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Chloride transporters and GABA polarity in developmental, neurological and psychiatric conditions

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ABSTRACT

Neuronal chloride regulation is a determinant factor for the dynamic tuning of GABAergic inhibition during and beyond brain development. This regulation is mainly dependent on the two co-transporters K^+/Cl^- co-transporter KCC2 and $Na^+/K^+/Cl^-$ co-transporter NKCC1, whose activity can decrease or increase neuronal chloride concentrations respectively. Altered expression and/or activity of either of these co-transporters has been associated with a wide variety of brain disorders including developmental disorders, epilepsy, schizophrenia and stroke. Here, we review current knowledge on chloride transporter expression and activity regulation and highlight the intriguing potential for existing and future interventions to support chloride homeostasis across a wide range of mental disorders and neurological conditions.

1. Introduction

The principle component in shaping neural activity is the neuronal synapse, where electric signals from the presynaptic neuron are transmitted to the postsynaptic cell via specialized chemical signaling. At the synapse, activation of a presynaptic neuron causes the release of neurotransmitters into the synaptic cleft, which can subsequently activate target receptors on the postsynaptic membrane. Many postsynaptic receptors are ion channels whose activation alters permeability for specific ions resulting in a positive or negative change on the membrane potential of the postsynaptic neuron. For instance, glutamate receptors at excitatory synapses are permeable for sodium (Na^+) and potassium (K^+) ions inducing postsynaptic depolarization (towards the firing threshold). At inhibitory synapses, the neurotransmitter, γ-aminobutyric acid (GABA) activates chloride-permeable $GABA_A$ receptors. The direction of the resulting chloride (Cl^-) ion flow will depend on the intracellular chloride concentration ($[Cl^-]_i$) of the postsynaptic neuron. As we will review, the regulation of this intraneuronal chloride concentration through cation-chloride cotransporters is indeed essential for neural activity homeostasis in the brain. In the mature brain, $[Cl^-]_i$ is generally low and postsynaptic GABAergic signaling leads to chloride influx causing a postsynaptic hyperpolarizing effect (away from firing threshold). However, in certain pathophysiological conditions fine-tuning of $[Cl^-]_i$ can become dysregulated and affect GABAergic inhibition (Ben-Ari, 2017). An increasing body of evidence shows that altered chloride regulation is a mechanistic factor in a wide variety of neurological and psychiatric conditions (Doyon et al., 2016). It should be noted that, besides regulating chloride levels, neuronal cation-chloride cotransporters also regulate cell volume in the central nervous system (Glykys et al., 2017; Kahle et al., 2015) and spine morphology (Fiumelli et al., 2013; Gauvain et al., 2011; Li et al., 2007), which will not be discussed here.

In this review, we introduce the main chloride transporters in relation to GABA signaling and discuss their regulatory dynamics in general and in particular contexts such as in development. Following these accounts, we will review which of these mechanisms are known to be dysregulated in developmental, neurological, and psychiatric conditions. Finally, we discuss how chloride transporter dysregulations can serve as pharmacotherapeutic targets based upon existing or yet to be discovered agents.

2. Chloride concentration homeostasis in neuronal cells

In mature neurons, baseline levels of $[Cl^-]_i$ are maintained at a relatively low concentration (~5 mM), while the extracellular chloride level is typically

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around ~110 mM. The resulting chloride equilibrium potential is more negative than the membrane potential so that chloride currents will have a hyperpolarizing (inhibitory) effect on the membrane potential. The maintenance of the $[Cl^-]_i$ is performed by cation-chloride cotransporter proteins in the neuronal membrane.

The main chloride 'exporter' is the K^+/Cl^- co-transporter KCC2, which can extrude chloride from the neuron against its concentration gradient (Hübner et al., 2001; Rivera et al., 1999). The ATP-dependent K^+/Na^+-ATPase maintains the high intracellular K^+ concentration required for this shuttling process (Payne et al., 2003). KCC2 therefore behaves like a chloride pump, creating the driving force for chloride entry and postsynaptic membrane hyperpolarization upon GABA release (Blaesse et al., 2009; Chamma et al., 2012; Payne et al., 2003).

In opposite direction, the $Na^+/K^+/Cl^-$ co-transporter NKCC1 is regarded as the most active chloride 'importer'. This transporter can shuttle Cl^-, K^+ and Na^+ into the neuron, using the electrochemical gradient of Na^+. Together, KCC2 and NKCC1 are the two main transporters responsible for regulating $[Cl^-]_i$ and their relative activity controls the intraneuronal chloride level, which in turn determines the postsynaptic effect of GABAergic transmission. Under normal conditions, $[Cl^-]_i$ is sufficiently low, so that $GABA_A$ receptor activation leads to an increase in membrane conductance and a chloride flow into the neuron, causing relative hyperpolarization of the postsynaptic membrane potential. This decreases the probability that the postsynaptic neuron fires an action potential and is therefore referred to as the inhibitory effect of GABA signaling.

Repeated GABA signaling induces repeated chloride influx, which imposes an increasing chloride load onto the neuron (Doyon et al., 2016). Chloride entry in the neuron can therefore be enhanced if the postsynaptic cells are depolarized during sustained excitatory activity. With increasing levels of intracellular chloride, the electrochemical gradient becomes more reduced, which in turn decreases the inhibitory effect of GABA signaling on the postsynaptic neuron. As the reversal potential for GABA and the resting membrane potential are in relatively close proximity, relatively small increases in $[Cl^-]_i$ can shift the polarity of $GABA_A$ currents from hyperpolarizing to depolarizing, emphasizing the importance of maintaining a low $[Cl^-]_i$ (Raimondo et al., 2017). To recover from high chloride load and to maintain hyperpolarizing GABA effects, chloride ions are continuously extruded from the neuron by KCC2. This chloride clearance rate through KCC2 activity thus determines the recovery rate of the $[Cl^-]_i$ of a neuron after a period of intense GABA signaling. Small reductions in KCC2 activity already result in prolonged elevation of $[Cl^-]_i$ after GABAergic signaling and compromise the inhibitory capacity of GABAergic synapses. Under normal circumstances, however, KCC2-mediated chloride extrusion provides an efficient pathway in developed neurons for chloride recovery.

In striking contrast to mature neurons, $[Cl^-]_i$ levels are kept high in developing neurons, which results in an electrochemical gradient of chloride in opposite direction. In immature neurons, activation of $GABA_A$ receptors leatds a outward flow of chloride ions, causing a depolarization effect in the postsynaptic neuron (Ben-Ari et al., 1989; Sulis Sato et al., 2017). The depolarizing effect of GABAergic signaling in immature neurons is thought to be trophic/growth promoting and essential for the early establishment of neuronal circuits (Ben-Ari et al., 2007; Wang and Kriegstein, 2010). Although the effect of $GABA_A$ receptor activation in immature neurons is predominantly depolarizing, its effect on the neuronal network is not necessarily excitatory. A recent study in the neonatal neocortex demonstrated that GABA-mediated depolarization still imposes an overall inhibitory control on cortical network activity in vivo (Kirmse et al., 2015), presumably via shunting inhibition (Heigele et al., 2016).

