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Cation-chloride cotransporters in neuronal development, plasticity and disease

Kai Kaila[1,2], **Theodore J. Price**[3], **John A. Payne**[4], **Martin Puskarjov**[1,2], and **Juha Voipio**[1]

[1]Department of Biosciences, University of Helsinki, 00014 Helsinki, Finland [2]Neuroscience Center, University of Helsinki, 00014 Helsinki, Finland [3]University of Texas at Dallas, School of Behavior and Brain Sciences, Dallas, Texas 75093, USA [4]Department of Physiology and Membrane Biology, School of Medicine, University of California, Davis, California 95616, USA

Abstract

Electrical activity in neurons requires a seamless functional coupling between plasmalemmal ion channels and ion transporters. Although ion channels have been studied intensively for several decades, research on ion transporters is in its infancy. In recent years, it has become evident that one family of ion transporters, cation-chloride cotransporters (CCCs), and in particular K^+–Cl^- cotransporter 2 (KCC2), have seminal roles in shaping GABAergic signalling and neuronal connectivity. Studying the functions of these transporters may lead to major paradigm shifts in our understanding of the mechanisms underlying brain development and plasticity in health and disease.

Most mature neurons in the CNS actively extrude Cl^- and thus exhibit a low intracellular Cl^- concentration ($[Cl^-]_i$). This unique specialization is a necessary, but not sufficient, condition for the generation of hyperpolarizing inhibitory postsynaptic potentials (IPSPs)[1] by $GABA_A$ receptors ($GABA_A$Rs) and glycine receptors (GlyRs). The low $[Cl^-]_i$ comes about owing to an upregulation of the neuron-specific K^+–Cl^- cotransporter 2 (KCC2) during CNS neuron maturation and is maintained in adult central neurons[2–6] (FIG. 1; TABLE 1). This developmental decrease in $[Cl^-]_i$ does not occur in other cell types[6,7]. Thus, although neurobiologists generally regard immature neurons, which have a high $[Cl^-]_i$ (REFS 8–11) and exhibit depolarizing $GABA_A$R responses, as aberrant, it is actually the adult CNS neurons with their low internal levels of Cl^- that are exceptional.

As a substrate for plasmalemmal Cl^- transporters, Cl^- is used to maintain basic cellular parameters, such as cell volume and intracellular pH (pH_i). The low $[Cl^-]_i$ in mature central

Correspondence to K.K. Kai.Kaila@helsinki.fi.

Note added in proof

Our recent comment[281] on Glykys *et al.*[280] explains why immobile anions do not account for DF_{GABA}. In their response[297] they now suggest that DF_{GABA} is due to Donnan-equilibration of Cl^- plus an energy-requiring V_m shift. If so, Cl^- leaks would dissipate DF_{GABA}, thereby necessitating never-ending V_m shifts. As described here, DF_{GABA} and its spatial gradients are maintained by CCCs.

Competing interests statement

The authors declare no competing interests.

neurons means that their capacity to respond effectively to volume or pH_i disturbances is compromised[1,12]. There is also a high metabolic cost associated with maintenance of low $[Cl^-]_i$ and the generation of hyperpolarizing Cl^- currents during neuronal signalling, particularly when the neuron faces an energy crisis, such as that associated with recurrent seizures or stroke[13–15]. Clearly, these important trade-offs must be taken into account when judging whether a given alteration in $[Cl^-]_i$ regulation has a disease-promoting (maladaptive) or an adaptive role[15]. For instance, the frequently reported downregulation of KCC2 following neuronal trauma[16–23] may be part of a general adaptive cellular response that facilitates neuronal survival by reducing the energetic costs that are needed to preserve low $[Cl^-]_i$ (REFS 14,15) and by facilitating functional recovery through the removal of GABAergic inhibitory constraints on neuroplasticity[24,25].

The solute carrier 12 (SLC12) family of electroneutral cation-chloride cotransporters (CCCs) includes four K^+– Cl^- cotransporters (KCCs), KCC1 (encoded by *SLC12A4*), KCC2 (encoded by *SLC12A5*), KCC3 (encoded by *SLC12A6*) and KCC4 (encoded by *SLC12A7*); two Na^+–K^+–$2Cl^-$ cotransporters (NKCCs), NKCC1 (encoded by *SLC12A2*) and NKCC2 (encoded by *SLC12A1*); and an Na^+–Cl^- cotransporter (NCC; encoded by *SLC12A3*). Two members of the SLC12 family, KCC2 and NKCC1, are known to have cell-autonomous functions in central neurons, including a major role in setting the reversal potential and driving force of the anion currents mediated by $GABA_A$Rs and GlyRs [6] as well as by non-ligand-gated Cl^- channels[26,27]. Based on their developmental, cellular and subcellular patterns of functional expression, CCCs have turned out to be highly versatile factors in the control of GABAergic and glycinergic signalling. The importance of CCCs in brain function is underscored by the recent discovery of an ion-transport-independent structural role for KCC2 in the morphological and functional maturation of cortical dendritic spines[28–32]. Thus, KCC2 is in a key position to control and coordinate the development of both GABAergic and glutamatergic transmission. It is not surprising that CCC dysfunctions are likely to be associated with a wide range of neurological and psychiatric disorders[15,33–41].

Here, we summarize recent progress in our understanding of the roles of CCCs in neuronal development, plasticity and disease, with a particular focus on the functions of KCC2 and NKCC1 in synaptic signalling. Of the wide range of diseases to which CCCs have been linked, we focus on epilepsy and chronic pain because research on these disorders has shed light on the fundamental roles of CCCs in neuronal signalling.

Expression of CCCs in the CNS

CCCs are glycoproteins that have a core molecular weight of ~110–130 kDa. Each CCC has a similar predicted structure of 12 transmembrane segments and intracellular amino- and carboxy-terminal domains[33,42] (FIG. 1d). With the exception of a high-resolution structure that has been obtained for the C-terminal domain of a bacterial CCC protein[43], the tertiary structures of CCCs are not known. In terms of quaternary structure, the CCCs are likely to form dimers[42]. However, the available data are not conclusive regarding the manner in which multimerization affects CCC functions[6,42,44]. CCCs are expressed in all organ systems and, in addition to being instrumental in neuronal signalling, they are involved in a

range of physiological processes, including cell volume regulation (BOX 1), transepithelial ion transport, neuroendocrine signalling and blood pressure regulation[6,7,33,45–48].

Box 1

Control of neuronal and glial volume by CCCs

Following an acute perturbation, mechanisms that restore neuronal and glial volume are needed to cope with cell swelling. Isosmotic swelling results from an activity-dependent increase in the ionic load and is often seen in dendrites as a result of channel-mediated net influx of Na^+, Cl^- and osmotically obliged water. As the intracellular Na^+ concentration ($[Na^+]_i$) and $[Cl^-]_i$ are low under normal conditions, such an influx leads to a large relative increase in the concentrations of these ions, despite the accompanying water influx; and to a significant rise in the equilibrium potential of chloride (E_{Cl}). In some pathological states (such as water intoxication or hyponatraemia), neurons and glia are subject to hyposmotic swelling, as water flows from the vasculature into the extracellular space (ECS). In this case, cell swelling results exclusively from net water influx and intracellular ion concentrations will decrease.

The membrane lipid bilayer cannot expand elastically beyond 3%[260], and acute swelling is therefore largely attributable to unfolding of the cell plasmalemma[261]. In neurons, with their highly complex morphology, local differences in hydrostatic pressure effects as well as in Ca^{2+}-sensitive cytoskeletal mechanisms will lead to differential susceptibility to swelling in distinct subcellular compartments.

The ability of neurons to recover volume differs dramatically depending on whether the volume change results from isosmotic or hyposmotic swelling. One important volume regulatory mechanism involves net K^+ and Cl^- efflux via K^+–Cl^- cotransporters (KCCs). Volume recovery in mature CNS neurons upon hyposmotic swelling is severely compromised because of their low $[Cl^-]_i$, especially if mechanisms that replenish intracellular Cl^- without generating a further osmotic load (such as plasmalemmal HCO_3^-–Cl^- exchange) are inefficient. By contrast, recovery from isosmotic swelling by KCCs is facilitated by the increased $[Cl^-]_i$.

