Rs). Using the BEST1 knockout (KO) and MAOB KO mouse models, GABA release from BGs is blocked and tonic inhibition is lost. WT, wild type. *E*: in the absence of tonic inhibition mediated by BGs in the BEST1 KO and MAOB KO mouse models, and when MAOB is inhibited in the control mice using selegiline (Sele), the latency to fall is significantly increased indicative of improved motor learning on the rotor rod task. Error bars show SE; *P<0.05, **P<0.01. Image from Ref. 265, with permission from *Proceedings of the National Academy of Sciences USA*. Figure created with BioRender.com, with permission.

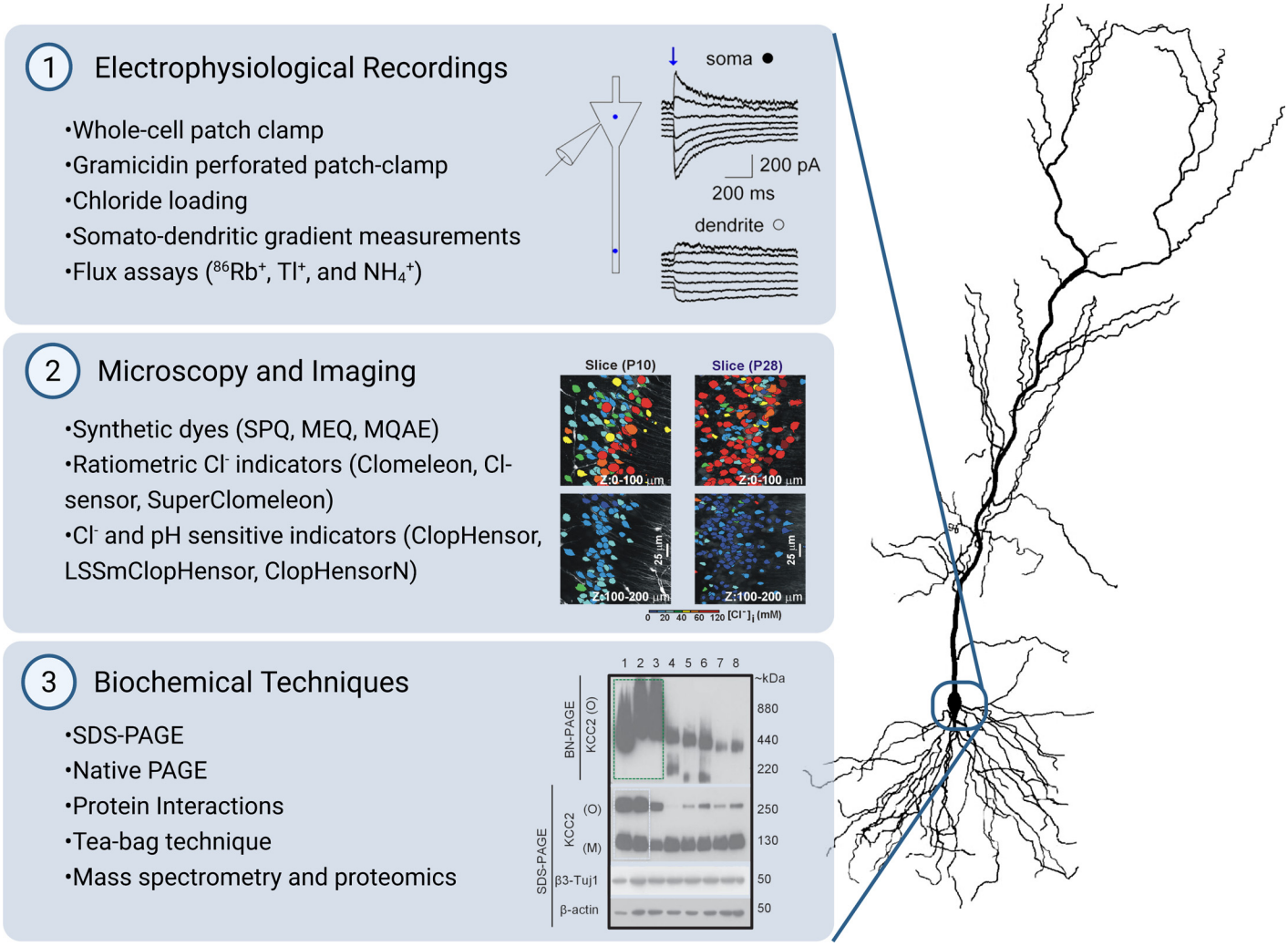

FIGURE 9. Summary of techniques commonly used to assess Cl^- regulation. Strategies include electrophysiological recordings (*1*), microscopy and imaging (*2*), and biochemical assays (*3*). Images reproduced from Ref. 193, with permission from *Proceedings of the National Academy of Sciences USA*; Ref. 267, with permission from the *Journal of Neuroscience* (licensed under Creative Commons CC-BY 3.0 license); and Ref. 208, with permission from *eLife*. Image of neuron reproduced from Ref. 268, with permission *from Journal of Physiology*. Figure created with BioRender.com, with permission.

simultaneous measurements of both $[Cl^-]_i$ and pH, but detecting small changes in $[Cl^-]_i$ remains challenging; ClopHensor also has relatively complex imaging and data analysis requirements. A future generation of this indicator, LSSmClopHensor, has been adapted for use with in vivo two-photon imaging, which facilitated the direct and simultaneous measurement of neuronal $[Cl^-]_i$ and pH_i at the single-cell level (283), but detecting small changes in $[Cl^-]_i$ remains difficult (including with further variants, e.g., ClopHensorN). Despite these tools having various limitations, they have been used in a wide variety of in vitro and in vivo studies providing valuable insights; however, neuroscientists still await next-generation Cl^- imaging tools with high Cl^- sensitivity in the physiological range (independent of changes in pH).