The difference in chloride regulation between immature and mature neurons is predominantly caused by a difference in expression and activity of the main chloride transporters NKCC1 and KCC2. Levels of NKCC1 are high in early developmental phases, while the expression of KCC2 is kept low (Ben-ari, 2002). During neuronal maturation, KCC2 expression is upregulated and NKCC1 levels are reduced, resulting in lower levels of neuronal chloride (Yamada et al., 2004). This shift in chloride level occurs during the second postnatal weeks in rodents (Ben-Ari et al., 2012). In rat hippocampi, an increased KCC2 mRNA expression is observed after postnatal week 2 (Dzhala et al., 2005; Rivera et al., 1999), whereas a decrease of NKCC1 expression is observed between P14 and P21 (Dzhala et al., 2005). In comparison, in humans, KCC2 expression increases around postconceptional week (PCW) 40, whereas NKCC1 expression reaches adult levels around PCW 50 (Dzhala et al., 2005). These findings indicate big differences of expression patterns across species, which should be kept in mind when comparing studies using different models. The characteristic upregulation of KCC2 and downregulation of NKCC1 during neurodevelopment is often referred to as the excitatory-inhibitory GABA sequence. KCC2 upregulation is crucial for functional brain development to endow mature neurons with an ability to rapidly restore $[Cl^-]_i$ after loading. The onset of developmental upregulation of KCC2 has been linked to increased postsynaptic calcium signals, as a result of activation of $GABA_A$ receptors in immature neurons (Fiumelli et al., 2005; Ganguly et al., 2001). Disturbances in the excitatory-inhibitory GABA sequence have been implicated in developmental disorders, such as childhood epilepsy and autism spectrum disorder (ASD) (Ben-Ari et al., 2012; Merner et al., 2015) but the mechanistic underpinnings of the polarity shift in GABAergic signaling during development are not fully understood.

A loss of excitatory actions of GABA in NKCC1 knockout mice has been shown to induce a compensatory increase in the intrinsic excitability of glutamatergic neurons (Sipilä et al., 2009) and delayed maturation of the glutamatergic and GABAergic synapses (Pfeffer et al., 2009). The importance of KCC2 for neuronal chloride homeostasis and GABA function was demonstrated by genetic knockout of KCC2 in animal models (Hekmat-Scafe et al., 2006; Hübner et al., 2001; Khalilov et al., 2011; Rinehart et al., 2009; Tanis et al., 2009; Zhu et al., 2008, 2005) leading to elevated neuronal $[Cl^-]_i$, depolarization of the equilibrium potential of GABA (EGABA) and reduced inhibition. Indeed, KCC2-deficient mice exhibited frequent generalized seizures and died shortly after birth (Hübner et al., 2001; Woo et al., 2002), whereas mice that were heterozygous for KCC2 deletion displayed altered seizure threshold and increased seizure susceptibility to seizure-inducing agents (Zhu et al., 2008). The effects of KCC2 or NKCC1 deficiency are reminiscent of electrophysiological phenotypes seen in multiple models of neurological and psychiatric disease, including autism (He et al., 2014; Tyzio et al., 2014), Down's syndrome (Deidda et al., 2015), schizophrenia (Hyde et al., 2011), epilepsy (Kahle et al., 2015; Silayeva et al., 2015; Woo et al., 2002), and neuropathic pain (Chen et al., 2014; Coull et al., 2003; Cramer et al., 2008; Kahle et al., 2014a). These studies underpin the importance of NKCC1 and KCC2 regulation for the homeostasis of neuronal $[Cl^-]_i$ and appropriate function of GABA signaling. As we will outline below, chloride homeostasis implies the existence of sensing and responding mechanisms to enable a dynamic and rapid equilibrium maintenance.

3. Mechanisms of chloride regulation

3.1. Regulation of KCC2 expression

KCC2 is encoded by the solute carrier family 12 member 5 (SLC12A5) gene. There are two major isoforms of KCC2. In neonatal mouse central nervous system, KCC2a and KCC2b are present in similar (low) levels, whereas in the adult brain KCC2b is the major isoform (Uvarov et al., 2009). The isoforms are differently regulated by alternative promoter and first exon usage (Uvarov et al., 2007). KCC2 is exclusively expressed in neuronal cells, a phenomenon that is mainly ascribed to the presence of a neuron-restrictive silencer element (NRSE) in the first intron of the gene (Karadsheh and Delpire, 2001; Schoenherr and Anderson, 1995). However, a 1.4 kb promoter fragment is also involved in the neuron-restricted expression of KCC2 (Uvarov et al., 2005), as KCC2 expression was still restricted to neurons in a transgene model lacking the NRSE for KCC2 but with the 1.4 kb promoter fragment active. One of the transcription factors that can bind to the 1.4 kb promotor region of *SLC12A5* is early growth response 4 (Egr4), which can regulate the expression of KCC2. This was shown by Uvarov et al. (2006), who demonstrated that co-transfection of KCC2 and Egr4 in N2a cells resulted in enhanced expression of KCC2. A second important binding site in this promoter region is the E-box region, which can bind the upstream stimulating factors

(USF-) 1 and 2. Amyloid precursor protein (APP) has been shown to downregulate KCC2 expression while simultaneously downregulating USF-1, suggesting that by downregulating USF-1, APP decreases KCC2 expression (Doshina et al., 2017). At present, the functions of the other binding sites located in the promoter region of *SLC12A5* are unknown.

Brain-derived neurotrophic factor (BDNF) has also been indicated as a modulator of KCC2 expression, but with diametrical effects in immature versus mature neurons. In mature neurons, BDNF binds to the tyrosine kinase receptor B (TrkB), which results in a decreased expression of KCC2 mRNA (Rivera et al., 2002; Shulga et al., 2008) as well as a decrease of total KCC2 protein translation (Rivera et al., 2004) and surface expression (Wake et al., 2007). It was shown that the signaling molecules src homology domain containing transforming protein/FGF receptor substrate 2 (Shc/FRS-2) and phospholipase C γ (PLC-γ) are required downstream of the TrkB receptor activation by BDNF mediating this decrease in KCC2 protein (Rivera et al., 2004). In immature neurons, BDNF signaling results in a strong increase in KCC2b mRNA expression and protein levels via activation of the ERK1/2 pathway and the transcription factor Egr4 (Ludwig et al., 2011; Puskarjov et al., 2015). In addition, it was recently shown that the molecular precursor of BDNF, proBDNF, which is mostly abundant during development, inhibits KCC2 expression and its function via its receptor p75NTR, resulting increased seizure susceptibility (Riffault et al., 2018). These findings show that regulation of KCC2 expression depends on the maturational state of the neurons.

Little is known about the regulation of KCC2 mRNA translation, except for the alternative splicing that results in KCC2a and KCC2b (Uvarov et al., 2009). It was reported that microRNA-92 negatively affects KCC2 translation, reducing the amount of KCC2 protein (Barbato et al., 2010). Future research should elucidate other factors influencing the translation of KCC2 mRNA.

3.2. Regulation of KCC2 activity

An important activating phosphorylation site is serine-940 (S940), which is located on the intracellular C-terminal domain of KCC2 (Payne et al., 1996). S940 phosphorylation enhances KCC2 cell surface stability and activity (Lee et al., 2010, 2007). KCC2 is directly phosphorylated at S940 by protein kinase C (PKC) leading to an overall increase in transporter activity. PKC suppression resulted in a strong, significant decrease of S940 phosphorylation in cultured hippocampal neurons (Lee et al., 2007). Similarly, dephosphorylation of S940 has been linked to an activity-dependent reduction of KCC2 function through a decrease in transporter stability (Lee et al., 2010). Pathways influencing PKC activity can therefore indirectly regulate KCC2 activity and chloride homeostasis. For example, the neuropeptide oxytocin increases KCC2 activity and is involved in the control of GABAergic signaling, as knockout of the oxytocin receptor gene resulted in a delayed GABA shift in mice (Leonzino et al., 2016). This effect of oxytocin on KCC2 activity signaling is mediated by promoting S940 phosphorylation via PKC, as PKC-inhibitors neutralize the stimulating effect of oxytocin on KCC2 activity (Leonzino et al., 2016). In addition to increasing KCC2 activity, oxytocin signaling also increases the level of KCC2 expression in immature neurons. Both effects of oxytocin may be restricted to a specific time window during development. Mice lacking oxytocin signaling not only displayed a delayed GABA shift, but also showed a disrupted E/I balance due to reduced GABAergic inhibition in mature neurons (Leonzino et al., 2016).