All KCC isoforms are thought to be activated by hyposmotic swelling, largely based on data obtained using heterologous expression in *Xenopus laevis* oocytes[33,141,262]. However, the intracellular volume-sensitive cascades and their coupling to cation-chloride cotransporter (CCC) functions are unlikely to be identical in amphibian oocytes[263,264] and neurons. In addition, a distinction should be made between an ion transporter that is volume-sensitive and one that is genuinely volume-regulatory. In the latter case, the net ion flux will be a graded function of the volume perturbation[265]. We do not yet have sufficient data to judge whether CCCs are genuinely volume-regulatory in mammalian neurons[12]. This is also true for astrocytes, which are thought to control the volume of the ECS[266]. The ECS volume has an important modulatory effect on neuronal excitability[267]. Somewhat surprisingly, recent work has shown that Na^+–K^+–$2Cl^-$ cotransporter 1 (NKCC1) is not a major factor in the recovery of ECS volume following activity-induced cellular swelling[268].

Central neurons are probably devoid of water-permeable aquaporins[266], and it has been shown that their soma and dendrites do not swell in response to a 20-minute exposure to moderately hypotonic solutions[269]. By contrast, peripheral nervous system neurons express water-permeable aquaporins and maintain a high $[Cl^-]_i$ (REF. 270), which implies that they have a higher sensitivity to swelling but also a better capacity for Cl^--dependent volume regulation[271]. Moreover, water can also move passively through ion channels[272], thereby influencing short-term and long-term homeostasis of volume.

There are two functional branches of the CCC phylogenetic tree[46]: the Na^+-dependent and the Na^+-independent CCCs. Uptake of Cl^- by NKCC1, NKCC2 and NCC is fuelled by the inwardly directed plasmalemmal Na^+ gradient, which is maintained by the Na^+/K^+ ATPase. By contrast, KCC1, KCC2, KCC3 and KCC4 typically mediate net Cl^- efflux, which is driven by the respective outwardly directed K^+ gradient (BOX 2). NCC and NKCC2 are predominantly expressed in the kidney[49,50], whereas all other CCCs exhibit varied spatiotemporal patterns of expression in the mammalian CNS (FIG. 1a) and some, such as NKCC1 and KCC3, are also expressed in the peripheral nervous system (PNS)[6,51,52].

Box 2

Energetics of chloride regulation

K^+–Cl^- cotransport is at thermodynamic equilibrium when all the energy available from the outward movement of K^+ across the membrane is used to extrude Cl^- (see the figure)[6]. This can be expressed in terms of ion concentrations or of equilibrium potentials (*E*) (see Supplementary information S2 (text)):

$$[Cl^-]_i = \frac{[K^+]_o}{[K^+]_i}[Cl^-]_o \iff E_{Cl} = E_K \qquad (1)$$

where o and i refer to extracellular and intracellular. Equation 1 also defines the equilibrium conditions at which net K^+–Cl^- transport by K^+–Cl^- cotransporters (KCCs) will reverse. In a resting neuron with constitutively active KCC2, E_{Cl} will be close to E_K, and an increase in $[K^+]_o$ will drive Cl^- into the neuron. Conversely, if a conductive influx of Cl^- increases intraneuronal $[Cl^-]$, thereby promoting K^+–Cl^- efflux via KCC2, $[K^+]_o$ will increase[101].

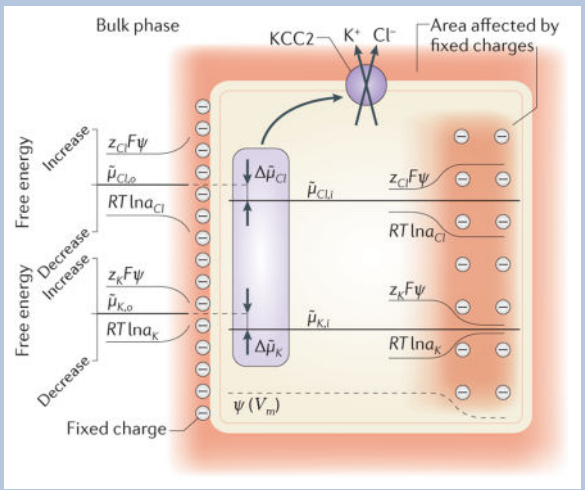
The equilibrium condition for Na^+–K^+–$2Cl^-$ cotransporters (NKCCs) is obtained in an analogous manner:

$$[Cl^-]_i = [Cl^-]_o \sqrt{\frac{[Na^+]_o[K^+]_o}{[Na^+]_i[K^+]_i}} \iff E_{Cl} = \frac{1}{2}(E_K + E_{Na}) \qquad (2)$$

With typical values of E_K = −90 mV and E_{Na} = +74 mV[164,224], KCCs could reduce $[Cl^-]_i$ down to 4.4 mM (E_{Cl} = −90 mV), and NKCCs could increase $[Cl^-]_i$ up to >90 mM (E_{Cl} of −8 mV). However, such theoretical limits can be achieved only in the absence of opposing Cl^- fluxes such as ‘leaks’ across channels. For instance, in the simultaneous

presence of KCC-mediated Cl^- extrusion and $GABA_A$ receptor ($GABA_AR$)-mediated Cl^- influx, a dynamic steady state will be established in which changes in Cl^- conductance or KCC activity will be reflected in $[Cl^-]_i$. This classical pump–leak relationship[273] is made use of in experiments addressing the transport capacity of KCC2 and NKCC1 by experimentally inducing an exogenous excess or deficit of Cl^-, respectively, in a neuron[5,146,185,274,275].

Equations 1 and 2 depend on bulk ion concentrations. However, binding of ions to transporters and channels takes place in the aqueous layer, where local ion concentrations may differ substantially compared with those in the bulk phase because of fixed charges located on the phospholipid or protein surfaces[272,276,277]. This has been shown to increase transport rates (conductance) in ion channels by increasing the availability of permeant ions at the pore mouth[272] but has no effect on thermodynamic equilibria. This is because, at equilibrium, a local electric field has an equal but opposite effect on the local concentration-dependent (more precisely, ion activity-dependent) and electrical potential-dependent components ($RT\ln a$ and $zF\psi$, respectively) of the free energy of mobile ions. Therefore, there is no change in the electrochemical potential of Cl^- or K^+ ($\tilde{\mu}_{Cl,o}$, $\tilde{\mu}_{K,o}$) in the vicinity of, for instance, a negatively charged external membrane surface (see the figure). With regard to KCCs, the local electrostatic effects on $[Cl^-]$ and $[K^+]$ are inversely proportional, keeping the product, $[K^+]\cdot[Cl^-]$, constant. Thus, there is no change in the KCC equilibrium as defined by equation 1. An analogous situation arises within the cell if a spatial gradient of immobile charge density generates an electric field (a shift in the local membrane potential (V_m)) along which mobile ions such as Cl^- and K^+ equilibrate (see the figure). Again, there is no change in the electrochemical potential of mobile ions ($\tilde{\mu}_{Cl,i}$, $\tilde{\mu}_{K,i}$); and their transmembrane gradients ($\Delta\tilde{\mu}_{Cl}$, $\Delta\tilde{\mu}_{K}$), the product $[K^+]\cdot[Cl^-]$ and the KCC equilibrium remain unaffected. In other words, the local shifts in V_m and E_{Cl} (and E_K) are equal, which keeps the transmembrane driving force of Cl^- (and K^+) constant (see Supplementary Information S2 (text))[278,279]. Similarly, the equilibrium of NKCCs is insensitive to local electrostatic effects (equation 2). Thus, in contrast to a recent study[280], the equilibrium and reversal of Cl^- transport by cation-chloride cotransporters are correctly predicted on the basis of bulk ion concentrations[281].



KCC2

The main Cl^- extruder to promote fast hyperpolarizing postsynaptic inhibition in the brain is KCC2 (TABLE 1). It is abundantly expressed in most mature mammalian central neurons, with very little or no expression in PNS neurons and non-neuronal cell types[2,6,53–55]. KCC2 is the only KCC isoform that is not expressed in glia[53,56,57]. The two N-terminally spliced variants, KCC2a and KCC2b, have similar ion-transport properties when expressed in human embryonic kidney (HEK) cells[58]. The expression of KCC2a remains relatively low throughout life, whereas KCC2b is strongly upregulated during postnatal life in mice and rats[3,58,59] and constitutes up to ~90% of total KCC2 protein in the adult murine cortex[58,59]. Of note, most studies on KCC2 expression have used mRNA probes and antibodies that detect both KCC2a and KCC2b[58]. Thus, unless stated otherwise, 'KCC2' here refers collectively to both splice variants. Certain subpopulations of adult CNS neurons, such as the dopaminergic neurons of the substantia nigra[60] and most neurons of the thalamic reticular nucleus[61–63], lack KCC2.