7.2. *Electrophysiological Estimates and Flux Assays for the Measurement of Intracellular Chloride and Transporter Activity*

Neuronal $[Cl^-]_i$ can be measured indirectly with whole cell patch-clamp or perforated patch-clamp electrophysiology. For both methods, the experimenter determines E_{Cl} and then calculates $[Cl^-]_i$ from the Nernst equation. E_{Cl} can be determined in voltage-clamp mode by evoking GABAergic postsynaptic currents while step depolarizing the membrane potential (51). A linear regression of the current amplitudes against the membrane potential is plotted, and the intercept of this line with the abscissa is taken as E_{Cl} (as explained above, $E_{Cl} \neq E_{GABA}$, but if bicarbonate is buffered then $E_{Cl} \approx E_{GABA}$). Whole cell patch clamp is the most straightforward electrophysiological

Table 2. ***The advantages and limitations of techniques for measuring intracellular chloride and transporter activity***

		Advantages	Limitations
		Imaging	
Dyes	SPQ MEQ MQAE	Relatively insensitive to bicarbonate concentration and pH variations; fast kinetics for chloride association	Experimental variability due to inconsistent dye loading; susceptibility to photobleaching; regular calibration required; not ratiometric
Genetically encoded Cl^- indicators	Clomeleon	For Clomeleon, Cl^- sensor: can be targeted to specific subcellular compartments or cell types; amenable to repeated measurements in vivo; more photostable than chemical dyes; ratiometric measurements can be performed with or without FRET; more robust estimates of the calibration parameters	The signal is pH dependent; lacks Cl^- affinity in the physiological range.
	Cl^- sensor	Relative to Clomeleon: improved Cl^- affinity in the physiological range, improved signal-to-noise ratio	The signal is pH dependent.
	SuperClomeleon	Relative to Clomeleon: improved Cl^- affinity in the physiological range, improved signal-to-noise ratio	The signal is pH dependent.
	ClopHensor	Permits simultaneous measurements of both $[Cl^-]_i$ and pH, which results in more accurate $[Cl^-]_i$ measurements	Technically challenging to use because of imaging requirements (e.g. 3 excitation wavelengths are required; laser light sources are preferable); relatively complex data analysis
		Electrophysiology	
Perforated patch-clamp recording		Provides a good estimate of $[Cl^-]_i$	Technically challenging (e.g. to obtain sufficient electrical access); labor intensive
Whole cell patch-clamp recording		The most straightforward electrophysiological technique for measuring E_{Cl}	Does not provide an accurate estimate of $[Cl^-]_i$ because the intracellular neuronal Cl^- dialyzes with the Cl^- in the intracellular patch pipette solution; labor intensive.
Whole cell Cl^- loading		Can be used to measure the somatodendritic Cl^- gradient and CCC extrusion capacity	Endogenous KCC2 activity can be altered by Cl^- loading, via activation of SPAK and OSR1 kinases; not suitable for measuring $[Cl^-]_i$; labor intensive.
		Flux assays	
$^{86}Rb^+$		High sensitivity and selectivity	Largely restricted for use in heterologous expression systems; low temporal and spatial resolution; requires the use of a radioactive isotope.
Tl^+		Does not require radioactivity.	Largely restricted for use in heterologous expression systems; low temporal and spatial resolution; produces complex changes in pH_i in response to application of NH_4^+.
NH_4^+		Does not require radioactivity.	Largely restricted for use in heterologous expression systems; low temporal and spatial resolution

CCC, cation-chloride cotransporter; $[Cl^-]_i$, intracellular Cl^- concentration; E_{Cl}, equilibrium potential for Cl^-; FRET, fluorescence resonance energy transfer; pH_i, intracellular pH.

technique for measuring $[Cl^-]_i$, but its effectiveness is limited by the fact that the intracellular neuronal Cl^- dialyzes with the intracellular patch pipette solution. Perforated patch-clamp recordings can be used to overcome this limitation, because gramicidin as the perforating agent forms pores in the neuronal membrane that are impermeable to Cl^- (284); however, this technique is inherently more challenging than the whole cell technique, in large part because of the time required and the challenge in obtaining sufficient electrical access. Another disadvantage of gramicidin perforated patch recordings is that they dialyze ions, which is an important consideration given that the Na^+ and K^+ gradients provide the energy for Cl^- transport via NKCC1 and KCC2, respectively.

Multiple flux assays are used to measure CCC activity, including those based on $^{86}Rb^+$ (285), Tl^+ (286), and NH_4^+ (286); however, they are largely restricted to use in heterologous expression systems, which limits their utility for understanding neuronal function. Transporter activity can also be assessed with whole cell patch-clamp electrophysiology by "loading" the cell with a known higher concentration of Cl^- from the pipette (132). Although both flux assays and Cl^- loading are currently used to assess CCC activity, neither is sufficient or satisfactory, and thus the development of additional technical tools for assessing neuronal Cl^- and Cl^- transporters is eagerly anticipated by neuroscientists.

