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TOPICAL REVIEW

Regulation of neuronal chloride homeostasis by neuromodulators

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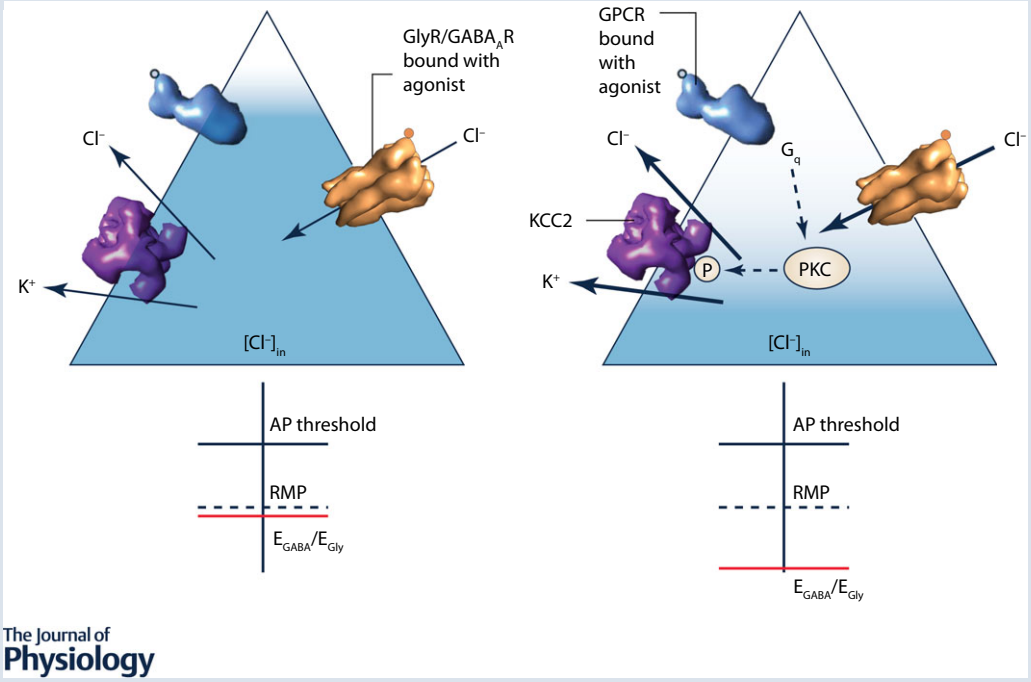

Abstract KCC2 is the central regulator of neuronal Cl^- homeostasis, and is critical for enabling strong hyperpolarizing synaptic inhibition in the mature brain. KCC2 hypofunction results in decreased inhibition and increased network hyperexcitability that underlies numerous disease states including epilepsy, neuropathic pain and neuropsychiatric disorders. The current holy grail of KCC2 biology is to identify how we can rescue KCC2 hypofunction in order to restore physiological levels of synaptic inhibition and neuronal network activity. It is becoming increasingly clear that diverse cellular signals regulate KCC2 surface expression and function including neurotransmitters and neuromodulators. In the present review we explore the existing evidence that G-protein-coupled receptor (GPCR) signalling can regulate KCC2 activity in numerous regions

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of the nervous system including the hypothalamus, hippocampus and spinal cord. We present key evidence from the literature suggesting that GPCR signalling is a conserved mechanism for regulating chloride homeostasis. This evidence includes: (1) the activation of group 1 metabotropic glutamate receptors and metabotropic Zn^{2+} receptors strengthens GABAergic inhibition in CA3 pyramidal neurons through a regulation of KCC2; (2) activation of the 5-hydroxytryptamine type 2A serotonin receptors upregulates KCC2 cell surface expression and function, restores endogenous inhibition in motoneurons, and reduces spasticity in rats; and (3) activation of A3A-type adenosine receptors rescues KCC2 dysfunction and reverses allodynia in a model of neuropathic pain. We propose that GPCR-signals are novel endogenous Cl^- extrusion enhancers that may regulate KCC2 function.

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Abstract figure legend Left, KCC2 sets the neuronal Cl^- gradient by extruding Cl^- from the neuron. This relatively low level of intracellular Cl^- results in the reversal potential for GABA (E_{GABA}) sitting hyperpolarized with respect to the resting membrane potential (RMP). Right, activation of G-protein-coupled receptors (GPCRs) can lead to activation of PKC, which can phosphorylate KCC2, resulting in increased Cl^- extrusion. As a result of this GPCR-mediated regulation of KCC2, E_{GABA} can hyperpolarize further with respect to RMP, resulting in increased GABA or glycinergic inhibition. Homology-modelled structure of the human KCC2 (hKCC2, purple), crystal structures of human adrenergic GPCR (PDB 2RH1, blue) (Cherezov *et al.* 2007), and human glycine receptor-α3 (PDB 5CFB, brown) (Huang *et al.* 2015) are used in this cartoon. Chimera 1.10.2 was used for molecular graphics rendering.

Abbreviations 5-HT, 5-hydroxytryptamine; 5-HT_2R, 5-HT type 2A serotonin receptor; A3AR, adenosine A3 receptor; AC, adenylyl cyclase; ASD, autism spectrum disorders; $[Ca^{2+}]_i$, intracellular calcium concentration; cAMP, cyclic adenosine 3′,5′-monophosphate; CCI, constriction injury; CCC, cation–chloride co-transporter; $[Cl^-]_i$, intracellular chloride concentration; DRG, dorsal root ganglion; E_{GABA}, equilibrium potential of GABAergic currents; E–I, excitation and inhibition; GABA, γ-aminobutyric acid; GPCR, G protein-coupled receptor; G proteins, guanine nucleotide-binding proteins; HCN, hyperpolarization-activated cyclic nucleotide-gated; KCC2, K^+–Cl^- cotransporter 2; mAChR, muscarinic acetylcholine receptor; mGluR, metabotropic glutamate receptor; mZnRs, metabotropic Zn^{2+} receptor; NKCC1, Na^+–K^+–$2Cl^-$ cotransporter 1; OXT, oxytocin; OXTR, oxytocin receptor; PKC, Ca^{2+}-dependent protein kinase C; PLC, phospholipase C; SCI, spinal cord injury.

Introduction

γ-Aminobutyric acid (GABA) acts primarily as an inhibitory neurotransmitter in the healthy mature brain. However, unlike glutamate, which maintains its function as an excitatory neurotransmitter throughout its lifetime, there is a change in the function of GABA across development and during pathophysiological states (Ben-ari *et al.* 2007). GABA is an excitatory neurotransmitter during embryonic development, and an inhibitory neurotransmitter in the adult brain. However, during neurological disorders including epilepsy, neuropathic pain, neurodevelopment and psychiatric disorders, hyperpolarizing inhibition is lost and GABAergic transmission can be depolarizing and even excitatory (Kaila *et al.* 2014).

The dynamic nature of GABAergic transmission originates from changes in the gradient for chloride (Cl^-), its most permeant ion (Kaila *et al.* 2014). The neuronal Cl^- gradient is primarily established and maintained by members of the secondarily active *SLC12A* family of cation–chloride cotransporters (CCCs). The Na^+–K^+–$2Cl^-$ cotransporter NKCC1 is the dominant Cl^- transporter during development and transports Cl^- into the neuron, thereby maintaining a relatively high level of intracellular Cl^-. While most CCCs are abundantly expressed in non-neuronal volume-regulatory cells, the evolutionarily conserved K^+–Cl^- cotransporter 2 (KCC2; *SLC12A5*) is the only CCC family member exclusively present in CNS neurons. KCC2 is a secondarily active cotransporter that uses energy from the K^+ gradient to extrude Cl^- from neurons; it is constitutively active in neurons under isotonic conditions, which results in a relatively low intracellular Cl^- concentration. Upon activation of the $GABA_A$ receptor ($GABA_A$R) Cl^- flows down its electrochemical gradient into the neuron, and this influx of negative charge results in membrane hyperpolarization.