The upregulation of KCC2 expression is part of neuronal differentiation[54,64–66], and this is consistent with a developmental gradient in the onset of KCC2 expression from caudal-to-rostral regions of the CNS[3,54,67]. In the spinal cord and brainstem of rodents, perinatal KCC2 expression levels are high and comparable to those observed in older animals[3,59,68,69], whereas in more rostral parts such as the cortex, robust upregulation of KCC2 commences by the time of birth[2,3,54,67,69] and undergoes a steep increase during the first postnatal month[2,10,67,70,71]. Interestingly, low levels of *Slc12a5* mRNA and protein can already be detected at early embryonic stages[54,65,72]. Indeed, knockout and overexpression studies point to a critical structural role of KCC2 in the maturation of neurons in the fetal nervous system[65,73,74].

In keeping with the timing of other milestones of CNS development[75], the developmental upregulation of KCC2 with regard to birth is both brain region- and species-specific[2]. While rats are born with very low KCC2 expression and depolarizing $GABA_AR$-mediated responses in cortical neurons, in the guinea pig KCC2 is upregulated *in utero* and cortical neurons show hyperpolarizing $GABA_AR$ responses at birth[2]. In the human neocortex, *SLC12A5* mRNA undergoes robust upregulation during the second half of gestation[37,41,76] (FIG. 1a), and there are immunohistochemical data indicating that from the 25th postconceptional week onwards, most cortical neurons express KCC2 (REFS 77,78). In contrast with the conclusions of a widely cited study that suggested that KCC2 is predominantly expressed postnatally[79], the above data collectively indicate that, unlike in rodents, massive upregulation of KCC2 in the human neocortex begins prenatally.

Neuron-specific expression of KCC2, as studied in mice, is ensured by multiple mechanisms, including two neuron-restrictive silencing elements (NRSEs) associated with *Slc12a5* (REFS 80–82) and transcription factors of the early growth response (EGR) family[70,83] (FIG. 2). Pathways regulating neuron-restrictive silencing factor (NRSF), which binds to NRSEs, may contribute to the downregulation of KCC2 (see below) during epileptogenesis[84] (BOX 3). Brain-derived neurotrophic factor (BDNF) and its receptor tropomyosin-related kinase B (TRKB) are thought to promote the developmental

upregulation of mRNA encoding KCC2b[83,85] (FIG. 2c) through extracellular signal-regulated kinase 1/2 (ERK1/2)-dependent expression of EGR4 (REF. 83). However, total KCC2 expression decreased by less than ~50% when EGR4 signalling was blocked[70], suggesting that KCC2 transcription is under the control of additional mechanisms, including upstream stimulating factor 1 (USF1) and USF2 (REF. 86). There is also evidence for steroid hormone-dependent effects on KCC2 expression, which has important implications for gender differences in the propensity to early-life seizures[87,88].

Box 3

Regulation of KCC2 by TRKB and calpain

Deficits in K^+–Cl^- cotransporter 2 (KCC2) expression, which are often associated with decreased efficacy of GABAergic inhibition and the emergence of depolarizing $GABA_A$ receptor ($GABA_AR$)-mediated currents, have been documented following experimental seizures[17,20,282,283] and in models of cerebral ischaemia[22,284], traumatic brain injury[16,19,285] and neuropathic pain[18,21,23]. The downregulation of KCC2 is part of a spectrum of changes associated with cellular de-differentiation following neuronal trauma[15,176]. In parallel with, or as a result of, changes in its serine 940 phosphorylation status[32,141], KCC2 is cleaved at its C-terminal domain by the Ca^{2+}- and brain-derived neurotrophic factor (BDNF)-activated protease calpain, leading to a loss of a ~20–40 kDa fragment[23,150,151,286] (FIG. 1d) and reduction of both total and surface-expressed KCC2 protein. The part of the C-terminal domain that is removed contains many of the sites that are critical for the function and regulation of KCC2 (REFS 141,215) (FIG. 1d), and it is thus not surprising that calpain-mediated cleavage of KCC2 compromises neuronal Cl^- extrusion capacity[150,151]. Furthermore, both serine 940 dephosphorylation and calpain activation have been suggested to regulate the lateral mobility of KCC2 within the plasma membrane, with potential consequences for the synaptic localization of AMPA receptors[29,32,151]. As the C-terminal domain of KCC2 is also important for its ion-transport-independent functions[30] (including formation of the mature spine phenotype[28,29]), calpain cleavage of KCC2 is likely to contribute to the changes in dendritic spines associated with calpain activation (REFS 32,287,288).

In cortical and spinal cord neurons, trauma- or seizure-induced downregulation of KCC2 involves activation of NMDA receptors (NMDARs), tropomyosin-related kinase B (TRKB), calpain and probably protein phosphatase 1 (REFS 17,23,149,150,152). Downregulation of KCC2 is absent in mice carrying a point mutation that uncouples TRKB from phospholipase Cγ1 (PLCγ1)[152], and, at least in the hippocampus, loss of KCC2 protein triggered by brain-derived neurotrophic factor (BDNF) also takes place in the presence of NMDAR inhibitors[17]. This is intriguing, as PLCγ1 activation seems to be one of the most well-defined ways in which TRKB activation promotes epileptogenesis[289,290]. In models of seizures and trauma, the most prominent loss of KCC2 mRNA and protein takes place in regions with the highest increase in BDNF and TRKB expression[17,92].

In immature neurons, experimentally-increased BDNF–TRKB signalling promotes KCC2 expression[83,85] (see also REF. 291). This contrast to the situation in the adult

brain is likely to be related to the qualitative difference in TRKB phosphorylation and activation of its downstream cascades, such as PLCγ1, in immature and mature cortical neurons[292,293]. Interestingly, in damaged mature neurons, BDNF may resume its ability to promote KCC2 expression soon after an acute insult[21,294]. This supports the idea that acute downregulation of KCC2 associated with excitotoxicity and an energy crisis, such as ischaemia and seizures, reflects an adaptive response to promote neuronal survival and potential for rewiring[15]. Indeed, recent work has shown that reinduction of synaptic plasticity by chronic exposure to the serotonin reuptake inhibitor fluoxetine involves upregulation of BDNF and downregulation of KCC2 (REF. 25).

An important question is whether developmental upregulation of KCC2 is influenced by neuronal activity[89]. Chronic exposure to glutamatergic, GABAergic or voltage-gated Na^+ channel antagonists had no major effect on KCC2 levels in cortical neurons *in vitro*[71,90] (see also REFS 10,91–95). Likewise, upregulation of KCC2 is unaffected in VIAAT (vesicular inhibitory amino acid transporter)-knockout mice, despite the complete absence of GABAergic synaptic transmission[96].

NKCC1

NKCC1 is the principal transport mechanism responsible for Cl^- uptake and for the depolarizing $GABA_AR$ responses of immature neurons[10,94,97] (TABLE 1). By contrast, in some other neuronal populations, such as the brainstem superior olivary complex, other — probably HCO_3^--dependent — mechanisms of Cl^- uptake are important[69]. For example, depolarizing GABA actions in dendrites of mature pyramidal neurons[98,99] are fully attributable to HCO_3^--mediated $GABA_AR$ currents and associated depolarizing extracellular K^+ concentration ($[K^+]_o$) transients[100,101] (see below).

In line with the nearly ubiquitous expression of NKCC1 (REFS 102,103), *Slc12a2* includes a promoter region that is characteristic of a housekeeping gene[104]. A substantial portion of *Slc12a2* mRNA in the adult rodent CNS and PNS is found in glial cells[105,106]. NKCC1 has been suggested to undergo a global downregulation during development in the rat brain and human brain[79,107,108]. However, other studies have reported a developmental upregulation of mRNA encoding NKCC1 in the CNS of rodents and humans[37,50,67,69,109–112] (FIG. 1a). The gene encoding NKCC1 produces two splice variants, NKCC1a and NKCC1b[104,112,113]. Expression of NKCC1b, which lacks exon 21, is higher than that of NKCC1a in the adult human brain[112,113]. Therefore, the apparent downregulation of NKCC1 (REFS 79,107,108) may be explained by the use of probes and antibodies targeting exon 21 and thus detecting NKCC1a but not NKCC1b (see REFS 41,50), of which the latter undergoes more robust developmental upregulation[112].

KCC3

KCC3 mRNA and protein are widely expressed throughout development in the rodent CNS[114,115]. In the mouse CNS, KCC3 is mainly, but not exclusively, expressed in neurons[115] and appears to undergo developmental upregulation in the rodent CNS in parallel with KCC2 (REFS 114,115). In the human cortex, mRNA encoding KCC3 is highly expressed throughout life (FIG. 1a). Nevertheless, the physiological roles of KCC3 in

neurons remain largely unknown[5,6,51,116,117] (TABLE 1). The idea that KCC3 has important roles in the healthy nervous system is strongly supported by the fact that peripheral neuropathy associated with agenesis of the corpus callosum (ACCPN; also known as Andermann syndrome) is caused by genetic impairment of KCC3 (REFS 46,116,118). Interestingly, neuron-specific knockout of *Slc12a6* in mice has been demonstrated to recapitulate most of the neuropathic features of ACCPN[119]. Of the two main splice variants, KCC3a and KCC3b, KCC3a is preferentially expressed in the CNS and PNS[56,114,115].