8. INTRANEURONAL CHLORIDE GRADIENTS

Intracellular Cl^- homeostasis is essential for the maintenance of the strength and efficacy of $GABA_A$R-mediated inhibition. However, there are intraneuronal differences in $[Cl^-]_i$ and E_{GABA}, which are not well understood. Some lines of evidence point toward differential subcellular localization of CCCs as an explanation for the presence of intracellular Cl^- gradients. Indeed, Szabadics et al. (174) found that GABA-mediated excitation at the axon initial segment (AIS) is due to the absence of KCC2 expression at this compartment, where E_{GABA} is more depolarized than the resting membrane potential (RMP). Thus, GABA-transmitting axo-axonic cells that exclusively innervate the AIS of pyramidal cells evoke a depolarizing (and perhaps even excitatory) GABA-mediated response in this compartment. In contrast, somatic and dendritic $GABA_A$R-mediated responses are hyperpolarizing. This compartmentalized E_{GABA} has been shown to result in part from differential subcellular localization of KCC2; KCC2 density is significantly lower at the AIS compared with the soma, suggesting increased intracellular Cl^- at the AIS and a consequent depolarizing effect of GABA.

NKCC1 has also been shown to maintain the axo-somatic Cl^- gradient. Using gramicidin perforated patch recordings of dentate gyrus cells, Khirug et al. (106) found that wild-type mice exhibit an axo-somatic ΔE_{GABA} of ~5 mV, whereas this gradient is absent in NKCC1$^{-/-}$ mice. In contrast, the somato-dendritic ΔE_{GABA} is similar between wild-type and NKCC1$^{-/-}$ mice. This suggests that NKCC1 plays a role in maintaining the Cl^- gradient at the AIS that results in depolarizing GABA, while not significantly contributing to dendritic E_{GABA}.

In contrast to the CCC-mediated maintenance of intracellular Cl^- gradients, Glykys et al. (287) reported in 2014 that local impermeant anions establish the neuronal $[Cl^-]$. This hypothesis was supported by their observation that $[Cl^-]_i$ (visualized by 2-photon imaging of Clomeleon) was broadly distributed over a wide millimolar range that was incongruent with the $[Cl^-]_i$ equilibria of both NKCC1 and KCC2 and that CCC inhibition did not significantly alter $[Cl^-]_i$. Instead, the authors argued that $[Cl^-]_i$ was mediated by cytoplasmic impermeant anions ($[A]_i$) and polyanionic extracellular matrix glycoproteins ($[A]_o$); changes in $[A]_i$ and $[A]_o$ resulted in corresponding changes in $[Cl^-]_i$, dictated by osmotic and ionic gradients.

Voipio et al. (288), however, strongly disputed the conclusion that immobile anions can generate the driving force for Cl^- through $GABA_A$Rs. They highlighted that immobile charges cannot affect the electrical potential gradient for Cl^- current, as no energy is consumed by these immobile anions, thus making it thermodynamically impossible for immobile charges to maintain a Cl^- driving force. Glykys et al. (289) responded to this theoretical concern by outlining that the Cl^- driving force is not solely established by the Cl^- equilibrium potential (to which fixed anions contribute), i.e., E_{Cl}. The difference between the membrane potential (MP) and E_{Cl} is what creates the driving force: $DF = MP - E_{Cl}$. Luhmann et al. (290) further disputed the results, raising multiple concerns including the reliance on Clomeleon without more appropriate controls, the limited range for reliably reporting $[Cl^-]_i$, and the use of an additional technique to substantiate the results. Glykys et al. (289) explained that the change in fluorescence ratio per change in $[Cl^-]_i$ is sufficiently sensitive between 1 and 20 mM, and thus sufficiently sensitive for their hypothesis.

9. CCC-MEDIATED NEURONAL EXCITABILITY CONTRIBUTES TO NEUROLOGICAL DISORDERS

CCC dysfunction and aberrant neuronal Cl^- homeostasis play a causative role in numerous neurological disorders, including epilepsy (291), chronic pain (292),

schizophrenia (92, 293), autism (294), Rett syndrome (295–297), fragile X syndrome (92, 293), Down syndrome (DS) (298), and Huntington's disease (HD) (299, 300). The CCC dysfunction normally leads to an increase in neuronal $[Cl^-]_i$, often due to KCC2 dysfunction or reduced KCC2 expression, which renders GABA and/or glycine as excitatory neurotransmitters. This destabilizes the excitation-inhibition and leads to the phenotypes observed with these disorders.