The concentration of intracellular Cl^- in neurons was once through to be static; however, we now realize that neuronal Cl^- homeostasis is highly dynamic due to the multifaceted regulation of KCC2. Not only is

KCC2 significantly upregulated during ontogeny, but in mature neurons KCC2 is dynamically altered in response to both physiological and pathophysiological levels of activity. Coincident pre- and postsynaptic physiological activity reduces KCC2 function, which weakens synaptic inhibition and facilitates disinhibition-mediated long term potentiation (Woodin *et al.* 2003; Ormond & Woodin, 2009, 2011). In contrast, pathophysiological activity associated with epilepsy, neuropathic pain, and neurodevelopmental disorders all involve a breakdown of KCC2-mediated Cl^- homeostasis (Coull *et al.* 2003; Tao *et al.* 2012; He *et al.* 2014; Hübner, 2014; Tyzio *et al.* 2014). Therefore it is of paramount importance to understand the critical regulators of KCC2 function in depth, with the goal of discovering how KCC2 function can be maintained or restored in the face of pathophysiological perturbations. Several excellent reviews have been recently published that comprehensively examine cellular KCC2 regulation (Chamma *et al.* 2012; Kahle *et al.* 2013; Kaila *et al.* 2014; Medina, 2014). A central theme in these reviews is the important role of Ca^{2+}-dependent protein kinase C (PKC)-mediated phosphorylation of KCC2. PKC belongs to a group of kinases that phosphorylate serine and threonine residues on a variety of proteins and therefore regulate numerous cellular responses. While there are numerous PKC-regulatory sites in KCC2, the S940 site is now established as the predominant PKC-phosphorylation site in neurons (Lee *et al.* 2007, 2011; Kahle *et al.* 2013; Silayeva *et al.* 2015). Thus, a compelling avenue for developing therapeutic treatments to enhance KCC2 function is to explore strategies for regulating neuronal PKC signalling.

In this review we provide the first cohesive summary of published evidence that G protein-coupled receptor (GPCR) activation is a key that opens the door to PKC-mediated KCC2 regulation. After briefly reviewing GPCRs, we first provide evidence that GPCR-mediated intracellular signalling can modulate CCCs in the hippocampus and cortex, and in models of neuropathic pain and neuropsychiatric disorders. Next we examine whether all G_q-GPCR signalling augments KCC2 function, and consider what KCC2 pharmacology may reveal about GPCR modulation of KCC2 function. Lastly we examine the physiological effects of GPCR-mediated neuromodulation of KCC2 function, and speculate that opioids, which act via a G_q-class of opioid GPCRs, may increase KCC2 function in dorsal root ganglion (DRG) neurons, and that this mechanism might account for opioid-induced hyperalgesia and tolerance.

GPCR modulation: a brief overview

GPCRs are a diverse family of proteins and represent the largest class of gene products targeted by therapeutic agents. Comprising ∼1000 genes encoding for seven-transmembrane receptor proteins, GPCRs are the largest superfamily of cell surface receptors (Pierce *et al.* 2002). The brain expresses about 90% of all known GPCRs (Vassilatis *et al.* 2003; Premont & Gainetdinov, 2007), and the majority of them recognize diverse physiological, signals such as hormones, neurotransmitters, ions, peptides, lipids and cellular metabolites, and convert them into a variety of meaningful cellular signals. Briefly, ligand binding to the extracellular portion of GPCRs structurally alters their intracellular domain allowing them to function as guanine nucleotide exchange factors for heterotrimeric guanine nucleotide-binding proteins (G proteins). Activated GPCRs subsequently mediate the transfer of GDP to GTP binding to activate the G protein. Regardless of the diversity of their activation, GPCRs connect to a group of heterotrimeric G proteins that are functionally segregated into four broad classes: G_s, G_i, G_q and G_{12}. When activated by GPCRs, these G proteins in turn regulate a relatively small number of effector molecules that determine the final cellular effect. For example, activation of G_s-coupled GPCRs results in stimulating adenylyl cyclase (AC), and a subsequent cascade of cAMP-dependent protein kinase A (PKA)-mediated cellular functions. In contrast, $G_{i/o}$-coupled GPCRs can inhibit AC activation and cAMP-dependent cellular functions. Activation of G_q-coupled GPCR results in stimulating phospholipase C (PLC)-β, a subsequent increase in $[Ca^{2+}]_i$ from intracellular stores, and a cascade of PKC-dependent cellular functions. Finally, G_{12}-coupled GPCR activation leads to increased function of several RhoGEFs resulting in cytoskeletal changes. Based on the important role of PKC in regulating KCC2 function, this review focuses on the G_q-coupled GPCR signalling that activates PKC (summarized in Table 1).

GPCR modulation of CCCs in the hippocampus and cortex: metabotropic glutamate receptors. Among the first GPCRs shown to positively regulate KCC2 function were the group I metabotropic glutamate receptors (mGluR1 and mGluR5). Banke & Gegelashvili (2008) discovered that tonic activation of group I mGluRs strengthens GABAergic inhibition in CA3 pyramidal neurons, through a regulation of KCC2. Pharmacological activation of group I mGluRs using the agonist (*RS*)-3,5-dihydroxyphenylglycine (DHPG) hyperpolarizes E_{GABA} (a common measure of KCC2 function in neurons), and this effect is sensitive to antagonists of mGluR1 (N-phenyl-7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxamide), mGluR5 (2-methyl-6-(phenylethylnyl)pyridine hydrochloride), Ca^{2+}-dependent PKC ($G_{Ö}6976$) and KCC2 (furosemide).

How does the tonic activation of group I mGluRs increase KCC2 function? Group I mGluRs can activate

Table 1. KCC2/NKCC1 regulation by GPCR signalling

Receptor type	Regulation of KCC2/NKCC1 function	Neuronal type	NKCC1 involvement?	Reference
mGluRI/mZnR (GPR39)	• Group I mGluR agonist DHPG (20 μM) hyperpolarizes E_{GABA} • Extracellular Zn^{2+} hyperpolarizes E_{GABA}, measured by NH_4^+ and Cl^-flux assays • Effect abolished by $G_{\alpha q}$ and ERK1/2 inhibitors, and in GPR39$^{-/-}$ neurons	• Hippocampal CA3 and cortical neurons	• Not abolished by NKCC1 inhibition with bumetanide	• Banke *et al.* 2008 • Chorin *et al.* 2011 • Saadi *et al.* 2012 • Gilad *et al.* 2015
	• Group I and II mGluR agonist *trans*-ACPD (10 μM) increases K^+ influx	• Cortical neurons	• Abolished by bumetanide indicating NKCC1 involvement	• Schomberg *et al.* 2001
$5HT_{2A}R$	• $5HT_{2A}$ agonist TCB-2 (0.1–10 μM) rescues Cl^- homeostasis defects in SCI model by hyperpolarizing EIPSP • Effect prevented by inhibiting KCC2 and PKC	• Spinal motoneurons	• NKCC1 inhibition not tested	• Bos *et al.* 2013
A_3AR	• A3AR agonist MRS5698 rescues Cl^-homeostasis defects in CCI model by increasing neuronal Cl^- transport	• Lamina II dorsal horn neurons	• NKCC1 inhibition not tested	• Ford *et al.* 2015
	• Broad spectrum adenosine receptor antagonist caffeine (10 mM) depolarizes E_{GABA}	• Hippocampal neurons	• NKCC1 inhibition not tested	• Fiumelli *et al.* 2005
OXTR	• Maternal oxytocin signalling shifts GABAergic transmission to inhibitory • OXTR antagonist SSR126768A elevates excitatory actions of GABA	• Hippocampal CA3 pyramidal neurons	• NKCC1 inhibition with bumetanide occludes SSR126768A-mediated excitatory GABA, indicating NKCC1 involvement	• Tyzio *et al.* 2006 • Tyzio *et al.* 2014
α_1-Adrenergic receptor	• α_1-AR agonist phenylephrine (100 μM) depolarizes E_{GABA}	• Parvocellularneuro-endocrine cells in the paraventricular nucleus of the hypothalamus	• NKCC1 inhibition not tested	• Hewitt *et al.* 2009
mAchR	• mAChR agonist CCh (10–20 μM) does not alter E_{GABA}; predicted to depolarize E_{GABA} at prolonged higher concentrations	• Hippocampal neurons	• NKCC1 inhibition not tested	• Lee *et al.* 2010 • Deeb *et al.* 2013 • Takkala and Woodin, 2013