KCC1 and KCC4

In the rodent CNS, mRNA encoding KCC1 is expressed in neurons and non-neuronal cells at relatively low levels[2,61]. In the embryonic brain, it is exclusively detected in the choroid plexus[54]. mRNA encoding KCC4 is abundant in the embryonic ventricular zone[54] but is present at low levels in the adult rodent CNS, with the exception of the suprachiasmatic nucleus in the rat[56]. In the adult mouse brain, KCC4 protein is mainly detected in cranial nerves, brainstem and spinal cord[120]. There is no obvious CNS phenotype in mice lacking KCC1 (REF. 121) or KCC4 (REF. 122). In the human cortex, the expression of both KCC1 and KCC4 is very low (FIG. 1a).

Post-translational regulation of CCCs

The mere presence of CCC protein, even in abundant quantities in the plasma membrane, does not imply that the transporter is active[11,68,69,123]. Like a closed ion channel, an ion transporter may be completely inactive despite the presence of a strong driving force. Once activated, a transporter's capacity is determined by the number of operational transporters in the plasma membrane as well as the unitary transport rate (also known as ion-turnover or ion-translocation rate). Membrane trafficking, (de)phosphorylation of key residues and proteolytic cleavage all kinetically regulate the transport capacity of CCCs (FIGS 1d,2b).

The kinetic regulation of NKCC1 has been extensively studied in secretory epithelia, where it has been shown to be under phosphorylation-dependent control by secretagogues[7]. NKCC1 is sensitive to changes in a cell's $[Cl^-]_i$ (REFS 7,124). A fall in $[Cl^-]_i$ below the physiological 'set point' leads to direct phosphorylation of NKCC1 at specific N-terminal residues, its functional activation and thereby restoration of $[Cl^-]_i$ (REFS 124,125). Conversely, an increase in $[Cl^-]_i$ promotes Cl^- extrusion by KCC2 (REF. 126), indicating that NKCC1 and KCC2 are reciprocally regulated by $[Cl^-]_i$. An interesting hypothesis is that WNKs (with no lysine kinases), acting through STE20-type kinases (SPAK and OSR1 (oxidative stress-responsive kinase 1)), may function as part of the Cl^--sensing[127] regulatory pathway that alters the phosphorylation states, and consequently the activities, of KCC2 and NKCC1 in a physiologically concerted manner[128–130]. Upon their activation by upstream kinases, such as WNKs, SPAK and OSR1 bind to and phosphorylate the cytosolic N-terminal tail of NKCC1 and stimulate Cl^- uptake, whereas protein phosphatase 1 (PP1)-mediated dephosphorylation of NKCC1 has the opposite effect[131–138] (FIG. 1d). C-terminal phosphorylation of KCC2 by SPAK and OSR1 is likely to decrease KCC2-mediated K^+–Cl^- cotransport[129,139,140]. The exact signalling pathways remain to be identified in neurons[44,141].

In immature cortical neurons, a substantial part of the total cellular KCC2 protein pool resides in cytosolic vesicles or in an inactive state in the plasmalemma[11,142,143]. Dephosphorylation of threonine residues 906/1007 in the C terminus[139] is likely to be involved in the activation of KCC2 and in the consequent development of hyperpolarizing $GABA_AR$ signalling [141] (see also REF. 144). In immature hippocampal neurons, neonatal seizures trigger a fast and pronounced enhancement of KCC2-mediated Cl^- extrusion, leading to 'precocious' hyperpolarizing $GABA_AR$ responses [11]. An analogous situation has been described in spinal cord injury[145]. This effect is caused at least in part by an activity-induced increase in the insertion of KCC2 into the plasma membrane and is likely to act as an endogenous safety mechanism in immature neurons to counteract the massive increases in intracellular Cl^- loads induced by seizures and trauma. Changes in KCC2 membrane trafficking involve TRKB, protein kinase C (PKC) and 5-HT_{2A} serotonin receptors[11,145,146], and phosphorylation of KCC2 by PKC at serine 940 decreases the rate of internalization of KCC2 from the plasma membrane[147].

By contrast, in mature neurons, prolonged intense neuronal firing results in sustained increases in $[Ca^{2+}]_i$ and leads to downregulation of KCC2 (REF. 148). The underlying mechanisms are likely to include PP1-dependent dephosphorylation of KCC2 at serine 940 and calpain-mediated cleavage[149–151] (see also REF. 148) (BOX 3). Plasmalemmal KCC2 undergoes rapid endocytosis and proteolysis in response to seizures, resulting in loss of hyperpolarizing responses to GABA[150,152]. The qualitatively opposite changes in KCC2 ion-transport functions in response to seizures in mature versus neonatal neurons are probably attributable to the age-dependence of TRKB activation (BOX 3) and of CNS energy consumption[75]. The basal turnover of total cellular KCC2 protein in mature neurons is slow, lasting several hours, as seen in hippocampal slices and cultures[2,150]. The rate of turnover of plasmalemmal KCC2 in healthy neurons has not been measured so far (see REF. 150) but, of note, following overexpression of KCC2 in HEK-293 cells, the entire functional cell-surface pool is recycled every 10 minutes[147].

Given that optimizing energy metabolism is a major determinant of the evolution and overall 'design' of the CNS[14,153], it is interesting that the brain-type creatine kinase (CKB) interacts with and activates both KCC2 and KCC3 (REFS 154,155). There is also evidence for co-regulation of and a direct interaction between KCC2 and the Na^+/K^+ ATPase[11,40,156], suggesting that they may form an ion-transport metabolon[15].

CCCs regulate inhibitory signalling

A CNS neuron typically receives input from thousands of excitatory and inhibitory synapses. An inhibitory synaptic input decreases the spiking probability of the target neuron, whereas an excitatory synapse has the opposite effect. The increases in the anion conductance that are gated by $GABA_ARs$ are spatially restricted according to the cell type-specific innervation patterns of distinct interneuron types[157]. Moreover, the GABAergic conductances — as well as the corresponding inhibitory postsynaptic currents (IPSCs) — are temporally restricted by the receptor channels' kinetics. There are two ways in which $GABA_AR$ activity can inhibit the target neuron. In a short-circuiting process known as shunting inhibition, which can take place at any level of the membrane potential (V_m), the

increase in conductance that results from $GABA_AR$ activation suppresses the spatial and temporal summation of depolarizing membrane responses to excitatory postsynaptic currents (EPSCs) and functionally excitatory intrinsic currents[158,159]. Voltage inhibition is caused by hyperpolarizing IPSPs and is enabled by KCC2. The hyperpolarizing IPSP outlasts the original conductance change and the associated shunting inhibition. As it spreads along the neuronal membrane, the IPSP is attenuated in a manner dictated by the membrane space and time constants[160,161].

If $GABA_AR$ channels were exclusively selective for Cl^-, all neurons with effective Cl^- extrusion would produce hyperpolarizing IPSPs because the equilibrium potential of Cl^- (E_{Cl}) would be more negative than the resting membrane potential (V_{rest}). However, $GABA_ARs$ and GlyRs mediate currents carried not only by Cl^- but also by HCO_3^-, with a HCO_3^-/Cl^- permeability ratio of 0.2–0.4 (REFS 161,162). The equilibrium potential of HCO_3^- (E_{HCO3}) is maintained at a much more positive level than E_{Cl}, at around −10 to −20 mV by pH-regulatory mechanisms (Supplementary information S1 (figure)). Therefore, the reversal potentials (E_{GABA} and E_{Gly}) of $GABA_AR$ currents and GlyR currents are more positive than E_{Cl}, and this deviation becomes progressively larger with a decrease in $[Cl^-]_i$ (REFS 1,161). In neurons with high $[Cl^-]_i$, the HCO_3^- component has little influence on E_{GABA}, and the driving force of the $GABA_AR$-mediated current (DF_{GABA}) is depolarizing. In a neuron with functionally active KCC2 and a consequent low $[Cl^-]_i$, E_{Cl} is more negative than V_{rest} and DF_{GABA} can either be hyperpolarizing or depolarizing, depending on the levels of the internal and external Cl^- and HCO_3^- concentrations[163,164] (Supplementary information S1 (figure), Supplementary information S2 (text, equation 9)). Certain types of adult neurons, such as neocortical pyramidal neurons and dentate granule cells, equipped with highly active KCC2-mediated Cl^- extrusion, exhibit moderately depolarizing HCO_3^--dependent IPSPs, even under resting (quiescent) conditions[163,164].