9.1. Epilepsy

Epilepsy is a continuous and often progressive condition in which the brain circuitry becomes susceptible to spontaneous seizures (301). Although the underlying causes of epilepsy are diverse, risk factors for the development of the disease include genetic conditions, exposure to environmental risk factors, and brain injury (113, 302). The etiology of epilepsy is complex, with initiators of epileptic events including *1*) increased cellular excitability, *2*) reduced network inhibition, and *3*) network hypersynchronization. Regardless of the driver of the event, these periods of increased network activity can increase GABA neurotransmission, which leads to intracellular Cl^- accumulation; the net result of this increase in $[Cl^-]_i$ is a reduction in the strength of inhibition and sometimes a switch in the polarity of GABA from inhibitory to excitatory (7, 303–305). In addition, KCC2 mutations have been identified in human patients suffering from different types of epilepsy (113, 255, 305, 306) (**FIGURE 10**). Thus, there is a clear link between Cl^- dysregulation, CCCs, and this major neurological disorder. However, it remains unclear whether changes in the Cl^- gradient are causative of epilepsy or a consequential phenotype. Like many big medical mysteries, the answer likely lies somewhere in between.

Epilepsy research uses a plethora of methods for recapitulating epileptic events in vitro and in vivo, including regulating network excitability with conditions of low magnesium, high K^+, application of 4-aminopyrimidine (4-AP) (307, 308), or chemical induction paradigms including kainic acid, pilocarpine, and pentylenetetrazol (PTZ) to induce chronic seizures (309). More "physiological" strategies include the in vivo kindling model, where an electrode is implanted into a brain region and used to repeatedly induce regional seizures with electrical current (310, 311), and optogenetic methods using light to regulate the activity of excitatory or inhibitory neurons (312). These induction methods are used widely to mimic

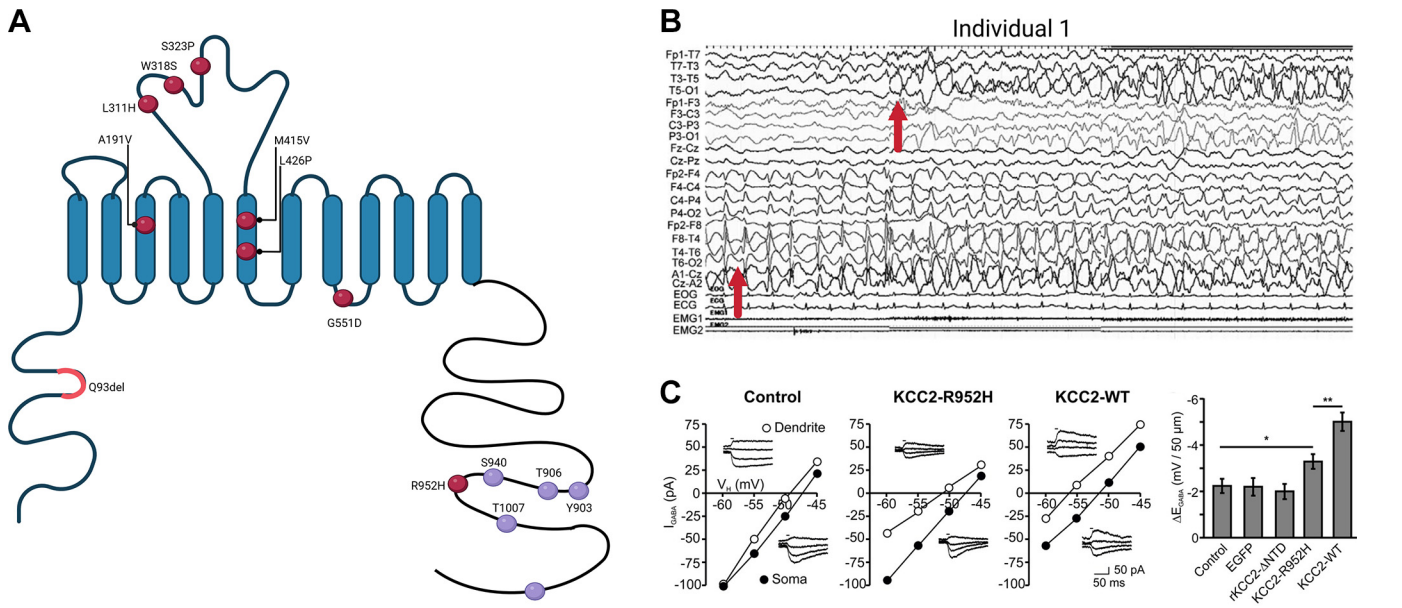

FIGURE 10. KCC2 in epilepsy. *A*: mutations in KCC2 identified in various models of epilepsy. Red points demonstrate point or deletion mutations identified in epilepsy. Purple points highlight KCC2 residues whose phosphorylation status variations have been implicated in epilepsy. *B*: ictal EEG of an individual with biallelic *SLC12A5* mutations. Spikes over the right frontal area emerged first (*bottom* arrow) with accompanying eye deviations to the left. Subsequent spikes over the left temporal area (*top* arrow) were accompanied by eye deviations to the right. Image modified from Ref. 306, with permission from *Scientific Reports*. *C, left*: whole cell patch-clamp GABA equilibrium potential (E_{GABA}) recordings and corresponding current-voltage (*I-V*) curves at the soma and dendrite of cortical layer 2/3 pyramidal neurons transfected with a cosegregating variant of *SLC12A5* (KCC2-R952H) implicated in febrile seizures, wild-type KCC2 (KCC2-WT), or nontransfected enhanced green fluorescent protein (EGFP)-negative control neurons (control). Neurons were somatically loaded with Cl^-. V_H, holding potential. *Right*: Cl^- extrusion capacity of pyramidal neurons expressing EGFP, KCC2-WT, KCC2-R952H, or a transport-deficient NH_2-terminally deleted KCC2 (rKCC2-ΔNTD) was quantified as the somatodendritic E_{GABA} gradient ($\Delta E_{GABA} = E_{GABA\text{-soma}} - E_{GABA\text{-dendrite}}$). The Cl^- extrusion capacity of KCC2-R952H neurons is significantly lower than that of KCC2-WT neurons. Image taken from Ref. 255, with permission from *Epilepsia*. Figure created with BioRender.com, with permission.