PLC, producing inositol trisphosphate (IP_3) and diacylglycerol (DAG), which can lead to mobilization of Ca^{2+} from intracellular stores, as well as PKC activation (Anwyl, 1999). PKC phosphorylates S940-KCC2 (pS940), which increases KCC2 activity by potentially increasing KCC2 plasma membrane stability and/or by slowing transporter endocytosis (Lee *et al.* 2007). In addition, pS940-KCC2 can also prevent a calpain-induced proteolytic cleavage of the KCC2 C-tail and a subsequent downregulation of transporter efficacy (Lee *et al.* 2011; Puskarjov *et al.* 2012; Zhou *et al.* 2012). Banke & Gegelashvili (2008) found that promoting PKC activity (with phorbol 12-myristate 13-acetate, a broad activator of protein kinase C) resulted in a hyperpolarizing shift in E_{GABA}, and inhibition of PKC (using Gö6976) resulted in a depolarizing shift in E_{GABA}. However it remains untested whether the PKC-mediated hyperpolarization of E_{GABA} is pS940-dependent (Table 2). What is the physiological significance of this mGluR-mediated activation of KCC2? Altering mGluR activity leads to a dynamic regulation of E_{GABA} across an ∼16 mV range from hyperpolarizing to depolarizing, and in some cases GABA may even become excitatory. The authors suggest that this mechanism ensures robust inhibition during intense synaptic activity when glutamate spill-over activates mGluRs. Moreover, in another recent intriguing discovery, it was demonstrated that chloride ions themselves act as allosteric activators of mGluRs (Tora *et al.* 2015). Therefore, one could envision a model whereby Cl^- extrusion by KCC2 provides extracellular anions required for mGluR activity within a functional KCC2–mGluR complex. However this hypothesis needs to be systematically tested.

KCC2 is critical for inhibitory synaptic transmission, which invites the question: how can a glutamate receptor normally localized to excitatory synapses regulate KCC2 function? Canonical KCC2 function is the extrusion of Cl^- to maintain neuronal osmolarity and hyperpolarizing inhibition. However, in recent years it has become clear that KCC2 is also highly localized to excitatory synapses (Gulyás *et al.* 2001). In fact, we know that KCC2 plays a structural role in dendritic spine formation during development (Li *et al.* 2007; Llano *et al.* 2015), and in mature excitatory synapses KCC2 restricts AMPAR (AMPA-type glutamate receptor) lateral diffusion in dendritic spines (Gauvain *et al.* 2011). Not only is KCC2 highly enriched at excitatory synapses, but quantum dot imaging has revealed that KCC2 dwell time is 1.3-fold longer at excitatory *vs.* inhibitory synapses, suggestive of stronger confinement of KCC2 to excitatory synapses (Chamma *et al.* 2013). The stronger confinement may result from interactions between KCC2 and proteins at excitatory synapses. Considering the abundant expression of KCC2 at glutamatergic synapses, it is perhaps unsurprising that mGluRs (Farr *et al.* 2004) and other members of the excitatory synapse (Ivakine *et al.* 2013; Mahadevan *et al.* 2014) are physically coupled with KCC2.

GPCR modulation of CCCs in the hippocampus and cortex: metabotropic Zn^{2+} receptors. A series of recent studies have demonstrated that activation of metabotropic Zn^{2+} receptors (mZnRs) regulate KCC2 function (Chorin *et al.* 2011; Saadi *et al.* 2012; Gilad *et al.* 2015). In the CA3 region, the mossy fibre (MF) terminals form specialized synapses onto CA3 pyramidal neurons. MF terminals co-release Zn^{2+} from their massive boutons during glutamatergic neurotransmission (Frederickson *et al.* 2000) where it acts via postsynaptic metabotropic Zn^{2+} receptors (mZnRs) on CA3 pyramidal neurons (Chorin *et al.* 2011). At this synapse both extracellular application and synaptic release of Zn^{2+}, which activate mZnRs, increases KCC2 surface expression and transporter efficacy. Interestingly, the authors demonstrate that upregulation of KCC2 function is sensitive to the $G_{\alpha q}$ inhibitor YM-254890 and the PLC inhibitor U73122, both of which block the mZnR-mediated intracellular Ca^{2+} increase and PKC activation. A loss of mZnR/GPR39 also abolishes the Zn^{2+}-induced increase in KCC2 activity. Similar to mGluR activation in CA3 pyramidal neurons, mZnR activation is also furosemide sensitive and PKC inhibitor sensitive. But unlike the mGluR experiments, the authors suggest that this mZnR phenotype is also extracellular signal-regulated kinase (ERK)–mitogen-activated protein kinase (MAPK) sensitive, and that mZnR activation results in increased surface levels of KCC2 in a soluble (*N*-ethylmaleimide-sensitive) factor attachment protein receptor (SNARE)-dependent manner. Although the precise KCC2 residue(s) that mediate the Zn^{2+}-dependent increase in KCC2 activity are currently untested, based on the requirement of PKC activation following mZnR activation, it is reasonable to predict that the mechanism occurs via phosphorylation of S940 (see Table 2).

It is important to consider whether the above reports of GPCRs on KCC2 are specific to this transporter, or also involve NKCC1 regulation. Activation of a GPCR that hyperpolarizes E_{GABA} can result from an increase in KCC2 function and/or a decrease in NKCC1 function. To test which transporter has been modulated by the GPCR it is important to specifically inhibit one of these transporters during the GPCR activation; this is most easily accomplished using the NKCC1-specific inhibitor bumetanide (Table 1). This is an important consideration because there are reports that GPCRs can also regulate NKCC1 function. For example, activation of mGluRs using the group I and II agonist 1-aminocyclopentane-*trans*-1,3-dicarboxylic acid (*trans*-APCD) enhances bumetanide-sensitive K^+ influx in heterologous cells and cultured cortical neurons (Sun & Murali, 1999; Schomberg *et al.* 2001). This effect can be abolished by chelating Ca^{2+} using BAPTA-AM, indicating

Table 2. Post-translational regulation of KCC2 by GPCR signalling

Receptor type	Role of PKC and pS940-KCC2	Surface expression	Neuronal type	Reference
mGluRI/mZnR (GPR39)	• Direct effect untested • Predicted to increase pS940 KCC2 since mGluR/mZnR activation results in increased PKC and ERK signal	• Untested for Group I mGluRs, but since mGluR activation increase PKC signal it could increase KCC2 pS940 • Extracellular Zn^{2+} increases surface KCC2 in wild-type neurons, but abolished in GPR39$^{-/-}$	• Hippocampal CA3 neurons • Cortical neurons	• Banke *et al.* 2008 • Chorin *et al.* 2011
$5HT_{2A}R$	• Effect depends on PKC signalling • pS940 was not specifically tested	• Increased surface KCC2	• Spinal motoneurons	• Bos *et al.* 2013
A_3AR	• Causal role for PKC not tested • The A3AR agonist increases KCC2 pS940	• Predicted to increase surface KCC2	• Lamina II dorsal horn neurons	• Ford *et al.* 2015
OXTR	• Causal role for PKC not tested • Possibly via increased PKC signal and increase KCC2 pS940	• Untested • Oxytocin rescue could increase surface KCC2 since autism models have high $[Cl^-]_i$	• Hippocampal CA3 pyramidal neurons	• Tyzio *et al.* 2006 • Tyzio *et al.* 2014
α_1-Adrengic receptor	• Untested	• Untested	• Parvocellular neuroendocrine cells in the paraventricular nucleus of the hypothalamus	• Hewitt *et al.* 2009
mAchR	• Lee *et al.* (2010) suggested that a direct phosphorylation of Y903, Y1087 may be involved in mAchR- mediated regulation of KCC2	• Decreased surface expression upon prolonged CCh treatment (100 μM) decreased total KCC2 due to increased KCC2 degradation	• Hippocampal neurons	• Lee *et al.* 2010

that the increase in intracellular Ca^{2+} is critical for the mGluR-mediated increase in NKCC1 activity.