Slightly depolarizing postsynaptic $GABA_AR$ responses can sometimes exert a stronger inhibitory action than hyperpolarizing ones as a result of the inactivation of voltage-gated conductances. Strongly depolarizing GABAergic synaptic responses can be functionally excitatory, and they are termed ‘GABA PSPs’. Such responses are based on a high $[Cl^-]_i$, and they are mainly seen in immature or diseased neurons[40,48,165–168].

Neurons also harbour various extrasynaptic $GABA_ARs$ and GlyRs, which have an exceptionally high agonist affinity. They are found in all subcellular compartments of neurons, and current data suggest that the axon proper (the entire axon with the exception of the axon initial segment (AIS) and presynaptic boutons) contains only such high-affinity $GABA_ARs$ [169]. Extrasynaptic $GABA_ARs$ and GlyRs mediate tonic inhibition[170,171], which is often considered to be of a purely shunting type. However, NKCC1-dependent depolarizing and excitatory tonic $GABA_AR$ currents have been identified in somatic recordings in immature neurons[172,173] and in axons of adult neurons[169].

Development and subcellular targets of inhibition

Endogenous network activity promoted by NKCC1-dependent depolarizing $GABA_AR$ signalling is thought to be crucial for the development of neuronal connections before the maturation of sensory inputs[6,9,174,175]. Depolarizing $GABA_AR$ signalling, typically acting in

concert with glutamatergic mechanisms and intrinsic excitatory currents[172], generates intracellular Ca^{2+} transients that activate downstream cascades with trophic functions[9,174]. The developmental upregulation of KCC2 reduces the depolarizing action of GABA, ending its trophic effect; however, this may be resumed during post-traumatic dedifferentiation of neurons[15,176]. The mechanisms and consequences of depolarizing GABA actions during neuronal maturation have been extensively reviewed[6,9,174,175].

The causal role of KCC2 in the ontogenesis of $GABA_AR$-mediated hyperpolarization was first demonstrated by *Slc12a5* knockdown in cortical pyramidal neurons[2]. This finding was corroborated in subsequent studies[4,5,29,73,151,177–179] (TABLE 1). Furthermore, early over-expression of KCC2 is sufficient to establish precocious hyperpolarizing $GABA_AR$-mediated signalling [148,180–182] (TABLE 1). Work on adult cerebellar Purkinje neurons demonstrated no change in Cl^- extrusion capacity in mature neurons with a targeted loss of KCC3, whereas cell-specific knockout of the gene encoding KCC2 dramatically reduced the ability of Purkinje neurons to extrude Cl^- in the absence and presence of experimental Cl^- loading[5].

Recent *in vitro* studies demonstrated the existence of spatial differences in the values of E_{GABA} and DF_{GABA} (REFS 183,184) that are attributable to subcellular heterogeneity of CCC localization[185,186]. In neocortical and hippocampal principal neurons, E_{GABA} and DF_{GABA} shift towards more hyperpolarizing values from the AIS to the soma as a result of high NKCC1 levels and negligible levels of KCC2 in the AIS[183,185–187] (but see REF. 188). Although the AIS-targeting axo-axonal interneurons have a depolarizing effect, this does not immediately imply that they are functionally excitatory. A moderately depolarized E_{GABA} value in the AIS will render inhibition purely shunting near the action potential threshold. Accomplishing inhibition with minimal voltage responses may explain the functional significance of NKCC1 expressed in the AIS. Notably, $GABA_ARs$ at the AIS have been shown to act as gatekeepers that control pyramidal cell firing[189].

The above data indicate that NKCC1 and KCC2 are targeted to different compartments during neuronal maturation. Exon 21, which is not present in NKCC1b, contains a dileucine motif that is implicated in the basolateral versus apical targeting of NKCCs in polarized epithelial cells[190]. In neuronal differentiation, this would tentatively suggest (see REF. 191) that NKCC1a and NKCC1b are targeted to the dendrites and axons, respectively. Moreover, silencing the expression of the cytoskeletal scaffold protein ankyrin G dismantles the AIS and causes axons to acquire properties of dendrites, including the expression of KCC2 and, strikingly, even the formation of ‘axonal spines’ (REF. 187).

Why hyperpolarizing inhibition?

Merely shunting the postsynaptic membrane in the absence of any voltage change will decrease the probability of spiking[192]. Therefore, an obvious question that emerges is: what is achieved by equipping neurons with a Cl^- extruder such as KCC2 that enables hyperpolarizing IPSPs? Most of the data on hyperpolarizing IPSPs have been obtained in experiments on quiescent *in vitro* preparations and, therefore, two important factors must be considered. First, the continuous network activities of neuronal circuits lead to activity-

dependent Cl^- loading; and, second, there is no well-defined V_{rest} under *in vivo* conditions. Both of these factors will have a powerful impact on $DF_{GABA,}$ as explained below.

By definition, a shunt reduces input resistance and excitation of the plasma membrane by providing a route for outwardly directed current. Thus, DF_{GABA} always drives inhibitory outward currents when E_{GABA} is more negative than the momentary V_m. In a neuron engaged in network activity, inward currents generated by excitatory synapses and voltage-gated channels induce profound membrane depolarizations that are transient in space and time. For $GABA_AR$ signalling to be functionally inhibitory at a given moment, the local level of E_{GABA} must therefore be maintained by active extrusion of Cl^- at a level that is sufficiently negative to promote outwardly directed $GABA_AR$ currents. If the membrane conductance were to be increased by opening $GABA_ARs$ in the absence of Cl^- extrusion, the depolarizing drive of excitatory inputs would lead to an unremitting $GABA_AR$-mediated uptake of Cl^- (REF. 14), rendering E_{GABA} progressively more depolarizing and, finally, resulting in an excitatory action of GABA. Thus, extrusion of Cl^- by KCC2 is necessary for the maintenance of efficient $GABA_AR$-mediated inhibition during network activity. This also means that the amplitudes of hyperpolarizing IPSPs or hyperpolarizing E_{GABA} values observed in quiescent neurons do not provide valid estimates of their counterparts and of DF_{GABA} under the dynamic conditions *in vivo*.

The above discussion leads to further important corollaries. The non-uniform plasmalemmal distribution of ion channels and transporters[6] implies that the temporal dynamics of DF_{GABA} must be distinct in different subcellular compartments of a neuron, as dictated by the local ionic loads and leaks; by the diameter (or surface-to-volume ratio) of a given compartment; and by the presence or absence of cytosolic carbonic anhydrase activity, which affects the rate of the channel-mediated Cl^-–HCO_3^- shuttle and thereby the local-level E_{GABA} and DF_{GABA} (REFS 6,176,193) (Supplementary information S2 (text)). Various types of interneurons show astonishingly variable, anatomically distinct patterns of innervation of their targets[157]. Therefore, it is likely that the subcellular localization and efficacy of plasmalemmal KCC2 and other anion-regulatory proteins add to the multitude of mechanisms that are known to underlie the integration of excitatory and inhibitory synaptic signals. Because of technical reasons, intracellular recordings *in vivo* are typically carried out in cell bodies, and therefore they do not provide correct estimates of E_{GABA} and DF_{GABA} in dendrites during network activities[194]. Thus, at the moment, tackling the above ideas is largely restricted to using computational models[195]. Experimental approaches would require reliable dyes for *in vivo* imaging of neuronal Cl^-, combined with simultaneous measurements of the V_m. As pointed out by several researchers, including pioneers on its development, Clomeleon[196] — a widely used first-generation optogenetic sensor[197] — has a Cl^- affinity that is far beyond the physiologically relevant concentration range[196]. Fortunately, there are promising methodological developments to solve such problems[196,198].

CCCs control ionic plasticity

Neurons in the living CNS are never at rest, and their plasmalemmal Cl^- and HCO_3^- gradients are determined moment by moment by the pump–leak relationship between CCCs

and $GABA_A$Rs, as well as other channels and transporters (Supplementary information S2 (text))[1,15]. These mechanisms underlie the phenomenon of ionic plasticity[6,15,176,199,200], a hallmark of $GABA_A$R-mediated transmission that is based on the dynamic nature of DF_{GABA} (REFS 100,201,202).