Activation of *5-hydroxytryptamine type 2A* (5-HT2A) receptors by serotonin has been reported to also increase KCC2 activity, as serotonin application restored the cell membrane expression of KCC2 and restores endogenous inhibition in a spinal cord injury mouse model (Bos et al., 2013). As PKC inhibitors prevented the effect of 5-HT2A activation on KCC2 activity, it is suggested that this regulation is also PKC-dependent (Bos et al., 2013). Altogether, these results indicate that S940 phosphorylation is influenced by various pathways that ultimately activate PKC, and thus result in enhanced KCC2 activity.

KCC2 is enriched at excitatory synapses and its activity is directly regulated by activation of glutamate receptors. KCC2 surface expression is regulated by kainate receptors, through the formation of molecular complexes between kainate receptor subunit GluK2 and KCC2 (Mahadevan et al., 2014; Pressey et al., 2017). PKC-mediated phosphorylation of GluK2 enhances KCC2 activity. PKC can also phosphorylate KCC2 itself as a result of activation of group I metabotropic glutamate receptors (mGluRs), which elevate intracellular Ca^{2+} levels by inducing calcium release from internal stores. Since Ca^{2+} can activate PKC, group I mGluRs are indirectly involved in the PKC-dependent stimulation of KCC2 activity (Banke and Gegelashvili, 2008). Thus, activation of mGluR by enhanced levels of excitatory neurotransmitter glutamate can indirectly strengthen the inhibitory effect of GABAergic signaling by increasing KCC2 activity. This mechanism is suggested to play an important role in maintaining the local balance between excitatory and inhibitory signals (Banke and Gegelashvili, 2008). Interestingly, calcium influx via NMDA receptors is associated with a decrease in KCC2 activity through dephosphorylation of S940 by protein phosphatase 1 (PP1) (Lee et al., 2011). Moreover, it was shown that NMDA-receptor mediated calcium influx also results in cleavage of KCC2 by the calcium-activated protease calpain, strongly decreasing surface expression and therefore KCC2 activity (Puskarjov et al., 2012; Zhou et al., 2012). BDNF signaling may enhance calpain activity during synaptic plasticity (Zadran et al., 2010). The link between neuronal activity and KCC2 function may play an important role in neurological pathologies, in which enhanced NMDA signaling would lead to a decrease in KCC2 activity and an elevated $[Cl^-]_i$.

Besides PKC-dependent regulation and calpain-dependent cleavage, KCC2 activity can be influenced by additional factors. For example, transforming growth factor β2 (TGF-β2) enhances KCC2 activity and KCC2 levels on the cell surface in both immature and mature neurons (Roussa et al., 2016), which is achieved via PKC-independent phosphorylation and activation of cAMP-responsive-element-binding protein (CREB) (Fukushima et al., 2007; Roussa et al., 2016). The protein RAB11B, which is under transcriptional control of CREB and thus indirectly of TGF-β2, is required for the TGF-β2-dependent increase in KCC2 activity (Roussa et al., 2016). Insulin-like growth factor 1 (IGF-1) can also activate KCC2, as transient stimulation of IGF-1 receptors activates KCC2. This process is believed to involve the protein tyrosine kinase c-Src, as the addition of c-Src in cultured hippocampal neurons combined with IGF-1 increases KCC2 activity. Active tyrosine kinase was required for the regulation of KCC2 activity, as heat-inactivated c-Src was unable to support the effect of IGF-1 on KCC2 (Kelsch et al., 2001). In addition, APP has been found to be involved in the regulation of KCC2 activity in mature neurons by stabilization of KCC2 on the cell membrane. APP acts by directly binding to KCC2, which blocks the phosphorylation of tyrosine residues, which normally promotes internalization and ultimately lysosomal degradation of KCC2 (Chen et al., 2017). All these pathways described above enhance KCC2 protein activity and facilitate chloride extrusion.

In parallel to the upregulating signaling pathways, there are also factors that are known to downregulate KCC2 activity. There are two threonine residues, T906 and T1007, on the intracellular C-terminal domain of KCC2 that strongly decrease KCC2 activity when phosphorylated (Rinehart et al., 2009). One of the pathways involved in the phosphorylation of KCC2, is the pathway consisting of kinases with-no-lysine kinase (WNK) and SPS1-related proline/alanine-rich kinases (SPAK) or the SPAK homolog oxidative stress-responsive kinase 1 (OSR1). WNKs phosphorylate and activate SPAK/OSR1, which in turn bind to the CCCs at their amino terminus and phosphorylate Thr or Ser residues at the carboxy terminus of the KCCs. The protein initiating this signaling cascade, WNK, directly binds to chloride, which prevents autophosphorylation of WNK and thus activation of WNK itself (Piala et al., 2014). Importantly, the activity of WNK is coupled to the $[Cl^-]_i$. If the concentration is high, WNK is inactive and thus unable to inhibit KCC2 activity. When $[Cl^-]_i$ concentration falls below a certain threshold, the WNK-SPAK/OSR1 pathway will be activated, and KCC2 activity will be reduced. Via this mechanism, the WNK-SPAK/OSR1 pathway adjusts the chloride extrusion rate to the variable chloride load via regulation of KCC2 phosphorylation. A recent study showed that enhancement of $GABA_A$ receptor-mediated currents increases KCC2 surface expression and confinement in the membrane is mediated by T906 and T1007 phosphorylation via the WNK-SPAK/OSR1 pathway (Heubl et al., 2017). This indicates a direct effect of increased chloride influx on KCC2 activity, in turn maintaining chloride

concentrations within physiological limits. SPAK has been shown to specifically decrease KCC2a activity. In contrast to KCCb, KCC2a has a binding site present for and is able to bind SPAK, which decreases KCC2a activity (Markkanen et al., 2017).

Phosphorylation of tyrosine residues in KCC2 can also reduce its activity. There are two tyrosine residues located on the intracellular C-terminus of KCC2, Y903 and Y1087, which increase KCC2 internalization and ultimately lysosomal degradation when phosphorylated (Lee et al., 2010). The exact pathways involved in phosphorylation of these residues are yet to be discovered. It was shown that direct binding of APP to KCC2 prevents phosphorylation of these residues and thereby its internalization effects (Chen et al., 2017). Phosphorylation of Y1087 has also been suggested to increase KCC2 activity, as an Y1087D mutation shifted the GABA reversal potential to more depolarizing values, a process associated with a more uniform distribution of KCC2 over the plasma membrane (Watanabe et al., 2009). The loss of KCC2 clustering at synapses is suggested to be a form of rapid regulation of KCC2 activity at the plasma membrane, which is modulated by neuronal activity (Chamma et al., 2013). To summarize, KCC2 expression and its activity levels are dynamically regulated by multiple molecular pathways in both directions.

3.3. Regulation of NKCC1 expression

The regulation of neuronal NKCC1 has been far less investigated compared to KCC2. The important characteristic of NKCC1 is that its expression is highly dynamic during neuronal maturation (Ben-Ari et al., 2007). In the early stages of brain development, elevated expression level of NKCC1 results in high $[Cl^-]_i$. However, during maturation of the brain, the developmental dynamics of NKCC1 expression are dependent on the brain region (Watanabe and Fukuda, 2015). The precise mechanisms underlying the developmental changes in NKCC1 expression are currently unknown.