What is the physiological necessity of regulating KCC2 transporter function during glutamatergic transmission at MF–CA3 synapses? The CA3 region of the hippocampus is among the primary epileptogenic regions of the brain (McCormick & Contreras, 2001). While a breakdown of the fine balance between excitation and inhibition (E–I) is often considered to underlie epileptogenesis, the molecular mechanisms that underlie the maintenance of E–I balance under physiological conditions are largely unknown. We propose that an increase in ambient glutamate release could increase the strength of GABAergic inhibition by increasing KCC2 activity via mGluR1 and/or mZnR signalling; and this neuromodulation could mediate E–I regulation within CA3 pyramidal neurons during normal physiological levels of activity. It has been previously demonstrated that excitotoxic Ca^{2+} influx via NMDA receptors (NMDARs) contributes to dephosphorylation of S940, a calpain-mediated cleavage of the C-terminus and a downregulation of transporter activity (Lee *et al.* 2011; Puskarjov *et al.* 2012). It is unlikely that the neuromodulatory enhancement of KCC2 function by mGluRs could overcome the NMDAR-mediated decrease in KCC2 that occurs during pathophysiological states. However, by identifying the neuromodulatory ability of mGluRs to increase KCC2 transporter function, these pathways can serve as pharmacological targets that can be exploited to reduce aberrant Cl^- homeostasis subsequent to excitotoxicity.

GPCR modulation of KCC2: the role of serotonin and adenosine receptors in neuropathic pain. KCC2 dysfunction often results in neuronal and circuit hyperexcitability, which underlies numerous diseases including epilepsy, spasticity, neuropathic pain and neurodevelopmental disorders (Kaila *et al.* 2014). During neuropathic pain pathogenesis, multiple cellular mechanisms converge to down-regulate KCC2 expression and function, ultimately resulting in increased neuronal Cl^-. Therefore the identification of cellular mechanisms capable of increasing KCC2 activity during pain pathogenesis is of paramount importance for the developmental of neuropathic pain treatments.

In a well-established spinal cord injury (SCI) model of spasticity, Bos *et al.* (2013) demonstrated that a pharmacological activation of the 5-hydroxytryptamine (5-HT) type 2A serotonin receptors (5-HT_2Rs) using a broad-spectrum 5-$HT_{2A/2B/2C}$R agonist, (±)-2,5-dimethoxy-4-iodoamphetamine hydrochloride, or a high-affinity 5-HT_{2A}R agonist (4-bromo-3,6-dimethoxybenzocyclobuten-1-yl)methylamine hydrobromide (TCB-2) upregulates KCC2 cell surface expression. Moreover, this increase in KCC2 surface expression and function can restore endogenous inhibition in motoneurons and reduce spasticity in rats. These effects are abolished by the 5-HT_2R antagonist ketanserin. 5-HT_2Rs are G_q-coupled GPCRs and upon their activation they stimulate PLC leading to activation of PKC; it is likely that the increase in KCC2 cell surface expression results from a PKC-mediated reduction in KCC2 endocytosis. In confirmation of this hypothesis, inhibition of Ca^{2+}-dependent PKC (with nanomolar concentrations of Gö6976) abolished the 5-HT_2R-dependent hyperpolarization of E_{GABA} in neurons. It is also notable that serotonin activation of 5-HT_2Rs was previously reported to modulate Cl^- homeostasis in zebrafish spinal cord neurons (Brustein and Drapeau 2005).

In a chronic constriction injury (CCI)-induced model of neuropathic pain, pharmacological activation of A3A-type adenosine receptors (A3ARs) using agonists IB-MECA and MRS5698 rescues KCC2 dysfunction and reverses allodynia (Ford *et al.* 2015). The authors report that KCC2 surface expression, phosphorylation of S940-KCC2, and KCC2 transporter efficacy all significantly increase during A3A activation. The authors suggest that this increase in KCC2 expression and function may underlie the ability of MRS5698 to restore inhibitory neurotransmission during neuropathic pain. Although a causal role for PKC signalling remains untested in this model, it is notable that A3ARs are also Gq-coupled GPCRs that mediate the release of intracellular Ca^{2+} and increase PKC activity. Thus G_q-GPCR activation increases KCC2 function via a PKC-mediated signalling cascade in two different regions of the nervous system.

GPCR modulation of CCCs: the role of oxytocin signalling in neuropsychiatric disorders. An emerging concept in translational therapies for neurodevelopmental disorders is the use of the neuropeptide oxytocin (OXT) (Meyer-Lindenberg *et al.* 2011). Decreased levels of OXT have been reported in several neuropsychiatric disorders including autism, schizophrenia and major depression (Modahl *et al.* 1998; Scantamburlo *et al.* 2007; Goldman *et al.* 2008). Moreover, exogenous OXT can restore various aspects of social behaviour and cognitive dysfunction associated with neuropsychiatric disorders including autism spectrum disorders (ASDs) (Gordon *et al.* 2013; Peñagarikano *et al.* 2015).

Neurodevelopmental disorders are thought to arise from E–I imbalances during development (Rubenstein & Merzenich, 2003). Since the shift of GABA polarity from excitation to inhibition in the brain is a fundamental developmental landmark, any aberration of this shift in GABAergic polarity could contribute to neurodevelopmental disorders. In line with this hypothesis Tyzio *et al.* (2014) and He *et al.* (2014) demonstrated that in different mouse models of ASD, neuronal Cl^- homeostasis and GABA polarity are fundamentally altered, such that neurons retain high $[Cl^-]_i$ and depolarizing E_{GABA}. In both

ASD models the authors reveal that the developmental upregulation of KCC2, which drives the hyperpolarizing GABA shift, is delayed or dysfunctional. Moreover the authors established that the maternal hormone OXT is a critical regulator of the GABA polarity shift (Tyzio *et al.* 2006, 2014). OXT treatment of ASD animals reversed several cellular, physiological and behavioural phenotypes of ASD, and this phenotypic rescue could be blocked with the OXT receptor (OXTR) antagonist SSR126768A. In addition, NKCC1 blockade with bumetanide occludes the effect of the OXTR antagonist and restores the developmental delay. OXTR is a G_q-type GPCR that is abundantly expressed in the hippocampus and neocortex during the perinatal period (Tyzio *et al.* 2006). Although the role of NKCC1 is firmly established in this phenotype, it will be important to determine the mechanism by which OXTR activation regulates NKCC1 transport. It is plausible that oxytocin signalling could act via this pathway to increase the surface expression and activity of KCC2, which could occur in parallel with a decrease in NKCC1 function to increase the driving force of GABAergic currents (the difference between resting membrane potential and E_{GABA}).

Several additional questions arise from these studies that warrant future experimental interrogation. Does OXT signalling contribute to the GABA polarity shift across different brain regions? Does other G_q-GPCR signalling collude with OXT signalling to contribute to the GABA polarity shift?

Does all G_q-GPCR signalling augment KCC2 function? It is likely to depend on intracellular Ca^{2+}. Do all G_q-GPCRs act as KCC2 activators? The answer is likely to be no. Muscarinic acetylcholine receptors (mAChRs), another important class of G_q-GPCRs, mediate cholinergic modulation of fast neurotransmission in several brain regions including the hippocampus. While relatively low concentrations of the mAChR agonist carbachol (CCh; 10–20 μM) failed to alter basal E_{GABA} in hippocampal neurons (Deeb *et al.* 2013; Takkala & Woodin, 2013), CCh at higher concentrations (100 μM) was reported to downregulate KCC2 function (Lee *et al.* 2010). This implies that there may be an effective range of Ca^{2+} release from intracellular stores that could have varying impacts on KCC2 activity. In accordance with this hypothesis, Hewitt *et al.* (2009) demonstrated that a robust activation of α_1-adrenergic receptors using high concentrations of the agonist phenylephrine (100 μM) in parvocellular neuroendocrine cells within the paraventricular nucleus (PVN) of the hypothalamus downregulates KCC2 function and depolarizes E_{GABA}. Furthermore, relatively high concentrations of caffeine bath application (10 mM; acting via adenosine receptors) robustly downregulated KCC2 function and depolarized E_{GABA} (Fiumelli *et al.* 2005). Together these results highlight the idea that $[Ca^{2+}]_i$ elevation from intracellular stores may be a critical checkpoint that determines the direction of KCC2 regulation downstream of G_q-GPCR signalling. Furthermore, future studies should systematically test whether nanomolar concentrations of adenosine receptor agonists and adrenergic receptors can increase KCC2 function.