Repetitive stimulation of GABAergic inputs in hippocampal pyramidal neurons reduces the amplitude of hyperpolarizing IPSPs or IPSCs, and this is often followed by a change in their polarity[100,202,203]. Dendrites, with their high surface-to-volume ratio, are highly prone to such fast activity-dependent E_{Cl} shifts. In hippocampal CA1 neurons that show robust hyperpolarizing IPSPs *in vitro*, functionally excitatory GABAergic responses can be induced by high-frequency stimulation[100]. Such stimulation causes intense interneuronal firing, a consequent HCO_3^--dependent increase in $[Cl^-]_i$ and a large depolarizing shift in DF_{GABA} in the target pyramidal neurons, which is sufficient for the activation of NMDA receptors (NMDARs)[1,202]. Because CO_2 readily permeates neuronal membranes, intraneuronal HCO_3^- that is lost owing to net efflux through $GABA_A$Rs is replenished by the activity of cytosolic carbonic anhydrases[193,204] (Supplementary information S2 (text)). Under these conditions, the increase in $[Cl^-]_i$ leads to enhanced K^+–Cl^- extrusion by KCC2, and to a consequent increase in $[K^+]_o$, which further depolarizes both neurons and glia in a non-synaptic manner[101] (FIG. 2a).

The mechanisms described above are likely to promote tetanus-induced long-term potentiation[203] as well as highly synchronized spontaneous network events, including seizures (see REFS 15,204), and may contribute to neuropathic pain[205]. Notably, carbonic anhydrase inhibitors, which are well known for their anticonvulsant actions, strongly inhibit activity-dependent E_{Cl} shifts and GABAergic excitation in slice preparations[100,101] and reduce neuropathic pain[206,207].

A large increase in membrane conductance leads to a fall in the membrane time constant, which can change a neuron's integrative properties from an integrate-and-fire mode to coincidence detection[208]. An intriguing, novel scenario that emerges here is that the high shunting conductance and the consequent rapid positive shift in E_{Cl} that are caused by pronounced activity of GABAergic inputs may counteract each other: a high inhibitory conductance can silence the cell, but this effect is opposed by the functionally pro-excitatory positive shift in E_{Cl}. Thus, fast ionic plasticity (FIG. 2a) may turn out to be a key parameter in the dynamic control of coincidence detection.

In addition to fast ionic shifts, E_{GABA} and E_{Gly} are subject to transcriptional and post-translational modifications of CCCs (see above)[6], thereby extending the temporal domains of ionic plasticity to a lifelong timespan that is relevant to neuronal development, ageing and trauma[15] (FIG. 2).

CCCs in dendritic spine formation

Most excitatory synapses are formed on dendritic spines, and most inhibitory inputs are made onto dendritic shafts[157,209]. The steep developmental increase in KCC2 expression in the rodent cortex[3,71,143,210] is associated with intense synaptogenesis[30,143,211,212], and a considerable proportion of the KCC2 in cortical neurons is located in, or in the vicinity of,

dendritic spines[29,142,143,186]. In humans, the onset of massive cortical synaptogenesis[213,214] and robust upregulation of KCC2 commences at the third trimester of pregnancy[76–78]. Recent work has demonstrated a role for KCC2 in the formation of dendritic spines that is not dependent on its ability to transport ions[28,30,31] (FIG. 1c). Neurons in cortical cultures from *Slc12a5*$^{-/-}$ mice exhibited elongated filopodium-like dendritic protrusions and a reduced number of functional excitatory synapses[28]. Strikingly, transfection of *Slc12a5*$^{-/-}$ neurons with either wild-type or an N-terminally deleted transport-deficient KCC2 construct (KCC2-ΔNTD) rescued spines[28,31]. Furthermore, *in vivo* overexpression of KCC2, KCC2-ΔNTD or the cytosolic C-terminal domain of KCC2 during the late embryonic period induced a lifelong increase in the number of spines in cortical pyramidal neurons[30,31]. Although both the intracellular N- and C-terminal domains of KCC2 are necessary for its function as a K^+–Cl^- cotransporter[28,31,150,215], the above data indicate that the structural role of KCC2 in spine formation is mediated by its C-terminal domain. It has been suggested that the actin-associated protein 4.1N (also known as band 4.1-like protein 1) directly interacts with the C terminus of KCC2 (REFS 28,65). Preventing expression of 4.1N or of actin depolymerization in mature neurons increased the lateral diffusion of KCC2 away from excitatory synapses in cultured hippocampal neurons[151]. Changes in the localization of KCC2 between spines and the dendritic shaft may regulate the efficacy of excitatory synapses by physically constraining AMPA receptors in spine heads[29,151] (FIG. 1c).

The molecular cascades that underlie the developmental upregulation of KCC2 in spines remain poorly understood[6,32,44,216]. Embryonic overexpression of BDNF results in precocious upregulation of KCC2 and in an increased number of GABAergic and non-GABAergic synapses[85]. The cell adhesion molecule neuroligin 2 (NLGN2)[217,218] appears to maintain dendritic spines by promoting KCC2 expression[71]. Alterations in the regulation of NLGN2 and/ or KCC2 may turn out to be relevant in developmental neuropsychiatric disorders, such as autism and schizophrenia[37–39,219]. Whether the recently discovered interactions between KCC2 and the kainate-type glutamate receptor (KAR) auxiliary subunit NETO2 (REF. 220), as well as between KCC2 and KARs themselves[221], are important for the morphogenetic roles of KCC2 is not known.

Some interneurons have been shown to target spines[222]. In particular, somatostatin-expressing interneurons exert a $GABA_AR$-mediated inhibitory effect on glutamatergic Ca^{2+} transients in individual spines[223]. Although much of the KCC2 in this location may have a structural role, there are no data available on the presence of KCC2-mediated Cl^- extrusion in spines. Notably, activity-dependent increases in $[Na^+]_i$ within spines can reach levels of up to 100 mM[224], which must be associated with comparable decreases in intraspine $[K^+]$. This would immediately reverse the direction of net Cl^- transport by KCC2 from extrusion to uptake, thereby producing a positive-feedback loop between the amount of excitation and net accumulation of both Cl^- and Na^+, as well as net influx of water. Thus, it is possible that the spinogenesis-promoting KCC2 protein fraction is not transport-active. This is an important novel hypothesis that has to be tested in future research.

CCCs in seizures and epilepsy

Experimentally induced defects in GABAergic transmission can provoke seizures *in vivo* and seizure-like activity *in vitro*. However, as discussed recently[15], the standard concept of excitation/inhibition balance (E/I balance) is unable to explain the aetiology and manifestation of epilepsies, which encompass a wide and heterogeneous spectrum of brain malfunctions and adaptive mechanisms[225]. Although the E/I balance concept has turned out to be useful in, for instance, understanding neuronal firing characteristics during distinct cortical states[226], it is obvious that suggesting an E/I imbalance as a cause of epileptogenesis and epilepsy is based on a circular argument (see REFS 15,227): the presence of seizures is taken as an indicator of an altered E/I ratio[228].

Because of the key functions of KCC2 and NKCC1 in controlling the efficacy of inhibition, many studies have addressed the roles of these ion transporters in the acute generation of seizures and in epileptogenesis (reviewed in REFS 15,36,40,41,228). The above E/I misconception has extended to the use of the 'NKCC1-to-KCC2 ratio' (as measured in quantifications of total mRNA and protein levels in samples of brain tissue) as a parameter of proneness to epilepsy and other neurological disorders. However, among other caveats (see REF. 15), the total amounts of CCC mRNA or protein do not directly translate into functional efficacy.

The multiple and context-dependent roles of CCC functions in epilepsy are evident in light of the enhanced susceptibility to seizures that follows genetic impairment of KCC2 expression in mouse models[229,230] and in human patients[31,231], resulting in the loss of both its ion-transport-dependent and ion-transport-independent functions[28,31]. Observations on adult rat dentate granule cells show that, under normal conditions, spiking of these cells is strongly suppressed by KCC2-dependent GABAergic mechanisms[20,100,101]. Progressive downregulation of KCC2 after pilocarpine-induced status epilepticus decreased the efficacy of inhibition and abolished the function of the dentate gyrus as a hippocampal barrier against seizure activity arising in the entorhinal cortex[20]. In sharp contrast to the above two examples, a seizure-promoting action by KCC2 is seen in mature neurons under conditions that lead to GABA-mediated excitation paralleled by an increase in $[K^+]_o$, that are sufficient to trigger ictal events[100,101,204]. Notably, almost all interneurons that target perisomatic regions of the hippocampal CA1 area are activated during epileptiform discharges[232].

In hippocampal brain slices from adult patients with temporal lobe epilepsy, downregulation of KCC2 and upregulation of NKCC1 leads to depolarizing $GABA_AR$ responses in a subpopulation of subicular principal neurons. This has been implicated in the generation of spontaneous interictal-like (but not ictal-like) activity[233–235] (see also REF. 167). Inhibition of NKCC1 by bumetanide blocks interictal activity *in vitro* (for example, see REF. 234), but this does not imply that the drug would block ictal events. Indeed, the evidence for an anticonvulsant effect of bumetanide in neonates and adults is meagre[40,41]. Novel compounds, perhaps including CNS-permeant prodrugs of bumetanide[236] as well as activators of KCC2 (REF. 237), are needed for further research on CCCs as pharmacotherapeutic targets.