3.4. Regulation of NKCC1 activity

The N-terminus of the NKCC1 protein, a highly-conserved region across species, comprises three threonine residues, T212, T217 and T230 (Darman and Forbush, 2002; Flemmer et al., 2002). Phosphorylation of these threonine residues greatly determines the activity of NKCC1. Especially the phosphorylation on T217 is essential for NKCC1 activation, whereas the other two residues have a more modulating role (Darman and Forbush, 2002). The chloride dependent WNK-SPAK/OSR1 pathway involved in KCC2 activity regulation also mediates regulation of NKCC1 activity. With a specific antibody against phosphorylated T212 and T217, WNK3 activation has been shown to enhance NKCC1 phosphorylation on these threonine residues, thereby promoting NKCC1 activity (Kahle et al., 2005). The protein phosphatase PP1 is involved in dephosphorylation of NKCC1, resulting in inactivation of the co-transporter (Gagnon and Delpire, 2010). These findings further emphasize the importance of the WNK-SPAK/OSR1 pathway as one of the major pathways involved in regulating the $[Cl^-]_i$. The activation of this pathway increases import of chloride by enhancing NKCC1 and reducing KCC2 activity, whereas inactivation of the pathway stimulates chloride export by suppressing NKCC1 and enhancing KCC2 activation.

Additional factors that regulate NKCC1 activity are estradiol and PI-3-kinase/Akt. Estradiol enhances NKCC1 activity by phosphorylation of T212 and T217 (Perrot-Sinal et al., 2007). Knockdown of SPAK and OSR1 prevents this increase in NKCC1 phosphorylation (Nugent et al., 2012). Furthermore, phosphorylation of NKCC1 is regulated by Akt via WNK3 in glioblastoma cells (Garzon-Muvdi et al., 2012).

These findings on KCC2 and NKCC1 regulation show that a delicate, sensitive and reciprocal system is in place for fine tuning and maintenance of cellular chloride homeostasis. Fig. 1 summarizes the main regulatory components as reviewed above.

The cotransporter activity regulatory system has been referred to as a molecular “rheostat” (Kahle and Delpire, 2016). The coupling of chloride sensitive regulatory kinases with chloride transporters would comprise a system that senses changes in $[Cl^-]_i$, and transduces this signal to the transporter activity to modulate GABA neurotransmission and mediate the developmental excitatory-inhibitory GABA sequence (Kahle and Delpire, 2016). Specific disruptions of this chloride ‘rheostat’ may be involved in neurological and psychiatric disorders.

4. Chloride transporter (dys)regulation in neurodevelopmental, psychiatric and neurological disorders

As discussed in the previous section, disturbances in the pathways regulating chloride transporters in development or at later stages can affect neuronal functioning. For this reason, we review available evidence for involvement of specific and more common disruptions of chloride transporter systems across neurological and psychiatric brain disorders.

4.1. Epilepsy

An involvement of KCC2 and NKCC1 mechanisms in the pathology of various epilepsy syndromes has been indicated through multiple human tissue studies as well as animal model studies. First and foremost, KCC2 knockout mice display enhanced seizure susceptibility and epileptiform changes (Hübner et al., 2001; Woo et al., 2002). Conversely, animal models of various epilepsies report that reductions in KCC2 function, by decreasing the efficacy of hyperpolarizing inhibition, can increase excitability and seizure susceptibility. In addition, changes in NKCC1 expression have also been identified in various types of epilepsy in animal model and human specimen (Di Cristo et al., 2018). For instance, elevated NKCC1 protein levels in temporal lobe epilepsy (TLE) have been found in surviving neurons of human hippocampal sclerotic tissue as compared to unaffected contralateral hippocampus tissue (Sen et al., 2007). In hippocampal slices obtained from TLE patients, interictal activity as a result of depolarizing $GABA_A$ signaling was observed in a subset of neurons (Cohen et al., 2002; Köhling et al., 1998). A follow-up study showed that this depolarization was related to KCC2 downregulation (Huberfeld et al., 2007). Combining in situ hybridization results with interictal-like activity measurement, it was detected that multiple KCC2 mRNA negative subicular pyramidal cells in TLE patient tissue displayed depolarizations instead of hyperpolarizations during epileptiform bursts, which suggests altered chloride homeostasis in these neurons. Consistent with this notion, several studies reported that intracellular chloride accumulation and consequent depolarizing currents can sustain epileptiform after discharges and/or seizure activity (Alfonsa et al., 2015; Fujiwara-Tsukamoto et al., 2003; Krishnan and Bazhenov, 2011). Indeed, it is important to realize that $[Cl^-]_i$ will accumulate after prolonged seizures (Dzhala et al., 2010; Huberfeld et al., 2015). In turn, this may enhance depolarizing GABA signaling by further increasing $[Cl^-]_i$, which may lower seizure threshold and alter anticonvulsant efficacy as part of a cataclysmic set of mechanisms referred to as “seizures beget seizures” (Ben-Ari, 2006). In addition, exaggerated chloride entry into cells through NKCC1/KCC2 dysregulation is associated with water movement that results in an increase in both cellular volume and $[Cl^-]_i$ unless compensatory mechanisms are activated (Glykys et al., 2017).

In the lithium-pilocarpine induced status epilepticus mouse model, the expression of NKCC1 and KCC2 is also changed. In this model, NKCC1 mRNA and protein levels were found to be significantly upregulated 1 day, 14 days and 45 days after pilocarpine injection. KCC2 mRNA and protein levels on the other hand were significantly downregulated at these time points (Li et al., 2008). In drug-resistant TLE patients, increased calpain expression has been reported (Feng et al., 2011), suggesting an increase in calpain-mediated KCC2 degradation (Puskarjov et al., 2012) in these patients. These results further suggest that altered chloride regulation may contribute differentially to different types of epilepsy or neuronal hyperexcitability.

Genetic mutations affecting NKCC1/KCC2 activity have also been implicated in epilepsy syndromes. A loss-of-function mutation in *SLC12A5* was reported in an Australian family with familial febrile seizures with a mutation in the highly-conserved region of the C-terminus of KCC2 (R952H). This mutation resulted in a decreased KCC2 expression on the surface membrane of a mouse neural stem cell line and thus in a decline in the chloride extrusion