KCC2 pharmacology and neuromodulation. Since KCC2 function is central to GABAergic and glycinergic inhibition, identification of pharmacological tools to specifically and directly inhibit or activate the transporter activity has been an important quest in the KCC2 field. Among the first class of KCC2 antagonists discovered was VU0240551; it was identified in a high-throughput screen using a small-molecule library of several thousand compounds. Secondary screening identified that this compound may also inhibit several G_q-GPCRs including A1- and A3-type adenosine receptors (Delpire *et al.* 2009). More recently, a structural analogue of this KCC2 blocker was synthesized, termed VU0463271, which exhibits stronger nanomolar affinities to KCC2 and a robust blockade of KCC2 transport compared with VU0240551 (even at low concentrations; 100 nM) (Delpire *et al.* 2012; Sivakumaran *et al.* 2015). However, the authors report that VU0463271 also binds to α_1-adrenergic receptors (IC_{50} of $\sim$350 nM; (Sivakumaran *et al.* 2015). Therefore future studies must be performed to determine whether specific α_1-adrenergic receptor blockers could also impact KCC2 function in a cell-type specific manner.

A novel KCC2 agonist termed CLP257 was recently demonstrated to exhibit significant specificity towards KCC2 but not to other CCCs, and increase its transporter efficacy *in vitro* and in spinal cord lamina II neurons (Gagnon *et al.* 2013). Although the precise mechanism of the drug action on KCC2 is currently unknown, CLP257 was able to act as a chloride extrusion enhancer in a neuropathic pain model, and restore Cl^- transport in adult spinal cord slices with impaired KCC2 function. While the compound itself exhibited modest binding to adenosine receptors and other classes of GPCRs (Gagnon *et al.* 2013), future studies must systematically examine whether G_q-GPCR agonists could also work as novel Cl^- extrusion enhancers under physiological and/or pathological states.

What are the physiological effects of GPCR-mediated neuromodulation of KCC2 function?. Hyperpolarizing inhibition was once considered to simply be an inverse linear regulator of neuronal excitability, e.g. the less hyperpolarizing inhibition, the more action potential firing, and vice versa. This led to the concept that inhibition was simply a brake on excitation, but we now know that inhibitory functions are much more complex (reviewed in Isaacson & Scanziani, 2011). In addition to hyperpolarizing the membrane potential away

from the action potential threshold, GABAergic transmission can also be inhibitory by shunting concurrent excitatory inputs. Shunting inhibition occurs from the current-induced increase in membrane conductance, which reduces the amplitude and duration of excitatory postsynaptic potentials (EPSPs) (Staley & Mody, 1992). But what are the consequences of hyperpolarizing and shunting inhibition beyond a direct regulation of postsynaptic output? And how would G_q-GPCR-mediated alterations of KCC2 that alter the strength of inhibition affect the neurophysiological functions of inhibition?

The answers to these questions can be complex, as exemplified by the relationship between GABA and glycinergic inhibitory currents, and hyperpolarization-sensitive ion channels, such as the hyperpolarization-activated cyclic nucleotide-gated (HCN) channels and the T-type voltage-gated calcium channels. HCNs are activated by hyperpolarizing potentials and are permeable to cations (Shah, 2014); they mediate the h-current (I_h). HCN channels are activated by membrane potentials less that –50mV and mediate a conductance that contributes to neuronal excitability and sculpts the integration of synaptic inputs (Biel *et al.* 2009). Because HCN channels are sensitive to the level of hyperpolarization (Atherton *et al.* 2010), increases in KCC2 function that strengthen hyperpolarizing inhibition should amplify HCN-mediated neurophysiologic effects. Strong hyperpolarizing inhibitory postsynaptic potentials (IPSPs) due to strong inward Cl^- gradients activate I_h, which when combined with T-type calcium currents (hyperpolarization removes these channels from the inactivated state), can drive action potential firing. In neurons of the brainstem superior paraolivary nucleus (SPN), this IPSP–HCN-driven increase in firing encodes sound termination (Kopp-Scheinpflug *et al.* 2011). The relationship between inhibition and I_h is even more complex considering that I_h contributes to a depolarization of the resting membrane potential that maintains the driving force for Cl^- ions (Pavlov *et al.* 2011). Conversely, decreases in tonic G_q-GPCR signalling that lead to a decrease in KCC2 function and subsequent reduction in inhibition will decrease HCN activation. Based on genetic deletion and pharmacological blockade studies of HCN channels, we predict reduced GPCR signalling will in turn increase dendritic temporal summation (Shah *et al.* 2004; Huang *et al.* 2009; Pavlov *et al.* 2011). It is interesting to note that HCN channel

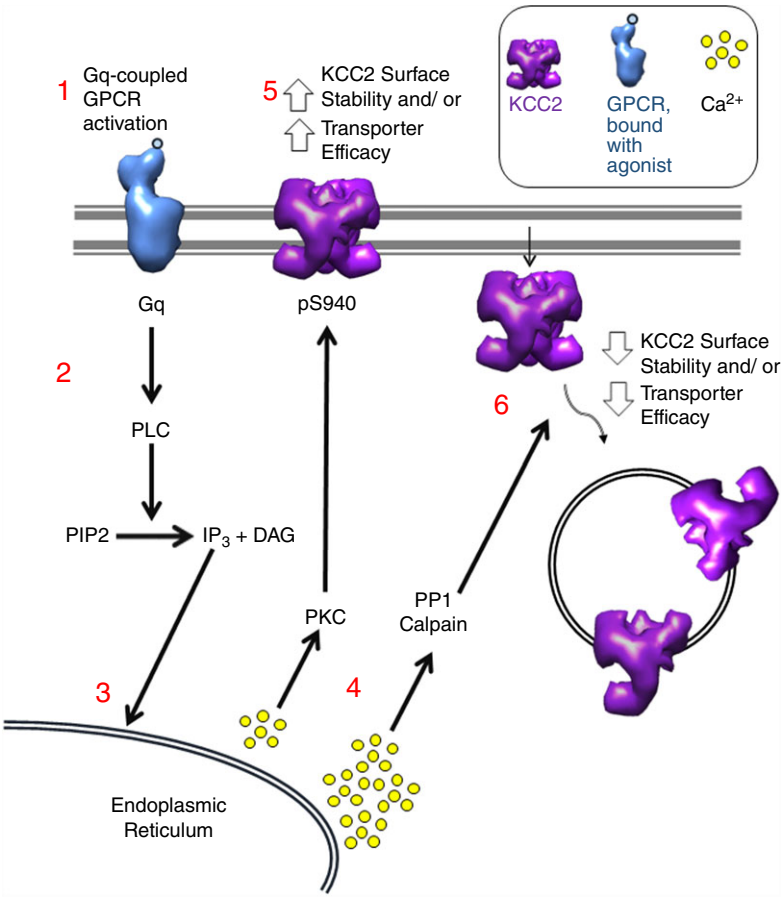

Figure 1. KCC2 regulation by GPCR signalling
Binding of diverse neuromodulators to their cognate G_q-coupled GPCRs result in the activation of G_q-proteins. (2) Activation of G_q-coupled GPCRs result in the stimulation of PLCβ), which hydrolyses PIP2 into the second messengers IP_3 and DAG. (3) IP_3 diffuses to the endoplasmic reticulum and contributes to the release of Ca^{2+}. (4) Relatively low increases in Ca^{2+} activate PKC, while higher concentrations activate protein phosphatase 1 (PP1). (5) PKC activation leads to the phosphorylation of several KCC2 residues (primarily S940), which contributes to increased surface stability and/or transporter efficacy. (6) PP1 activation results in dephosphorylation of several KCC2 residues (primarily S940), while calpain can cleave the C-terminus; both modifications can contribute towards decreased surface stability and/or transporter efficacy. The modelled structure of the human KCC2 (hKCC2, purple) was created based on homology modelling by I-TASSER (Roy *et al.* 2010; Yang *et al.* 2014) using available crystal structures of prokaryotic CCC cytoplasmic tail (PDB 3G40; Warmuth *et al.* 2009) and full-length prokaryotic amino acid transporters (PDB 3GI9; Shaffer *et al.* 2009). The *C*-score of the modelled hKCC2 CTD is ~ –1.0, indicating a reliable quality of the overall structural model. hKCC2 in this iteration is depicted as a dimer. Crystal structure of human adrenergic GPCR (PDB 2RH1, in blue; Cherezov *et al.* 2007) is used in this cartoon. Chimera 1.10.1 was used for molecular graphics rendering.

expression and function are associated with neuropathic pain and epilepsy (Biel *et al.* 2009). Ultimately, the complexity is determined by the proximity of appropriate GPCRs, KCC2 and hyperpolarization-sensitive ion channels within the same cellular compartment and cell type.