the events observed in a range of epileptic conditions; however, each method has different underlying mechanisms, which makes reconciling conflicting results challenging. When reconciling these results, it is important to differentiate between ictogenesis and epileptogenesis. Ictogenesis is a biochemical event that rapidly results in an electrical discharge of a brain circuit and is highly dependent on the movement of ions, whereas epileptogenesis is a long-term process occurring over months and years in which progressive anatomical circuit changes result in the development of epilepsy. Ictogenesis and epileptogenesis are mechanistically different, and therefore the development of therapies targeting these two disorders will diverge (313–315).

A potential link between CCCs and epilepsy has been intensively investigated since the discovery of KCC2, which quickly revealed a causative role in epilepsy-like phenotypes. Multiple research groups developed mouse lines expressing varying levels of KCC2 protein expression, which collectively revealed a strong correlation between the loss of KCC2 and hyperexcitability and the generation of spontaneous generalized seizures (316, 317). Mice expressing only 5% of KCC2 have severe phenotypes including the generation of spontaneous generalized seizures and do not survive past 3 wk postnatal development. Furthermore, the density of PV-positive interneurons in the hippocampus is significantly reduced in the homozygous KCC2 mutant mouse, likely because of the generalized seizure phenotype. The KCC2 "hypomorphic mouse" expresses 15–20% of the normal KCC2 compared with wild-type mice and exhibits significantly reduced threshold to PTZ-induced seizures (317). Thus, the loss of normal KCC2 function during development has been directly linked to the development of seizures and epileptic-like phenotypes.

Loss of KCC2 expression in adult animals and posttranslational regulation of KCC2 transport have also been identified in various models of epilepsy, including in mouse models of status epilepticus (SE), a state of continuous seizure in which brain circuits are altered at the biochemical and anatomical levels (318). The phosphorylation status of several KCC2 residues have been reported to be altered in SE, including S940 (300), T906/1007 (319), and Y903/1087 (62). Phosphorylation of KCC2-S940 increases transporter function and membrane expression (61), and thus this site is thought to be an efficient mechanism that could be hijacked to rapidly regulate KCC2 function during SE. Silayeva et. al. (320) investigated this prediction by injecting mice with the chemoconvulsant kainate and then subsequently producing acute brain slices to examine the impact on KCC2-S940 phosphorylation. The authors determined that KCC2 was rapidly dephosphorylated and internalized, resulting in less Cl^- extrusion, which consequently increased $[Cl^-]_i$. Furthermore, mice that were genetically modified to prevent S940 phosphorylation showed increased susceptibility and lethality in response to kainate injection (302).

Two additional KCC2 phosphorylation sites are also implicated in epilepsy: Y903/1087 and T906/1007. Using a model of SE induced with intraperitoneal injection of pilocarpine, researchers determined that the phosphorylation status of Y903/Y1087 is robustly increased, resulting in membrane KCC2 internalization and lysosomal degradation, which contributes to the hyperexcitable phenotype of epilepsy (62). The regulatory role of T906/1007 residues (117) in epilepsy was assessed with a genetically modified mouse expressing a nonphosphorylatable T906A/T1007A mutation. E_{GABA} measurements from these mutant mice were significantly hyperpolarized compared with wild-type mice, suggesting enhanced GABAergic transmission, and injection of kainate into wild-type and KCC2-T906A/T1007A mice showed that the nonphosphorylatable KCC2 mouse exhibited resilience against the induction of seizurelike activity and reduced death overall (319).

Global and cell type-specific NKCC1-knockout mice have also been used to study the role of Cl^- regulation in epilepsy. The global knockout NKCC1 mouse line has several deficits including deafness and balance perturbations (321, 322), as well as general increases in network activity around the time of the GABA switch (130). Knocking out NKCC1 specifically in hippocampal CA3 neurons resulted in increased intrinsic excitability of these neurons and increased susceptibility to seizure induction via 4-AP application compared with wild types. In addition to genetic strategies, injections of kainate (323) or pilocarpine (324, 325) in wild-type animals caused increased NKCC1 expression in the CNS, likely contributing to depolarizing GABAergic synaptic transmission and heightened network excitability. Moreover, although bumetanide holds the potential to be used as a therapeutic treatment for epilepsy (326, 327), several studies have demonstrated the inefficacy of this drug to rescue epileptogenesis (328, 329).

Alterations of other KCCs such as KCC3 can also contribute to seizures. KCC3-knockout mice had raised intraneuronal Cl^- concentration, especially in cell types that abundantly express KCC3, such as the cerebellar Purkinje cells. Even though spontaneous seizures were absent, these animals had reduced seizure threshold. Electrocorticograms from these animals also showed increased irregular electrical activity similar to those observed in patients with Andermann syndrome and certain forms of epilepsy (330). Therefore, even though the present review puts emphasis on KCC2 and NKCC1, the contributions of other CCCs in maintaining

intracellular Cl^- concentration and in the pathology of neurological disorders should not be neglected.