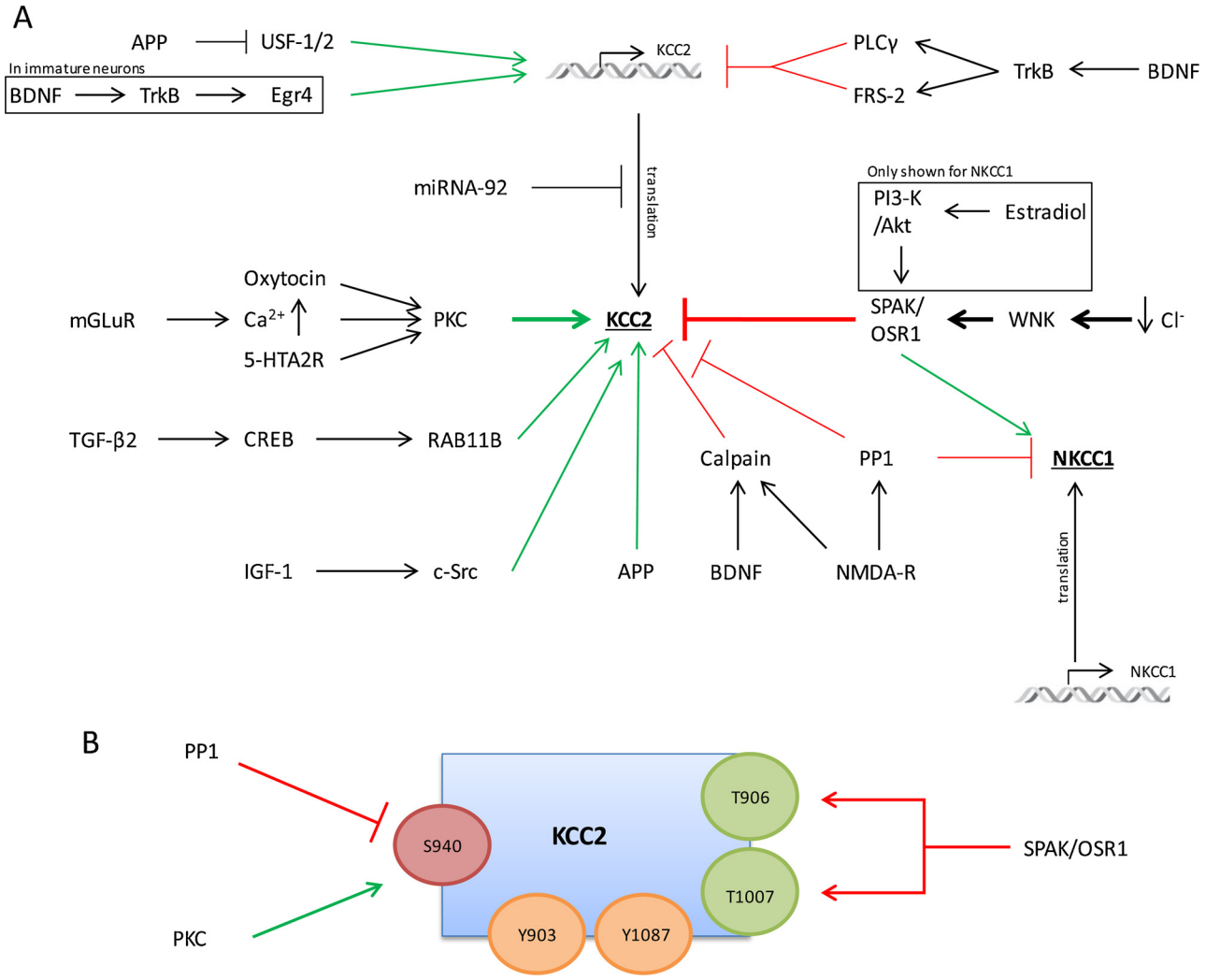

Fig. 1. Regulatory pathways involved in KCC2 and NKCC1 activity.
A) KCC2 and NKCC1 activity can be increased (green) and decreased (red) by multiple pathways in the mature neuron. Regulatory importance of the pathway is displayed by the weight of the arrow. B) Phosphorylation regulation of KCC2. Arrows indicate phosphorylation, blocked lines dephosphorylation. The effect of the interaction can be increasing (green) or decreasing (red) KCC2 activity.

capacity, as well as compromised formation of dendritic spines (Puskarjov et al., 2014). Such a decrease in spine formation is expected to compromise synaptic connectivity in the network, which then might also induce aberrant activity patterns (Kaila et al., 2014; Netoff and Schiff, 2002). The R952H mutation is also associated with idiopathic generalized epilepsy, similar to the R1049C mutation. Both KCC2 variants have been shown to decrease the extrusion of chloride, through a reduction of the above described S940 phosphorylation activation of KCC2 (Kahle et al., 2014b). A homozygous missense mutation in *SLC12A5* (L311H), located in the extracellular loop of KCC2, has been shown to result in a loss of KCC2 functionality and is also related to epilepsy of infancy with migrating focal seizures (Stödberg et al., 2015). In addition, L426P and G551D mutations in the transmembrane and intracellular domain, respectively, resulted in a reduced KCC2 expression at the cell surface and reduced protein glycosylation, both contributing to a loss of KCC2 activity (Stödberg et al., 2015).

The commonplace suggestion of increased excitability through enhanced NKCC1 or reduced KCC2 expression does not fit with all results. A recent review focussing on the role of KCC2 in epilepsy has summarized conflicting reports in the field as to the direction of KCC2 alterations and the consequent effects on GABAergic polarity (Di Cristo et al., 2018). Effects of seizures on KCC2 expression/function, and vice versa, the contribution of KCC2 alterations to the epileptic condition are age- and cell type dependent. Furthermore, the direction and extent of disease-related KCC2 alterations have been shown to be dependent on brain region, neuronal population and and/or subcellular compartments. It is also becoming clear that the consequences of alterations KCC2 in terms of pro- or anti-convulsive effect may depend on the stage of disease progression and on the state of cortical activity, e.g. during interictal or seizures (Di Cristo et al., 2018).

4.2. Schizophrenia

Schizophrenia is associated with hallucinations and delusions, in addition to impairments of cognitive functioning with sometimes devastating consequences (Elvevag et al., 2000). Notably, the cognitive deficits in schizophrenia have been associated with altered synchronization of neuronal oscillations (Uhlhaas et al., 2008) and changes in GABAergic neurotransmission (Gonzalez-Burgos and Lewis, 2008; Hashimoto et al., 2008).

Several post-mortem studies have investigated alterations in KCC2 and/or NKCC1 functionality. A study by Hyde et al., 2011 reported a 27% decrease in KCC2 mRNA in the hippocampus of schizophrenia patients, compared to controls, while the expression of NKCC1 in the hippocampus was not altered. This increased NKCC1/KCC2 ratio may indicate altered GABA signaling in hippocampal networks (Hyde et al., 2011; Mikawa et al., 2002). However, in the dorsolateral prefrontal cortex, a brain area of interest in schizophrenia research, NKCC1 and KCC2 expression levels were found unchanged (Arion

and Lewis, 2011; Hyde et al., 2011). In another study, the mRNA expression levels of two of the main regulators of NKCC1 and KCC2, WNK3 and OSR1 were upregulated in patients with schizophrenia (Arion and Lewis, 2011) suggesting reduced chloride extrusion activity and possibly altered GABAergic neurotransmission (Arion and Lewis, 2011).

Involvement of chloride transporters dysregulation in the pathology of schizophrenia has also been suggested from genetic variation in NKCC1 and KCC2. A coding variant in the KCC2 C-terminal regulatory domain R952H, has previously been associated with idiopathic generalized epilepsy (Kahle et al., 2014b) and familial febrile seizures (Puskarjov et al., 2014) have also been linked to schizophrenia (Merner et al., 2015). Variants in *SLC12A2*, the gene encoding for NKCC1, have also been associated with schizophrenia (Merner et al., 2016). A gain-of-function missense mutation, Y199C, is located in the important N-terminal regulatory domain, directly next to the crucial phospho-regulatory domain (T202/T207/T212) of NKCC1. Based on its locations, the authors hypothesize that this mutation enhances phosphorylation of NKCC1 by WNK-SPAK/OSR1 kinases, thereby increasing NKCC1 activity (Merner et al., 2016). Together, the loss-of-function mutation of KCC2 and gain-of-function mutations of NKCC1imply a role for altered chloride regulation in the pathology of schizophrenia.