What are the potential effects of GPCR-mediated alterations in inhibition at the circuit level? Sensory-driven and spontaneous cortical activity both produce concerted synaptic excitation and inhibition; simply speaking, you rarely have one without the other. Inhibitory synaptic transmission forms the basis of two fundamental neuronal circuits, feed-forward and feed-back inhibition, which are essential players in generating brain rhythms that underlie the cognitive functions of the brain. What would happen to these circuits if GPCR activation leads to a KCC2-mediated increase in hyperpolarizing inhibition? In feed-forward inhibitory circuits a presynaptic input excites an inhibitory interneuron, which in turn inhibits a postsynaptic principal cell; this circuit determines the coincidence detection and the temporal precision of firing (Buzsáki, 1984; Pouille & Scanziani, 2001). Thus, an increase in hyperpolarization would be expected to narrow the time window for synaptic integration, which would narrow the window for coincidence detection.

Conclusions and future directions

The holy grail of neuronal Cl^- homeostasis is to understand the mechanisms that increase KCC2 function. Researchers need to selectively activate KCC2 in order to restore Cl^- homeostasis and hyperpolarizing inhibition in patients affected with E–I imbalance-related neurological disorders. The selectivity needs to be compartment specific in order to prevent the deleterious consequences of strengthening inhibition in brain regions unaffected by the disorder. To achieve this selectivity we need diversity in KCC2 regulation. GPCRs are among the most important molecular targets for several neurological disorders including anxiety, depression, pain and addiction (Conn *et al.* 2009). Since GPCR diversity offers a unique window towards KCC2 regulation across different cell types, a systematic characterization of GPCR allosteric modulators that can modulate neuronal Cl^- homeostasis is required.

In this review we have revealed what is likely to be only the tip of the iceberg regarding the relationship between G_q-GPCRs and neuronal KCC2-mediated Cl^- homeostasis (summarized in Tables 1 and 2 and Fig. 1). While some of the concepts explored here have strong evidence from numerous studies, other concepts are still hypothetical. For example, it is currently unknown whether other GPCRs that have been functionally associated with KCC2 are also part of the KCC2 macromolecular complex. Adding validity to this possibility is that in addition to the mGluRs that exist in a complex with KCC2 (Farr *et al.* 2004; Kato *et al.* 2012), an orphan GPCR termed GPR50 was identified as a putative KCC2 interactor in a yeast two-hybrid screen (Grünewald *et al.* 2009). Physical association with these GPCRs with KCC2 might also provide a signalling-independent regulation of KCC2 protein expression, stability and/or trafficking. It is also largely unknown whether KCC2 is regulated by other classes of GPCRs – G_α/G_i including $GABA_BRs$ and dopamine receptors – and whether cAMP and PKA themselves regulate KCC2 function.

Another putative GPCR signal that might exert its physiological effect via KCC2 are opioids. Opioids are amongst the most widely used substances for analgesia (McQuay, 1999). In addition to pain alleviation, opioid administration is also paradoxically known to cause hyperalgesia and tolerance. While the paradoxical morphine-induced hyperalgesia occurs via brain-derived neurotrophic factor-mediated KCC2 downregulation (Ferrini *et al.* 2013), an analgesic mode of morphine function via KCC2 upregulation has not been examined. Opioids act via a cognate G_q-class of opioid GPCRs, and upon activation they enhance PKC signalling postsynaptically in DRG neurons (Mao *et al.* 1995; Mayer *et al.* 1999). Therefore, opioid-induced analgesia may occur via increasing KCC2 function and strengthening inhibition in DRG neurons.

In addition to determining the polarity of GABAergic transmission, KCC2 also plays important roles in both neuronal cell volume regulation (Alvarez-Leefmans *et al.* 2010) and dendritic spine morphogenesis (Li *et al.* 2007; Gauvain *et al.* 2011; Chamma *et al.* 2012). Whether GPCR-mediated regulation of KCC2 also modulates the ability of this transporter to regulate neuronal cell volume and/or its role in dendritic spine formation is likely to be an emerging avenue of investigation. Finally, neuromodulation of other CCC members, pumps and ion exchanges is also understudied, and thus in a nutshell, this field has the scope to grow rapidly in the coming years.

References

Alvarez-Leefmans FJ & Delpire E (2010). *Physiology and Pathology of Chloride Transporters and Channels in the Nervous System*. Academic Press/Elsevier.

Anwyl R (1999). Metabotropic glutamate receptors: electrophysiological properties and role in plasticity. *Brain Res Rev* **29**, 83–120.

Atherton JF, Kitano K, Baufreton J, Fan K, Wokosin D, Tkatch T, Shigemoto R, Surmeier DJ & Bevan MD (2010). Selective participation of somatodendritic HCN channels in inhibitory but not excitatory synaptic integration in neurons of the subthalamic nucleus. *J Neurosci* **30**, 16025–16040.

Banke TG & Gegelashvili G (2008). Tonic activation of group I mGluRs modulates inhibitory synaptic strength by regulating KCC2 activity. *J Physiol* **586**, 4925–4934.

Ben-ari Y, Gaiarsa J, Tyzio R & Khazipov R (2007). GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations. *Physiol Rev* **87**, 1215–1284.

Biel M, Wahl-Schott C, Michalakis S & Zong X (2009). Hyperpolarization-activated cation channels: from genes to function. *Physiol Rev* **89**, 847–885.

Bos R, Sadlaoud K, Boulenguez P, Buttigieg D, Liabeuf S, Brocard C, Haase G, Bras H & Vinay L (2013). Activation of 5-HT2A receptors upregulates the function of the neuronal K-Cl cotransporter KCC2. *Proc Natl Acad Sci USA* **110**, 348–353.

Brustein E & Drapeau P (2005). Serotoninergic modulation of chloride homeostasis during maturation of the locomotor network in zebrafish. *J Neurosci* **25**, 10607–10616.

Buzsáki G (1984). Feed-forward inhibition in the hippocampal formation. *Prog Neurobiol* **22**, 131–153.

Chamma I, Chevy Q, Poncer JC & Lévi S (2012). Role of the neuronal K-Cl co-transporter KCC2 in inhibitory and excitatory neurotransmission. *Front Cell Neurosci* **6**, 1–15.

Chamma I, Heubl M, Chevy Q, Renner M, Moutkine I, Eugène E, Poncer JC & Lévi S (2013). Activity-dependent regulation of the K/Cl transporter KCC2 membrane diffusion, clustering, and function in hippocampal neurons. *J Neurosci* **33**, 15488–15503.

Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SGF, Thian FS, Kobilka TS, Choi H-J, Kuhn P, Weis WI, Kobilka BK & Stevens RC (2007). High-resolution crystal structure of an engineered human β_2-adrenergic G protein-coupled receptor. *Science* **318**, 1258–1265.

Chorin E, Vinograd O, Fleidervish I, Gilad D, Herrmann S, Sekler I, Aizenman E & Hershfinkel M (2011). Upregulation of KCC2 Activity by zinc-mediated neurotransmission via the mZnR/GPR39 receptor. *J Neurosci* **31**, 12916–12926.

Conn PJ, Christopoulos A & Lindsley CW (2009). Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. *Nat Rev Drug Discov* **8**, 41–54.

Coull JAM, Boudreau D, Bachand K, Prescott SA, Nault F, Sík A, De Koninck P & De Koninck Y (2003). Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain. *Nature* **424**, 938–942.

Deeb TZ, Nakamura Y, Frost GD, Davies PA & Moss SJ (2013). Disrupted Cl^- homeostasis contributes to reductions in the inhibitory efficacy of diazepam during hyperexcited states. *Eur J Neurosci* **38**, 2453–2467.

Delpire E, Baranczak A, Waterson AG, Kim K, Kett N, Morrison RD, Scott Daniels J, David Weaver C & Lindsley CW (2012). Further optimization of the K-Cl cotransporter KCC2 antagonist ML077: Development of a highly selective and more potent in vitro probe. *Bioorganic Med Chem Lett* **22**, 4532–4535.