After the cloning of KCC2 (REF. 53), no variations in *SLC12A5* in human disease were reported for nearly two decades. Recently, a rare *SLC12A5* point variant (KCC2-R952H) was identified in an Australian family with early childhood onset of febrile seizures[31]. This missense variant led to a defect in neuronal Cl^- extrusion capacity and cortical dendritic spine formation in rodent neurons[31] (FIG. 1c,d). These data suggest that this is a *bona fide* susceptibility variant for febrile seizures, which might lead to other kinds of seizure disorders later in life. Support for this conclusion was gained from subsequent identification of KCC2-R952H and another point variant, KCC2-R1049H, in a French-Canadian cohort with idiopathic generalized epilepsy[231]. Although it is possible that an impairment of $GABA_AR$-mediated inhibition underlies the enhanced seizure susceptibility associated with KCC2-R952H, the decrease in the number of functional dendritic spines may lead to desynchronization at the level of neuronal networks, thereby promoting seizures[15,238].

CCCs and chronic pain

Epilepsy and neuropathic pain bear striking resemblances. Anticonvulsant agents are among the only effective treatments for neuropathic pain[239], and increasing evidence points to BDNF–TRKB- and calpain-mediated regulation of CCC function as common causes of epilepsy and neuropathic pain[23,240,241] (BOX 3).

The spinal dorsal horn is richly endowed with GABAergic and glycinergic interneurons, which provide inputs to dorsal horn neurons. The central terminals of primary afferents contain $GABA_ARs$ but are devoid of GlyRs[242]. The gate control theory of pain[243], which describes modulation of nociceptive processing at the first synapses of the pain pathway, was based, in part, on evidence that presynaptic inhibition of afferent fibres is mediated by interneurons in lamina I/II of the dorsal horn. This $GABA_AR$-mediated action results in depolarization of the presynaptic terminals of primary afferent fibres (primary afferent depolarization (PAD)) and the suppression of nociceptive signalling[244]. A small depolarization of the V_m, which is typical of PAD, produces presynaptic inhibition by inactivating voltage-gated Na^+ channels and consequently reducing transmitter release at the synapse[245].

Dorsal root ganglion (DRG) and trigeminal ganglion neurons express NKCC1, but not KCC2, providing an explanation for the high $[Cl^-]_i$ required for PAD[18,106]. Thus, CCCs are at the heart of the gate control theory of pain. NKCC1 expression in DRG neurons is also crucial for nociceptive functions that do not involve PAD. Notably, the Ca^{2+}-activated Cl^- channel anoctamin 1 depolarizes DRG neurons in response to nociceptive stimuli that evoke Ca^{2+} release from intracellular stores[246]. Plasticity of $GABA_AR$ responses in the presynaptic terminals of primary afferents may contribute to pathological pain in at least two ways: through a loss of $GABA_AR$-mediated inhibitory PAD caused by decreased expression of $GABA_ARs$ and through increased $[Cl^-]_i$ as a result of altered regulation of NKCC1, leading PAD to be converted to frank excitation of afferent fibres (FIG. 3a). In the case of peripheral nerve injury (PNI), a loss of $GABA_AR$-mediated responses has been observed in DRG neurons[247] and is paralleled by a reduction in PAD. There is also evidence for increased NKCC1 expression in response to peripheral inflammation[245], and inhibition of NKCC1 with intrathecally delivered bumetanide has been shown to reduce injury-induced

nociceptive hypersensitivity[248–250]. However, it has not been technically possible to measure $[Cl^-]_i$ in afferent terminals, and it is not known whether injury can lead to functional upregulation of NKCC1 at this critical subcellular site for nociception.

$GABA_AR$- and GlyR-mediated postsynaptic inhibition, which depends on KCC2 in projection neurons, is another feature of the dorsal horn pain gate. Following experimental PNI, a positive shift in E_{GABA} and E_{Gly}, which is attributable to loss of KCC2 activity in a subpopulation of lamina I and II neurons, generates depolarizing instead of hyperpolarizing GlyR- and $GABA_AR$-mediated responses[18]. Likewise, S*lc12a5* knockdown results in touch-evoked pain or allodynia[18]. An important question is whether allodynia is explained by touch-activated afferents gaining access to a nociception-specific pathway or by an amplification of postsynaptic responses in dorsal horn neurons that normally receive noxious and innocuous inputs. Recent evidence strongly suggests that nociception-specific neurons acquire a novel input in the innocuous range after PNI[251]. This demonstrates how innocuous sensory signals gain access to a pain- dedicated input pathway to the CNS (FIG. 3b). Interestingly, these effects are rapidly reversed by positive modulation of KCC2 (REFS 237,251). Other studies have pointed to changes in KCC2 expression in the spinal dorsal horn as a prominent feature of inflammation[245], metabolic nerve injury[252] and even opioid-induced hyperalgesia[253]. Similar changes in KCC2 expression have been demonstrated in spinal cord injury-induced spasticity and pain, suggesting a KCC2-based approach to therapeutics for these clinical conditions[21,145,254].

In PNI and opioid-induced hyperalgesia, there is strong evidence that microglial cell-derived BDNF has an essential role in modulating E_{GABA} and E_{Gly} through decreased KCC2 expression[240,255]. As in the post-traumatic cortex, the PNI-induced positive shift in E_{Gly}, and presumably E_{GABA}, parallels an influx of Ca^{2+} through NMDARs, resulting in calpain-mediated cleavage of KCC2 (REF. 23). Whether this involves activation of TRKB receptors by BDNF is unclear. However, BDNF signalling results in a loss of effective GABAergic inhibition in the spinal dorsal horn, resulting in nociceptive hypersensitivity[256]. Importantly, whereas blockade of spinal $GABA_ARs$ in naïve animals produces a neuropathic pain-like behavioural phenotype, $GABA_AR$ blockade following spinal application of exogenous BDNF reverses this BDNF-induced nociceptive hypersensitivity[257]. A similar mechanism may be at play in the brainstem, where chronic pain promotes a depolarization of E_{GABA}, through BDNF-mediated downregulation of KCC2, in descending pain facilitation neurons[258].

Summary and future directions

Beyond the view that functional CCCs are probably dimers of 12 transmembrane-domain monomers, there is little structural information about these transporters[42]. Although the high-resolution structure has been obtained for the C terminus of a bacterial CCC protein[43], the three-dimensional structure of the large and critically important C terminus of KCC2 has not been determined. There is also a striking lack of information about the intrinsic ion-transport rates of CCCs and their modulation by intracellular signalling cascades. Such data are crucial not only for understanding the fundamental molecular mechanisms and properties of CCC functions but also for the rational design of drugs targeting these transporters.

Accumulating evidence shows that CCCs are key molecules in shaping neuronal signalling and structure throughout an individual's lifespan. Research themes of particular future interest include CCC functions in short- and long-term plasticity. Explorations of the functions of excitatory synapses have yielded useful parameters such as synaptic weights that can be used in modelling of network events[158]. Much less analogous data are available for inhibitory signalling, and ionic plasticity based on CCCs adds a novel and exciting aspect to future research in this area. How does E_{GABA} change at subcellular sites in an individual neuron during network events? Do such changes have resonance frequencies of their own, thereby enhancing or suppressing various frequency bands during oscillatory activity? And would CCC-related abnormalities therein provide another link to neurological and psychiatric diseases, ranging from epilepsy to autism and schizophrenia? In addition, given the role of the hypothalamic–pituitary–adrenal axis in seizure disorders[259], research on the differential expression patterns of CCCs in neuroendocrine cells will also help to further clarify the molecular aetiologies of these and other types of CNS diseases.