By combining both neuroimaging and genetic data, a correlation was found between SNPs in *SLC12A2* and lower fMRI signal in the dorsolateral prefrontal cortex during a working memory task in schizophrenia patients as compared to healthy controls (Potkin et al., 2009), further implicating a genetic association of NKCC1 with cognitive symptoms of schizophrenia. In addition, a study performed in mice demonstrated a relation between the protein kinase SPAK, the NKCC1/KCC2 ratio and declarative memory function (Yang et al., 2015). SPAK knockout mice not only exhibited enhanced novelty exploration, generally used to determine declarative memory function in mental disorders, but also showed increased KCC2 expression, and thus a decreased NKCC1/KCC2 ratio in the PFC. Given that an increased NKCC1/KCC2 ratio might be involved in the pathogenesis of schizophrenia (Hyde et al., 2011), alterations of SPAK expression might provide a novel therapeutic strategy for schizophrenia patients. This suggestion is further supported by the associated increase in cognitive performance after SPAK knockout (Yang et al., 2015).

4.3. Autism spectrum disorder

Autism spectrum disorder comprises a highly heterogeneous group of neurodevelopmental disorders, characterized by deficits in the social interaction domain, restricted interests and repetitive patterns of behavior (Cellot and Cherubini, 2014). Although the precise mechanisms underlying the pathology of ASD are yet unknown, compelling evidence from both animal ASD models and human subjects indicates that deficits in GABAergic signaling, may contribute to the symptoms found in ASD patients (Cellot and Cherubini, 2014). This hypothesis is supported by the relatively high incidence of epilepsy in patients suffering from ASD as well as the high frequency of epileptiform activity observed in the electroencephalogram (EEG) of ASD patients (Robertson et al., 2015). Moreover, electrophysiological and behavioral ASD-like characteristics were induced in mice after blockade of maternal oxytocin signaling, which is important for the excitatory-to-inhibitory shift of GABAergic signaling in the offspring (Tyzio et al., 2006).

Studies are now beginning to investigate the involvement of NKKC1 and KCC2 in the pathology of ASD. The mutations in the C-terminal regulatory domain of KCC2 (R952H and R1049C) that were mentioned above have also been associated with ASD using a targeted sequencing approach (Merner et al., 2015). Furthermore, rare KCC2 variants that affect CpG sites were found in ASD patients, suggesting epigenetic dysregulation of KCC2 (Merner et al., 2015).

Several models of genetic disorders strongly associated with ASD have been linked to alterations in NKCC1/KCC2 regulation. One study demonstrated a significant decrease in KCC2 in the cerebrospinal fluid of patients suffering from Rett syndrome, while the expression of NKCC1 was found to be unaffected (Duarte et al., 2013). Confirming a possible functional interaction between MeCP2 and GABAergic signaling, MeCP2-deficiency in GABAergic neurons resulted in multiple behavioral features characteristic of Rett syndrome in mice (Chao et al., 2010). Furthermore, MeCP2 deficient mice exhibited alterations neural excitability (Dani et al., 2005), and deficits in BDNF expression (Wang et al., 2006). Alterations in GABA polarity have also been indicated in other genetic animal models of ASD. In mice with Fragile X syndrome (FRX) mutation and rats exposed *in utero* to valproate, the GABA shift is significantly delayed (He et al., 2014). Oral treatment with the selective NKCC1 antagonist bumetanide in pregnant VPA rats or FRX mice one day before delivery did not only restore the effects of elevated $[Cl^-]_i$ and excitatory GABA signaling at birth in the offspring, but also restored naïve behavior, respectively (Tyzio et al., 2014). Down syndrome (DS) is another example of a genetic disorder associated with ASD that has been associated with chloride dysregulation. A recent study found excitatory instead of inhibitory effects of GABA signaling in the hippocampus of adult DS mice. NKCC1 expression was found increased and bumetanide restored synaptic plasticity and hippocampus-dependent memory in these animals (Deidda et al., 2015). Finally, cellular and animal model studies of Tuberous Sclerosis Complex (TSC), a severe genetic disorder strongly associated with ASD and epilepsy, have also indicated alterations in KCC2/NKCC1 expression in and around tuber tissue (Ruffolo et al., 2016; Talos et al., 2012).

4.4. Ischemic stroke

Stroke is currently one of the major causes of death and disability worldwide and is mainly caused by the interruption of the blood flow to the brain, referred to as ischemia. The sudden depletion of oxygen and glucose results in irreversible neuronal death due to reduced ATP levels and an ionic imbalance across the neuronal cell membrane. The neuronal damage because of an ischemic stroke can cause severe impairments in stroke survivors.

Various lines of research have recently shown alterations in NKCC1 and KCC2 expression in the context of ischemic stroke. KCC2 mRNA and protein levels were downregulated in rats after experimentally induced by occlusion of the middle cerebral artery for 30 min and 120 min, leading to mild and severe injury respectively (Jaenisch et al., 2010). On the other hand, NKCC1 is upregulated in cortical neurons, as well as in lysates from cerebral and striatum samples in a rat model of focal cerebral ischemia/reperfusion injury (2-h middle cerebral artery occlusion and 24-h reperfusion) (Wang et al., 2014; Yan et al., 2003). It has been proposed that the observed NKCC1 upregulation activates the Raf/MEK/ERK cascade, CREB phosphorylation and hypoxia inducible factor 1α (HIF-1α), leading to stimulation of VEGF expression and ultimately to induction of neurogenesis (Lu et al., 2015). Additionally, upstream regulation of NKCC1 activity by the WNK3-SPAK/OSR1 pathway also contributes to the pathology of ischemic stroke, as WNK3 knockout mice exhibited decreased infarct volume and axonal demyelination, less cerebral edema, and accelerated behavioral recovery after middle cerebral artery occlusion, compared to WNK3 wild-type mice (Begum et al., 2015). Indeed, the absence of WNK3 was associated with decreased phosphorylation of NKCC1 as well as decreased levels of NKCC1 on the neuronal cell membrane (Begum et al., 2015). These findings might indicate new therapeutic strategies to improve outcome of stroke by targeting NKCC1 or regulatory proteins WNK3 and SPAK/OSR1.

4.5. Therapeutic agents influencing chloride transporters

Limitations of targeting the GABA system directly (e.g., with benzodiazepines or barbiturates) have been increasingly noted in the treatment of many brain disorders. Furthermore, paradoxical effects in response to GABA enforcing treatment have been observed in patients with developmental disorders that may point to dysregulated chloride and reversed GABA polarity (Bruining et al., 2015; van Tuijl et al., 2017). So far, the most used agent to treat chloride dysregulation at present is bumetanide, a selective antagonist of NKCC1 (Ben-Ari, 2017). This drug is already FDA approved as a safe loop diuretic with a mild adverse effect profile, which greatly facilitates the investigation of its effect in neurological disorders.

Application of bumetanide has shown conflicting results in epilepsy. Neonatal seizures have been most extensively studied in this respect. Indeed,

the neonatal brain is at high risk of developing seizures with the largest number of new-onset seizures is reported in the first few years of life (Cowan, 2002). Relative high $[Cl^-]_i$ in neonate neurons due to NKCC1 being still active may be responsible for a low seizure threshold (Dzhala et al., 2005). This notion has evoked studies into the application of bumetanide to this group of patients (Ben-Ari, 2017). Short-term prevention of epileptiform activity was established in vitro and in neonatal rats (Dzhala et al., 2005; Nardou et al., 2009). Application of bumetanide to hippocampal slices successfully suppresses epileptiform activity in vitro and attenuates electrographic seizures in neonatal rats (Dzhala et al., 2005). This was confirmed by another study, showing that application of bumetanide prevents the generation of spontaneous epileptic activity in neonatal hippocampal neurons in rats (Nardou et al., 2009).