Delpire E, Days E, Lewis LM, Mi D, Kim K, Lindsley CW & Weaver CD (2009). Small-molecule screen identifies inhibitors of the neuronal K-Cl cotransporter KCC2. *Proc Natl Acad Sci USA* **106**, 5383–5388.

Farr CD, Gafken PR, Norbeck AD, Doneanu CE, Stapels MD, Barofsky DF, Minami M & Saugstad JA (2004). Proteomic analysis of native metabotropic glutamate receptor 5 protein complexes reveals novel molecular constituents. *J Neurochem* **91**, 438–450.

Ferrini F, Trang T & Mattioli T (2013). Morphine hyperalgesia gated through microglia-mediated disruption of neuronal Cl^- homeostasis. *Nat Neurosci* **16**, 183–192.

Fiumelli H, Cancedda L & Poo MM (2005). Modulation of GABAergic transmission by activity via postsynaptic Ca^{2+}-dependent regulation of KCC2 function. *Neuron* **48**, 773–786.

Ford A, Castonguay A, Cottet M, Little JW, Chen Z, Symons-Liguori AM, Doyle T, Egan TM, Vanderah TW, De Konnick Y, Tosh DK, Jacobson KA & Salvemini D (2015). Engagement of the GABA to KCC2 signaling pathway contributes to the analgesic effects of A3AR agonists in neuropathic pain. *J Neurosci* **35**, 6057–6067.

Frederickson CJ, Suh SW, Silva D, Frederickson CJ & Thompson RB (2000). Importance of zinc in the central nervous system: the zinc-containing neuron. *J Nutr* **130**, 1471S–1483S.

Gagnon M, Bergeron MJ, Lavertu G, Castonguay A, Tripathy S, Bonin RP, Perez-Sanchez J, Boudreau D, Wang B, Dumas L, Valade I, Bachand K, Jacob-Wagner M, Tardif C, Kianicka I, Isenring P, Attardo G, Coull JAM & De Koninck Y (2013). Chloride extrusion enhancers as novel therapeutics for neurological diseases. *Nat Med* **19**, 1524–1528.

Gauvain G, Chamma I, Chevy Q, Cabezas C, Irinopoulou T, Bodrug N, Carnaud M, Lévi S & Poncer JC (2011). The neuronal K-Cl cotransporter KCC2 influences postsynaptic AMPA receptor content and lateral diffusion in dendritic spines. *Proc Natl Acad Sci USA* **108**, 15474–15479.

Gilad D, Shorer S, Ketzef M & Friedman A (2015). Homeostatic regulation of KCC2 activity by the zinc receptor mZnR/GPR39 during seizures. *Neurobiol Dis* **81**, 4–13.

Goldman M, Marlow-O'Connor M, Torres I & Carter CS (2008). Diminished plasma oxytocin in schizophrenic patients with neuroendocrine dysfunction and emotional deficits. *Schizophr Res* **98**, 247–255.

Gordon I, Vander Wyk BC, Bennett RH, Cordeaux C, Lucas MV, Eilbott JA, Zagoory-Sharon O, Leckman JF, Feldman R & Pelphrey KA (2013). Oxytocin enhances brain function in children with autism. *Proc Natl Acad Sci USA* **110**, 20953–20958.

Grünewald E, Kinnell HL, Porteous DJ & Thomson PA (2009). GPR50 interacts with neuronal NOGO-A and affects neurite outgrowth. *Mol Cell Neurosci* **42**, 363–371.

Gulyás AI, Sík A, Payne JA, Kaila K & Freund TF (2001). The KCl cotransporter, KCC2, is highly expressed in the vicinity of excitatory synapses in the rat hippocampus. *Eur J Neurosci* **13**, 2205–2217.

He Q, Nomura T, Xu J & Contractor A (2014). The developmental switch in GABA polarity is delayed in fragile X mice. *J Neurosci* **34**, 446–450.

Hewitt SA, Wamsteeker JI, Kurz EU & Bains JS (2009). Altered chloride homeostasis removes synaptic inhibitory constraint of the stress axis. *Nat Neurosci* **12**, 438–443.

Huang X, Chen H, Michelsen K, Schneider S & Shaffer PL (2015). Crystal structure of human glycine receptor-α3 bound to antagonist strychnine. *Nature* **526**, 277–280.

Huang Z, Walker MC & Shah MM (2009). Loss of dendritic HCN1 subunits enhances cortical excitability and epileptogenesis. *J Neurosci* **29**, 10979–10988.

Hübner CA (2014). The KCl-cotransporter KCC2 linked to epilepsy. *EMBO Rep* **15**, 732–733.

Isaacson JS & Scanziani M (2011). How inhibition shapes cortical activity. *Neuron* **72**, 231–243.

Ivakine EA, Acton BA, Mahadevan V, Ormond J, Tang M, Pressey JC, Huang MY, Ng D, Delpire E, Salter MW, Woodin MA & McInnes RR (2013). Neto2 is a KCC2 interacting protein required for neuronal Cl^- regulation in hippocampal neurons. *Proc Natl Acad Sci USA* **110**, 3561–3566.

Kahle KT, Deeb TZ, Puskarjov M, Silayeva L, Liang B, Kaila K & Moss SJ (2013). Modulation of neuronal activity by phosphorylation of the K-Cl cotransporter KCC2. *Trends Neurosci* **36**, 726–737.

Kaila K, Price TJ, Payne JA, Puskarjov M & Voipio J (2014). Cation-chloride cotransporters in neuronal development, plasticity and disease. *Nat Rev Neurosci* **15**, 637–654.

Kato AS, Knierman MD, Siuda ER, Isaac JTR, Nisenbaum ES & Bredt DS (2012). Glutamate receptor $\delta 2$ associates with metabotropic glutamate receptor 1 (mGluR1), protein kinase Cγ, and canonical transient receptor potential 3 and regulates mGluR1-mediated synaptic transmission in cerebellar Purkinje neurons. *J Neurosci* **32**, 15296–15308.

Kopp-Scheinpflug C, Tozer AJB, Robinson SW, Tempel BL, Hennig MH & Forsythe ID (2011). The sound of silence: ionic mechanisms encoding sound termination. *Neuron* **71**, 911–925.

Lee HHC, Deeb TZ, Walker JA, Davies PA & Moss SJ (2011). NMDA receptor activity downregulates KCC2 resulting in depolarizing GABAA receptor-mediated currents. *Nat Neurosci* **14**, 736–743.

Lee HHC, Jurd R & Moss SJ (2010). Tyrosine phosphorylation regulates the membrane trafficking of the potassium chloride co-transporter KCC2. *Mol Cell Neurosci* **45**, 173–179.

Lee HHC, Walker JA, Williams JR, Goodier RJ, Payne JA & Moss SJ (2007). Direct protein kinase C-dependent phosphorylation regulates the cell surface stability and activity of the potassium chloride cotransporter KCC2. *J Biol Chem* **282**, 29777–29784.

Li H, Khirug S, Cai C, Ludwig A, Blaesse P, Kolikova J, Afzalov R, Coleman SK, Lauri S, Airaksinen MS, Keinänen K, Khiroug L, Saarma M, Kaila K & Rivera C (2007). KCC2 interacts with the dendritic cytoskeleton to promote spine development. *Neuron* **56**, 1019–1033.

Llano O, Smirnov S, Soni S, Golubtsov A, Guillemin I, Hotulainen P, Medina I, Nothwang HG, Rivera C & Ludwig A (2015). KCC2 regulates actin dynamics in spines via interaction with β-PIX. *J Cell Biol* **209**, 671–686.

Mahadevan V, Pressey JC, Acton BA, Uvarov P, Huang MY, Chevrier J, Puchalski A, Li CM, Ivakine EA, Airaksinen MS, Delpire E, McInnes RR & Woodin MA (2014). Kainate receptors coexist in a functional complex with KCC2 and regulate chloride homeostasis in hippocampal neurons. *Cell Rep* **7**, 1762–1770.

Mao J, Price DD & Mayer DJ (1995). Mechanisms of hyperalgesian and morphine tolerance: a current view of their possible interactions. *Pain* **62**, 259–274.

Mayer DJ, Mao J, Holt J & Price DD (1999). Cellular mechanisms of neuropathic pain, morphine tolerance, and their interactions. *Proc Natl Acad Sci USA* **96**, 7731–7736.