Altering the extracellular $[K^+]$ is an additional confounding factor in studying the relationship between CCCs, Cl^- regulation, and epilepsy. As the interneuron firing rate increases to synchronize network activity at the start of an epileptic event, the extracellular $[K^+]$ is elevated (81, 331), which can directly regulate the function of CCCs. For example, high extracellular $[K^+]$ can reverse the direction of KCC2 (7, 332, 333), and thus enhancing KCC2 function in these conditions may further contribute to the severity of the event instead of ameliorating the symptoms (334).

9.2. Neuropathic Pain, Neuronal Trauma, and Spinal Cord Injury

Neuronal trauma in the form of physical injury, nonphysiological osmolarity, and increased temperature reverses the polarity of GABAergic signaling in cultured hypothalamic neurons, resulting in excitatory GABAergic action in mature neurons (335). It was predicted that trauma from injury or inflammation may also underlie disrupted GABA/glycinergic inhibition in the spinal cord. Specifically, the lamina I neurons in the dorsal horn of the spinal cord contain inhibitory circuits that are essential for gating the sensory perception of pain. When these circuits become damaged and no longer exert normal inhibition, patients experience sharp pain sensations, tingling, pins and needles, and allodynia (336). Using an in vivo model of peripheral nerve injury (PNI), a seminal publication by Coull et al. (337) identified that there was significant shift in the Cl^- gradient after PNI in the neurons of the dorsal horn. The researchers modeled PNI by chronically constricting the sciatic nerve in a rat and then used electrophysiological patch-clamp recordings and calcium imaging to identify the site of dysregulation. After PNI, E_{GABA} was significantly depolarized compared with sham-injured animals because of loss of KCC2 expression in the lamina I neurons of the dorsal spinal cord; and this KCC2 downregulation was demonstrated to be the mechanism for this increased excitability.

The causative role of KCC2 in neuropathic pain transcends different models of neuropathic pain, including after spinal cord injury (SCI) (338) and trigeminal neuropathic pain (339). One commonly used model of SCI includes transecting the spinal cord at different levels and assessing inhibition in the impacted circuitry. Using a thoracic and lumbar transection, Boulenguez et al. (338) discovered a progressive loss of KCC2 protein expression in the ventral horn of the spinal cord below the level of the SCI, with no change in NKCC1 expression found. This loss of KCC2 is triggered by excess brain-derived neurotrophic factor (BDNF) expression, which, via TrkB receptors, acts to negatively regulate KCC2 protein expression. Moreover, in a model of trigeminal neuropathic pain, chronic nerve constriction resulted in a combined increase in NKCC1 and decrease in KCC2 in neurons located at the site of the injury (339), resulting in excitatory GABA, which could be reversed with the NKCC1 antagonist bumetanide (339). Additionally, WNK1 phosphorylation of KCC2 in the spinal cord is a mechanism in a model of spared nerve injury and inflammation that mimics neuropathic pain (237). These models induce depolarization of E_{GABA} in lamina II neurons of the spinal cord, which can be reversed in the presence of WNK1 inhibitor or in the $WNK1^{-/-}$ mouse after SNI (237).

Can the loss of inhibition in spinal cord circuits as a result of downregulated KCC2 be rescued? Answering this question could identify novel therapeutic treatment of neuropathic pain and even paralysis as a result of SCI. This question was recently explored when researchers promoted the function of the remaining KCC2 in the spinal cord or exogenously reexpressed KCC2 at the site of injury. The authors discovered that application of the KCC2 agonist CLP290 significantly improved functional recovery after SCI (340). Furthermore, the authors identified that the specific reexpression of KCC2 in interneurons expressing vesicular GABA transporter (VGAT) at the site of the lesion was sufficient to improve scores on stepping abilities (340). This finding provides a clear demonstration that rescuing KCC2 expression or function after injury is sufficient to restore the excitation-inhibition balance at the level of the spinal cord and can ultimately promote recovery after injury. It will be interesting to know whether a similar regulation of KCC2 in additional models of peripheral injury and pain could restore neuronal inhibition in the spinal cord and rescue the symptoms associated with neuropathic pain.

9.3. Neurodevelopmental Disorders

Some forms of autism spectrum disorder (ASD) and intellectual disability have been linked to aberrant Cl^- gradient regulation. For example, Rett syndrome is an X-linked disorder causing a severe form of autism (341) due to mutations in the transcription regulatory protein methyl-CpG binding protein 2 (MeCP2). Normal development occurs for approximately the first 18 mo of postnatal life, and then motor, respiratory, and cognitive deficits appear and epilepsy develops (342, 343). Although deficits in GABAergic inhibition have been proposed as a mechanism underlying the ASD phenotypes observed in Rett syndrome (344), the first publication to link CCCs came a few years later. In 2013 Duarte et al. (295) discovered that there was reduced KCC2 protein found in CSF of human patients with Rett syndrome. The

authors predicted that the corresponding reduction of BDNF expression in Rett patients (345) caused a downregulation of KCC2, and ultimately a reduction in overall GABAergic inhibition leading to epilepsy. Furthermore, the motor cortex, cerebellum, Brodmann area, and the hippocampus all showed reduced transcript expression of KCC2 (346, 347).