Bumetanide was also able to reduce glutamatergic recurrent mossy fiber sprouting in the dentate gyrus of pilocarpine-treated rats (Kourdougli et al., 2017), which has been implicated in the formation of epileptiform bursts in both TLE patients and animal models (Golarai et al., 2001; Sutula et al., 1989; Tauck and Nadler, 1985). Indeed, the ability of bumetanide to constrain reactive glutamatergic network rewiring in adult epilepsy may strengthen its therapeutic potential and suggests that bumetanide has potential to prevent the recurrence of seizures. This prevention of seizures only succeeded after the epileptic focus had been established and that seizure prevention required continuous treatment. Bumetanide did not prevent the generation of seizures after a kainic acid (KA) injection, or the spreading to the healthy neighboring brain tissue (Nardou et al., 2009). However, increased the latency to the onset of epileptiform activity and decreased the average duration of epileptiform events in KA injected mice, compared to vehicle-treated controls (Sivakumaran and Maguire, 2016). In addition, bumetanide may also prevent pharmacoresistance in vivo, as KA injected mice resistant to the seizure-suppressing effects of the benzodiazepine diazepam showed reduced ictal activity when treated with both bumetanide and diazepam, compared to vehicle, only bumetanide, or only diazepam treatment (Sivakumaran and Maguire, 2016). Another study using Kv7 current-deficient mice, which develop an epileptic phenotype, has shown that treatment with bumetanide between P0 and P14 prevents structural and behavioral pathology and normalizes network activity in these mice (Marguet et al., 2015). The timing of bumetanide administration seems crucial, as treatment from P0 to P14 did not impair neurocognitive development (Marguet et al., 2015), whereas chronic bumetanide treatment (from E15 to P14) was far less favorable due to adverse side effects (Wang & Kriegstein, 2010).

To translate the effects of bumetanide from rodents to humans, a clinical phase I/II trial has been performed in the treatment of neonatal seizures caused by hypoxic ischemic encephalopathy (HIE), brain injury caused by oxygen deprivation to the brain (Pressler et al., 2015). Unfortunately, this trial was prematurely halted, as there was insufficient evidence for seizure reduction through the combination of bumetanide with phenobarbital, and an increased risk of hearing loss was detected (Pressler et al., 2015). These outcomes have been debated and it was argued that bumetanide had not been used as a primary agent to treat the neonatal seizures and that the effect of bumetanide as monotherapy for seizure reduction had not been tested (Ben-Ari, 2017; Pressler et al., 2015).

Bumetanide treatment has also been tested in the treatment of several neuropsychiatric disorders, most notably ASD. The accumulating evidence for chloride deregulation found in autism models (see above) has fueled the testing of bumetanide in human ASD (Hadjikhani et al., 2015; Lemonnier et al., 2013, 2017,2012; Lemonnier and Ben-Ari, 2010). Following a pilot study (Lemonnier and Ben-Ari, 2010), the group of Ben-Ari conducted two consecutive placebo controlled randomized trails testing bumetanide (1 mg/day for three months) in 60 and 90 patients respectively. Both trials showed a significant reduction in their primary endpoints (Lemonnier et al., 2017). Bumetanide administration in an adolescent with a typical FRX deletion reduced the severity of the syndrome (Lemonnier et al., 2013). The diuretic has also been show to improve recognition of emotions on both clinical and functional neuroimaging levels in seven males with ASD (Hadjikhani et al., 2015). In another study, bumetanide restored the EEG power spectrum changes after six months of bumetanide treatment of a 10-year old girl with ASD selected for treatment because of a previous paradoxical response to GABA enforcing drugs (Bruining et al., 2015). Another EEG-assisted case report showed significant improvement of both behavior and novel EEG markers relating to E/I ratios in an adolescent with ASD in the context of TSC (Vlaskamp et al., 2017). Together, these results show promise for bumetanide to ameliorate ASD symptomatology in subsets of ASD patients.

Bumetanide has also been tested in patients with schizophrenia. One case report showed effect of long-term treatment on hallucinations in a patient with schizophrenia (Lemonnier et al., 2016). However, a small double-blind randomized trial failed to show effect of bumetanide in patients with schizophrenia (Rahmanzadeh et al., 2017). Perhaps (genetic) stratification is warranted. Indeed, delayed GABA shift has been shown in a cellular model of the 22q11.2 microdeletion syndrome (22q11.2 DS) which is accompanied with strong risk of developing schizophrenia (Amin et al., 2017).

In animal stroke models, inhibition of NKCC1 by bumetanide administration before and during stroke induction resulted in reduced edema, reduced infarction volume and ischemic necrotic cell death, especially in the early stage of ischemic damage and promoted neurogenesis as well as increase sensorimotor recovery (Wang et al., 2014; Xu et al., 2017; Yan et al., 2003). In a rat model for hypoxia-ischemia brain damage induced during the neonatal period, treatment with bumetanide reduced the susceptibility of epilepsy induced by pentylenetetrazol (Hu et al., 2017). Bumetanide has also been shown to reduce seizure frequency in mice with traumatic brain injury (Wang et al., 2017). These results suggest that bumetanide may have a neuroprotective function in stroke and hypoxia-ischemia. It is also suggested that bumetanide can be used to reduce the effects of ischemic stroke, since bumetanide treatment might attenuate brain edema and infarct volume (Glykys et al., 2017; Yan et al., 2001).

Although bumetanide has potential to treat neurological disorders, it has disadvantageous pharmacokinetic properties that prevent it to efficiently cross the blood-brain barrier (BBB) (Römermann et al., 2017; Töllner et al., 2014). A possible way to increase its passage over the BBB is to mask the hydrophilic carboxyl group, something which is already being tested (Erker et al., 2016; Töllner et al., 2014) with promising results (Erker et al., 2016). It must also be considered that treatment with bumetanide does not turn GABAergic signals hyperpolarizing, but instead increases shunting inhibition, as it only decreases NKCC1 activity, but leaves KCC2 activity unaffected (Staley, 2006). Furthermore, an interesting aspect is that blocking NKCC1 has been shown to cause hyperpolarization of EGABA in fast-spiking interneurons but not principal neurons, suggesting that NKCC1 might be expressed at higher levels and/or be more active in GABAergic cells (Martina et al., 2001) and lead to so-called disinhibition of circuit output (Nelson and Valakh, 2015).

Therapeutics targeting other components involved in chloride regulation, e.g. KCC2, have gained increasing interest. A possible novel agent is IGF-1, which decreases the NKCC1/KCC2 expression ratio in developing neurons (Baroncelli et al., 2017) by upregulating the expression on KCC2 (Kelsch et al., 2001). The effect of IGF-1 on the hyperexcitability of the brain is suggested to be similar to bumetanide (Bou Khalil, 2017), although their molecular mechanisms differ. The increase in KCC2 activity by IGF-1 can likely lower chloride levels in affected neurons, increasing the effectiveness of inhibitory signals. Research has shown that a downstream target of IGF-1 is BDNF in both neonatal and mature brain (Carro et al., 2000; Landi et al., 2009). As previously mentioned, the effect of BDNF on chloride regulation is dependent on the maturational state of neurons, and as IGF-1 acts via BDNF, it is suggested that the effect of IGF-1 on chloride regulation is also dependent on the maturational state of the neurons. This needs to be considered when determining the therapeutic potential of IGF-1.