McCormick DA & Contreras D (2001). On the cellular and network bases of epileptic seizures. *Annu Rev Physiol* **63**, 815–846.

McQuay H (1999). Opioids in pain management. *Lancet* **353**, 2229–2232.

Medina I (2014). Current view on the functional regulation of the neuronal K-Cl cotransporter KCC2. *Front Cell Neurosci* **8**, 27.

Meyer-Lindenberg A, Domes G, Kirsch P & Heinrichs M (2011). Oxytocin and vasopressin in the human brain: social neuropeptides for translational medicine. *Nat Rev Neurosci* **12**, 524–538.

Modahl C, Green LA, Fein D, Morris M, Waterhouse L, Feinstein C & Levin H (1998). Plasma oxytocin levels in autistic children. *Biol Psychiatry* **43**, 270–277.

Ormond J & Woodin MA (2009). Disinhibition mediates a form of hippocampal long-term potentiation in area CA1. *PLoS One* **4**, e7224.

Ormond J & Woodin MA (2011). Disinhibition-mediated LTP in the hippocampus is synapse specific. *Front Cell Neurosci* **5**, 1–11.

Pavlov I, Scimemi A, Savtchenko L, Kullmann DM & Walker MC (2011). I_h-mediated depolarization enhances the temporal precision of neuronal integration. *Nat Commun* **2**, 199.

Peñagarikano O, Lázaro MT, Lu X-H, Gordon A, Dong H, Lam HA, Peles E, Maidment NT, Murphy NP, Yang XW, Golshani P & Geschwind DH (2015). Exogenous and evoked oxytocin restores social behavior in the Cntnap2 mouse model of autism. *Sci Transl Med* **7**, 271ra8.

Pierce KL, Premont RT & Lefkowitz RJ (2002). Seven-transmembrane receptors. *Nat Rev Mol Cell Biol* **3**, 639–650.

Pouille F & Scanziani M (2001). Enforcement of temporal fidelity in pyramidal cells by somatic feed-forward inhibition. *Science* **293**, 1159–1163.

Premont RT & Gainetdinov RR (2007). Physiological roles of G protein-coupled receptor kinases and arrestins. *Annu Rev Physiol* **69**, 511–534.

Puskarjov M, Ahmad F, Kaila K & Blaesse P (2012). Activity-dependent cleavage of the K-Cl cotransporter KCC2 mediated by calcium-activated protease calpain. *J Neurosci* **32**, 11356–11364.

Roy A, Kucukural A & Zhang Y (2010). I-TASSER: a unified platform for automated protein structure and function prediction. *Nat Protoc* **5**, 725–738.

Rubenstein JLR & Merzenich MM (2003). Model of autism: increased ratio of excitation/inhibition in key neural systems. *Genes Brain Behav* **2**, 255–267.

Saadi RA, He K, Hartnett KA, Kandler K, Hershfinkel M & Aizenman E (2012). SNARE-dependent upregulation of potassium chloride co-transporter 2 activity after metabotropic zinc receptor activation in rat cortical neurons in vitro. *Neuroscience* **210**, 38–46.

Scantamburlo G, Hansenne M, Fuchs S, Pitchot W, Maréchal P, Pequeux C, Ansseau M & Legros JJ (2007). Plasma oxytocin levels and anxiety in patients with major depression. *Psychoneuroendocrinology* **32**, 407–410.

Schomberg SL, Su G, Haworth RA & Sun D (2001). Stimulation of Na-K-2Cl cotransporter in neurons by activation of non-NMDA ionotropic receptor and group-I mGluRs. *J Neurophysiol* **85**, 2563–2575.

Shaffer PL, Goehring A, Shankaranarayanan A & Gouaux E (2009). Structure and mechanism of a Na^+-independent amino acid transporter. *Science* **325**, 1010–1014.

Shah MM (2014). Cortical HCN channels: function, trafficking and plasticity. *J Physiol* **592**, 2711–2719.

Shah MM, Anderson AE, Leung V, Lin X & Johnston D (2004). Seizure-induced plasticity of h channels in entorhinal cortical layer III pyramidal neurons. *Neuron* **44**, 495–508.

Silayeva L, Deeb TZ, Hines RM, Kelley MR, Munoz MB, Lee HHC, Brandon NJ, Dunlop J, Maguire J, Davies PA & Moss SJ (2015). KCC2 activity is critical in limiting the onset and severity of status epilepticus. *Proc Natl Acad Sci USA* **112**, 3523–3528.

Sivakumaran S, Cardarelli RA, Maguire J, Kelley MR, Silayeva L, Morrow DH, Mukherjee J, Moore YE, Mather RJ, Duggan ME, Brandon NJ, Dunlop J, Zicha S, Moss SJ & Deeb TZ (2015). Selective inhibition of KCC2 leads to hyperexcitability and epileptiform discharges in hippocampal slices and in vivo. *J Neurosci* **35**, 8291–8296.

Staley KJ & Mody I (1992). Shunting of excitatory input to dentate gyrus granule cells by a depolarizing GABAA receptor-mediated postsynaptic conductance. *J Neurophysiol* **68**, 197–212.

Sun D & Murali SG (1999). Na^+-K^+-$2Cl^-$ cotransporter in immature cortical neurons: a role in intracellular Cl^- regulation. *J Neurophysiol* **81**, 1939–1948.

Takkala P & Woodin MA (2013). Muscarinic acetylcholine receptor activation prevents disinhibition-mediated LTP in the hippocampus. *Front Cell Neurosci* **7**, 16.

Tao R, Li C, Newburn EN, Ye T, Lipska BK, Herman MM, Weinberger DR, Kleinman JE & Hyde TM (2012). Transcript-specific associations of SLC12A5 (KCC2) in human prefrontal cortex with development, schizophrenia, and affective disorders. *J Neurosci* **32**, 5216–5222.

Tora AS, Rovira X, Dione I, Bertrand H-O, Brabet I, De Koninck Y, Doyon N, Pin J-P, Acher F & Goudet C (2015). Allosteric modulation of metabotropic glutamate receptors by chloride ions. *FASEB J* **29**, 4174–4188.

Tyzio R, Cossart R, Khalilov I, Minlebaev M, Hübner CA, Represa A, Ben-Ari Y & Khazipov R (2006). Maternal oxytocin triggers a transient inhibitory switch in GABA signaling in the fetal brain during delivery. *Science* **314**, 1788–1792.

Tyzio R, Nardou R, Ferrari DC, Tsintsadze T, Shahrokhi A, Eftekhari S, Khalilov I, Tsintsadze V, Brouchoud C, Chazal G, Lemonnier E, Lozovaya N, Burnashev N & Ben-Ari Y (2014). Oxytocin-mediated GABA inhibition during delivery attenuates autism pathogenesis in rodent offspring. *Science* **343**, 675–679.

Vassilatis DK, Hohmann JG, Zeng H, Li F, Ranchalis JE, Mortrud MT, Brown A, Rodriguez SS, Weller JR, Wright AC, Bergmann JE & Gaitanaris GA (2003). The G protein-coupled receptor repertoires of human and mouse. *Proc Natl Acad Sci USA* **100**, 4903–4908.

Warmuth S, Zimmermann I, Dutzler R (2009). X-ray structure of the C-terminal domain of a prokaryotic cation-chloride cotransporter. *Structure* **17**, 538–546.

Woodin MA, Ganguly K & Poo MM (2003). Coincident pre- and postsynaptic activity modifies GABAergic synapses by postsynaptic changes in Cl^- transporter activity. *Neuron* **39**, 807–820.

Yang J, Yan R, Roy A, Xu D, Poisson J & Zhang Y (2014). The I-TASSER Suite: protein structure and function prediction. *Nat Methods* **12**, 7–8.

Zhou HY, Chen SR, Byun HS, Chen H, Li L, Han HD, Lopez-Berestein G, Sood AK & Pan HL (2012). N-methyl-D-aspartate receptor- and calpain-mediated proteolytic cleavage of K^+-Cl^- cotransporter-2 impairs spinal chloride homeostasis in neuropathic pain. *J Biol Chem* **287**, 33853–33864.

Additional information

Competing interests

The authors have declared no competing interests.

Author contributions

V.M. conceived of the review article topic and performed the detailed literature review, and V.M. and M.A.W. co-wrote the manuscript. Both authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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