Several mouse models of Rett syndrome have been created, including global and regional knockout models, conditional knockout, and overexpression models (348). Conditional knockout models include the removal of MeCP2 expression in VGAT-expressing neurons, which models many of the symptoms observed in the human form of the disorder (344). A more recent publication conditionally removed MeCP2 from PV and somatostatin (SST) interneurons to reveal that interneuron subtypes contributed to the disorder in different capacities (349). Specifically, selective loss of MeCP2 in PV interneurons resulted in the development of the motor and social abnormalities associated with Rett syndrome, whereas the loss of MeCP2 in SST interneurons resulted in the development of epilepsy and stereotyped behaviors. To further support the theory that disrupted neuronal inhibition underlies many phenotypes associated with this disorder, KCC2 expression has been shown to be reduced in human induced pluripotent stem cells (iPSCs), and the reexpression or upregulation of the function of existing KCC2 in a mouse model of Rett syndrome can rescue some of the phenotypes in these model systems (296, 297). Tang et al. (297) used a high-throughput screen of repurposed molecules to identify new drugs that could increase KCC2 expression and function, of which two identified compounds ameliorated the respiratory and locomotor phenotypes observed in the MeCP2 mutant ($Mecp2^{-/y}$).

The most common cause of intellectual disability is Down syndrome (DS), resulting from triplicate copies of chromosome 21 leading to abnormal fetal development. The Ts65Dn mouse model is commonly used to investigate symptoms of intellectual disability, as this model recapitulates symptoms of DS (350). Interestingly, some publications identified enhanced GABAergic signaling in the hippocampus as a mechanism for disrupted hippocampal learning in this model, whereas others reported contradictory data. This argument was resolved when Deidda et al. (298) discovered that Ts65Dn mice did in fact display heightened $GABA_A$-mediated signaling, and because of increased NKCC1 expression and no change in total KCC2, the $[Cl^-]_i$ was elevated and GABA action was depolarizing instead of inhibitory. When NKCC1 was inhibited with bumetanide to reduce $[Cl^-]_i$, neuronal inhibition was restored and the synaptic and cognitive deficits in this model were ameliorated. This publication highlights the significant potential of regulating NKCC1 activity in models where aberrant Cl^- gradients are observed, thus further supporting the need for optimizing the current molecules to target NKCC1 expressed in the CNS as opposed to systemic regulation, an endeavor that is currently underway (351, 352).

Although the major focus of loss of inhibition via dysregulation of the CCCs has been on models of autism spectrum disorders and intellectual disability, these transporters are being widely investigated in almost all neurological conditions. For example, Cl^- dysregulation has been observed in human patients with major depressive disorder and schizophrenia. Investigation of postmortem brain tissues has identified altered NKCC1:KCC2 expression profiles in adults diagnosed with these conditions (92), with several forms of truncated KCC2 found in some case studies (293). Postmortem human prefrontal cortices from schizophrenia and affective mood disorders also exhibit decreased expression of KCC2 transcripts. These data were recently substantiated in a mouse model of schizophrenia, where the authors demonstrate that cortical NKCC1 knockdown or bumetanide administration could ameliorate the depolarizing actions of GABA and behavioral manifestations associated with schizophrenia (353). Similar findings demonstrating altered CCC function have been observed in genetic neurodegenerative disorders such as Huntington's disease (HD) (300) and Alzheimer's disease (AD) (354). A recent publication investigating the cognitive deficits associated with HD elegantly demonstrated a specific loss of KCC2 and an upregulation of NKCC1 within the hippocampal region. Ultimately, this altered NKCC1-to-KCC2 ratio results in net Cl^- accumulation in neurons of the hippocampus, causing reduced inhibition and cognitive deficits in mice with HD (299, 300). A similar finding was observed in a mouse model of AD in which the amyloid precursor protein (APP) was genetically removed to mimic conditions in AD where APP is cleaved and lost from the synapse (354). Using this model, researchers observed reduced KCC2 expression and compromised neuronal inhibition in the hippocampus, but the authors demonstrated that synaptic inhibition could be rescued with the KCC2 activator CLP290. Although multiple possibilities for disrupted GABAergic neurotransmission in neurological disorders exist, including abnormal GABA receptor localization or altered interneuron targeting, changes in NKCC1 and KCC2 are prominent and consistently discovered. For more detail on this topic, we refer the reader to recent review articles (355).

9.4. *Bumetanide as a Therapeutic Treatment for Neurological Disorders*

Benzodiazepines, which bind to the $GABA_A$Rs and allosterically enhance their responsiveness to GABA, are

commonly prescribed to treat neurological disorders such as epilepsy. However, benzodiazepines have paradoxical effects when used to treat ASD because of the depolarizing and/or excitatory actions of GABA. Therefore, the use of loop diuretics such as bumetanide that can reestablish the hyperpolarizing effect of GABA was proposed for clinical trials (356). Early pilot studies in affected children indicated that bumetanide was effective in improving multiple measures evaluating the severity of the disorder, including through the Childhood Autism Rating Scale (CARS) and Clinical Global Impressions (357, 358). In addition to significantly improving conventional behavioral and clinical symptoms with few negative side effects, Hadjikhani et al. (356) showed that bumetanide administration in seven affected individuals ranging from adolescents to young adults significantly improved activation of brain regions involved in emotional processing.