In a study by Gagnon et al. (2013), a high-throughput screening of 92,500 drug-like compounds was performed to assess their ability to reduce intracellular chloride. This resulted in the finding of CLP257, a compound that lowered $[Cl\text{-}]_i$, presumably by increasing KCC2 activity (Gagnon et al., 2013). However, it was recently demonstrated that the effects of CLP257 on chloride levels are actually independent of KCC2 (Cardarelli et al., 2017). Further research on CLP257 is required to investigate its exact mechanism of action and its therapeutic potential, which ultimately may open treatment possibilities for diseases associated with chloride defects. Another target to

potentially increase the activity of KCC2 is the 5-hydroxytryptamine type 2 A (5-HT_{2A}) receptor, which when stimulated by serotonin increases both KCC2 activity and neuronal membrane expression (Bos et al., 2013). Selective serotonin reuptake inhibitors (SSRIs), which are already clinically used, can increase the extracellular serotonin concentration and thereby stimulate the 5-HT_{2A} receptor. Further research is required to investigate the potency of SSRIs for the treatment of disorders associated with KCC2 dysfunction.

The WNK-SPAK/OSR1 pathway constitutes a promising target for successful treatment of chloride dysregulation. The WNK-SPAK/OSR1 kinase system might represent a particularly effective mechanism to be pharmacologically exploited to restore GABAergic inhibition. Coincident inhibition of NKCC1-mediated chloride loading and activation of KCC2-mediated chloride extrusion via WNK-SPAK kinase inhibition has been put forward as an approach (Kahle et al., 2014b). A balanced correction of NKCC1/KCC2 activity could not only affect neuronal excitability directly but would also influence the efficacy of endogenous GABAergic inhibition, Moreover, it has been argued that since WNK kinases might act as chloride sensors (Piala et al., 2014), correcting WNK-SPAK kinase inhibition might circumvent feedback mechanisms that occur for instance when NKCC1 or KCC2 are targeted independently (Kahle and Delpire, 2016). Such an approach might be an elegant method of restoring GABAergic inhibition for a wide range of disorders.

Another possible therapeutic strategy is preventing maladaptive increases in KCC2 S940 dephosphorylation. Seizures affect GABA function of corticotropin-releasing hormone, which acts on the hypothalamic-pituitary-adrenal (HPA) axis (O'Toole et al., 2014). This has raised the possibility that antagonizing KCC2 S940 dephosphorylation may counter the hyperexcitability of the HPA axis in affective disorders like anxiety, major depression, and posttraumatic stress disorder. These are fascinating questions worthy of future investigation. These findings may also be relevant for seizure ontogeny; as mutations in human KCC2 at Arg952 and/or Arg1049 have been shown to disrupt the phosphorylation of KCC2 at S940 and have also been associated with idiopathic generalized epilepsy (Kahle et al., 2014b).

4.6. Final remarks

We have reviewed principles of the fascinating dynamic and flexible system of neuronal chloride concentration regulation in the brain. The study of its mechanisms is a relative new field for clinical neuroscience with great potential to unlock maladaptive brain states. Notably, we reviewed that several of the disruptions in expression, translation and/or regulation of NKLCC1/KCC2 cotransporters (Fig. 1) have been indicated across different brain disorders (summarized in Fig. 2).

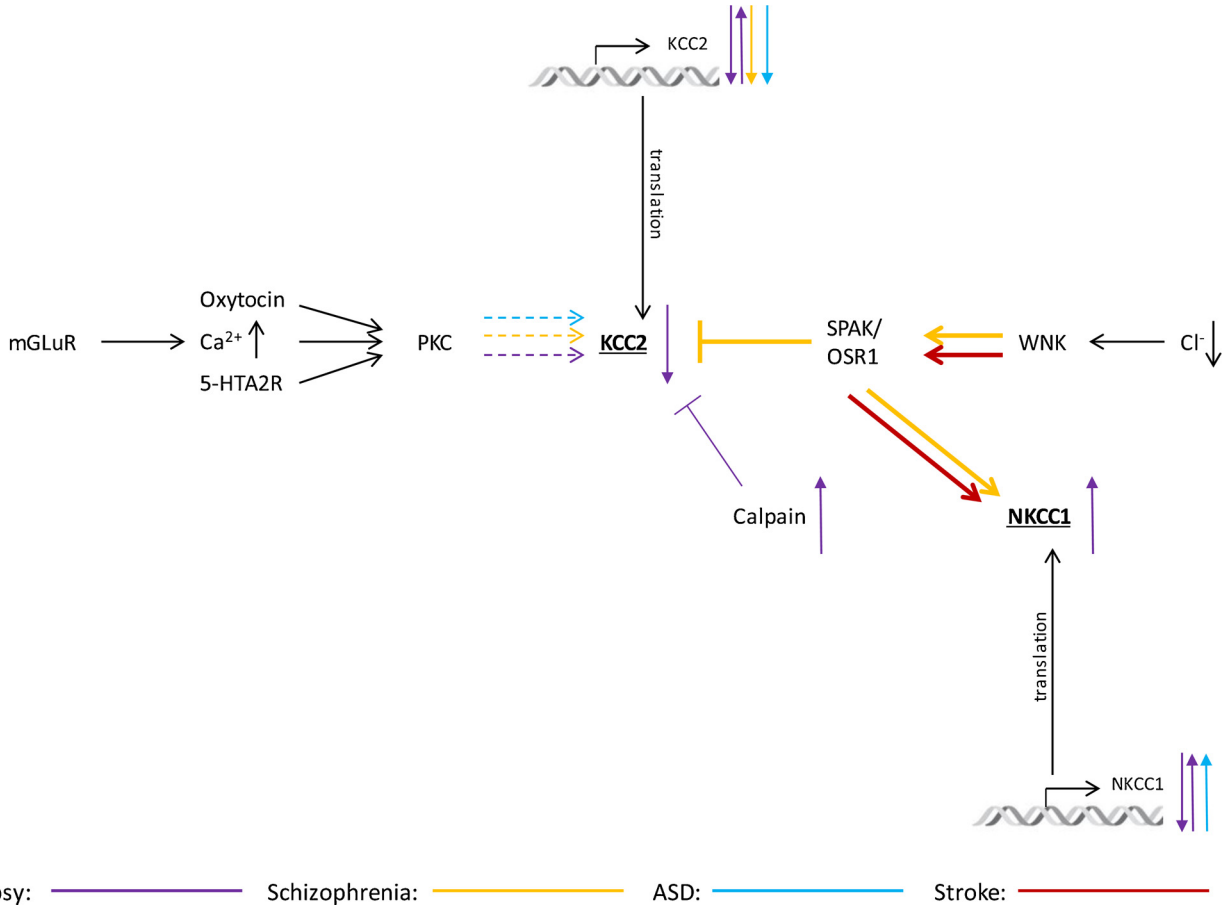

Fig. 2. Clinical associations of chloride cotransporter dysregulation.
Colored arrows correspond to disorders in which transporter mRNA expression levels, protein levels and/or designated regulatory pathways have been shown to be altered. Enhanced effect designated by continuous line, decreased effect by dashed line. Summarized findings are from animal model as well as human studies.

The activity of the main cotransporters NKCC1 and KCC2 seems closely related and partially these proteins act as a “yin-and-yang” balancing act to ensure chloride homeostasis regulation. We have highlighted that the (patho)physiological impact of NKCC1 and KCC2 cannot simply be estimated from its overall expression levels and that various posttranslational modifications and other regulatory elements also determine the chloride gradient. For now, the most investigated treatment targeting chloride deregulation is bumetanide for ASD (Bruining et al., 2015; Hadjikhani et al., 2015; Lemonnier et al., 2017, 2013, 2012; Lemonnier and Ben-Ari, 2010). The complexity of chloride regulation strongly suggests that other and perhaps more fine-grained strategies may be needed to correct chloride homeostasis and that these are well worth investigating. Their successful application will depend on many factors such as the developmental window and state of disease progression. This review supports the growing notion that disruption of chloride homeostasis constitutes a final common pathway in a wide range of brain disorders and mental conditions.

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