The reported improvement in associated symptoms with a favorable benefit-to-risk ratio (359) indicated that alterations in GABAergic signaling may underlie the etiology of autism and provided the rationale for large-scale randomized trials. A phase 2 clinical trial by Lemonnier et al. (358) in 60 autistic children (3–11 yr old) showed a reduction in associated symptoms following 3-mo administration of bumetanide. Similarly, a multicenter phase 2B trial by the same group in 88 ASD patients (2–18 yr old) also showed improvement in core symptoms of ASD following bumetanide administration (360). In another study by Zhang et al. (361) in 83 patients, 3-mo administration of bumetanide resulted in a significant reduction in the ratio of GABA and glutamate neurotransmitter concentrations, in addition to a previously reported reduction in symptom severity. Apart from being a stand-alone treatment, bumetanide also proved to be effective in reducing symptoms of autism when combined with other treatment methods, like applied behavior analysis (ABA), with no serious adverse effects (362). All of these trials also indicated that the frequency and incidence of side effects associated with bumetanide administration, including hypokalemia, dehydration, and loss of appetite, correlated with the dose of bumetanide (358, 360).

Contrary to previous trials, a single-center phase 2 trial by Sprengers et al. (363) showed that children (7–15 yr old) diagnosed with ASD did not show any improvement in Social Responsiveness Scale-2 (SRS-2) scale after the administration of bumetanide. The authors attributed the failure of this trial to the highly heterogeneous etiology of ASD, in which the reversed GABA polarity may not be the underlying cause in all ASD conditions (364). Currently, multiple phase 3 trials (365) (ClinicalTrials.gov Identifier NCT04766177, NCT03715153; EudraCT Number 2017–004420-30, 2017–004419-38) are evaluating the efficacy and safety of bumetanide in ASD, which provides promising prospects for its use in autism.

In addition to autism, the efficacy of bumetanide in treating various other neurological disorders is being clinically tested. In a pilot study examining adult patients with temporal lobe epilepsy (TLE), Eftekhari et al. (366) have found that bumetanide was efficient in reducing seizure frequency. Gharaylou et al. (367) reported that bumetanide administration downregulated NKCC1 protein levels in patients suffering from TLE and the reduction in NKCC1 levels may underlie the antiepileptic effect of bumetanide. Bumetanide was also found to be effective in reducing seizure burden in a multicenter, dose-escalation study when compared to a phenobarbital-treated control group, without increased serious side effects (368), but in another pilot study by Jullien et al. (369) bumetanide showed no effect in reducing seizure burden caused by hypoxic-ischemic encephalopathy, probably again because of the heterogeneity in etiology of seizures. The corresponding phase 1/2 trial (370) found that bumetanide did not improve seizure control in newborns with hypoxic-ischemic encephalopathy, although, importantly, this was an uncontrolled study. The efficacy of bumetanide in treating schizophrenia is debatable, with Rahmanzadeh et al. (371) reporting no significant improvement in the symptom severity, but bumetanide was found to be effective in reducing hallucinations in schizophrenic patients (372). Bumetanide was also found to be effective in treating neuropathic pain after spinal cord injury. The treatment also significantly increased the expression of KCC2 protein, indicating a role of GABAergic signaling in neuropathic pain (373).

10. CONCLUSIONS

Cl^- is arguably one of the most important neurophysiological ions, playing essential roles from neurodevelopment throughout the maturity of the CNS. Not only is Cl^- required for inhibitory synaptic inhibition in the mature nervous system, it is also a primary mechanism regulating neuronal excitability. The Cl^- gradients that permit these critical roles are generated and maintained by a variety of ion channels, exchangers, and cotransporters expressed in both neuronal and glial cells. In addition, Cl^- itself likely acts as a downstream signaling molecule used to initiate phosphorylation events to finely tune neuronal inhibition in response to changes in neuronal activity. Dynamically altering the Cl^- gradient provides a system to regulate the overall excitatory activity within a neuronal network, revealing the critical role of Cl^- regulators essential for the healthy brain.

Central to the maintenance of neuronal Cl^- gradients are the cotransporters NKCC1 and KCC2. Since their

discovery in the late 1990s, the structure, function, and expression profiles of these CCCs have been relatively well characterized, despite the relative lack of techniques available to directly measure cotransporter activity or Cl^- gradients. Advances in our understanding of CCC-mediated Cl^- regulation will be greatly facilitated by an enhanced technical toolbox for interrogating and regulating KCC2 and NKCC1 function and expression. These advances will identify new potential therapeutic treatments for the growing list of neurological disorders associated with CCC dysfunction. Given the expanded use of high-throughput screening strategies for the identification of KCC2-enhancing compounds, together with the development of next-generation Cl^- imaging tools, the future looks promising for the study of chloride transporters controlling neuronal excitability.

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AUTHOR CONTRIBUTIONS

M.A.W. interpreted results of experiments; J.C.P., M.d., V.A.R., and M.A.W. prepared figures; J.C.P., M.d., and M.A.W. drafted manuscript; J.C.P., V.A.R., and M.A.W. edited and revised manuscript; J.C.P. and M.A.W. approved final version of manuscript.

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