The above is but a brief list of possible directions for future studies on CCCs, intended to provoke creative, high-risk and high-impact approaches in this rapidly expanding field of research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Glossary

Inhibitory postsynaptic potentials (IPSPs)	Synaptic potentials elicited by GABA or glycine that inhibit postsynaptic excitation and the generation of action potentials
Reversal potential	The membrane potential at which a channel-mediated current reverses its polarity
Driving force	The electrical potential difference that drives a conductive current. The driving force is calculated as the difference between the membrane potential and either the equilibrium (Nernst) potential of a single ion species or the reversal potential of a channel-mediated current
Bulk ion concentrations	Ion concentrations in intracellular and extracellular compartments in which the vicinity of the membrane surface has no effect. All

	intracellular microelectrode measurements of membrane potentials yield data on the voltage between these bulk phases
Shunting inhibition	Suppression of postsynaptic excitation that results from an increase in neuronal membrane conductance that is caused by activation of $GABA_A$ receptors or glycine receptors
Excitatory postsynaptic currents (EPSCs)	Inward currents elicited by excitatory neurotransmitters (typically glutamate) that depolarize the neuron to enhance spiking probability
Space and time constants	Reflect the passive electrical properties of neurons. The time constant (τ_m) is the product of membrane resistance and capacitance ($\tau_m = R_m \cdot C_m$) and defines the rate of change of a passive membrane potential (V_m) response evoked by a current pulse. The space constant (λ) quantifies the spatial extent of passively spreading signals in an elongated structure, such as a dendrite. Note that inducing an inhibitory conductance produces a decrease in both τ_m and λ
Equilibrium potential	The membrane potential at which a single ion species is at equilibrium across the membrane; given by the Nernst equation
Axon initial segment (AIS)	A structurally and functionally specialized region between the neuronal soma and axon proper that has a low voltage threshold for action potential generation and therefore often acts as the main site of spike initiation
Tonic inhibition	Inhibition resulting from activation of extrasynaptic high-affinity $GABA_A$ receptors
Integrate-and-fire	A situation in which excitatory inputs impinging on a neuron's dendritic tree are summed up both spatially (from different locations) and temporally (during a high-frequency sequence of excitatory signals) to trigger spiking
Coincidence detection	A situation in which spiking occurs in response to near-simultaneous, spatially distinct excitatory signals. If a large increase in the neuron's conductance (a fall in time and space constants) takes place (for example, because of GABAergic inhibition), an integrate-and-fire mode of operation will change into coincidence detection
Excitation/ inhibition balance (E/I balance)	The relative quantitative contributions of excitatory and inhibitory synaptic signals at the level of a single neuron or a neuronal network

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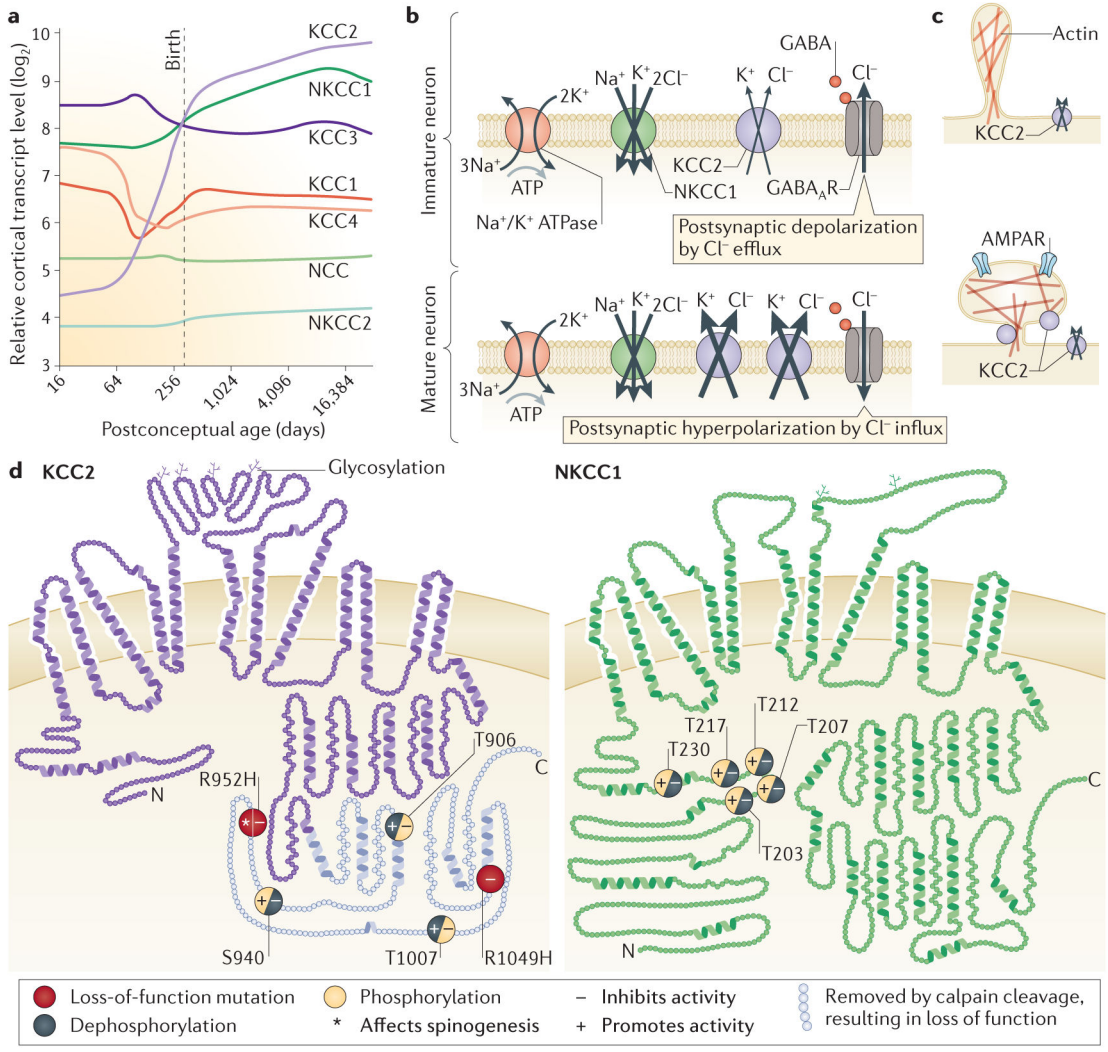

Figure 1. Developmental expression profiles, functions and secondary structures of CCCs

a | Line plots showing the average exon array signal intensity of cation-chloride cotransporter (CCC) transcripts in the human neocortex from the early fetal period to late adulthood. The profiles are qualitatively similar for the hippocampus, amygdala and cerebellar cortex. Of the five CCCs expressed in the human neocortex (K^+–Cl^- cotransporter 1 (KCC1), KCC2, KCC3, KCC4 and Na^+ – K_+–$2Cl^-$ cotransporter 1 (NKCC1)), only mRNA encoding KCC2 undergoes robust developmental upregulation. NKCC1 shows moderate postnatal upregulation, and KCC3 and NKCC1 are expressed at high levels throughout life while expression of KCC1 and KCC4 is low. The levels of NKCC2 and Na^+–Cl^- cotransporter (NCC) are below the level (6 on the *y* axis) for a gene to be considered as expressed according to the criteria described in REF. 295. Only the expression of KCC2 is neuron-specific. Expression data are from the data bank described in REF. 295 and accessible at the Human Brain Transcriptome website. **b** | NKCC1 and KCC2 control intracellular Cl^- concentration ($[Cl^-]_i$) in many central neurons. In immature neurons, the Na^+ gradient generated by the Na^+/K^+ ATPase drives cellular uptake of Cl^- via NKCC1 and KCC2 has a minor role. This generates a depolarizing Cl^- current across $GABA_A$ receptors

($GABA_AR$s). During neuronal maturation, functional KCC2 attains a high level of expression and the Cl^- current becomes hyperpolarizing. Cl^- extrusion by KCC2 is fuelled by the Na^+/K^+ ATPase-dependent K^+ gradient. NKCC1 and KCC2, like all CCCs, are electroneutral, in other words, they do not generate any current by themselves. **c** | Upregulation of KCC2 facilitates the structural and functional development of cortical dendritic spines in an ion-transport-independent manner, probably through effects on the actin–spectrin cytoskeleton. **d** | Secondary structures of human KCC2b and NKCC1b splice isoforms, highlighting residues critical for function. Dephosphorylation of KCC2 at threonine residues T906 and T1007 and phosphorylation at serine residue S940 are associated with functional activation. Phosphorylation by protein kinase C (PKC) at S940 promotes KCC2 membrane stability. Phosphorylation of NKCC1 by SPAK and OSR1 (oxidative stress responsive kinase 1) at the depicted N-terminal residues leads to functional activation. KCC2 transport function is inhibited by phosphorylation of T906 and T1007 and dephosphorylation at S940. Phosphorylation at T1007 and dephosphorylation at S940 are likely to be mediated by SPAK/OSR1 kinases, and protein phosphatase 1 (PP1), respectively. An arginine-to-histidine mutation at residue 952 (R952H), discovered in humans, results in loss of both transport activity and spinogenesis by KCC2. The approximate (~30 kDa) C-terminal fragment of KCC2 cleaved by calpain is shown in blue. Dephosphorylation of NKCC1 by PP1 at N-terminal threonine residues renders it transport-deficient. Putative glycosylation sites in KCC2 and NKCC1 are highlighted. AMPAR, AMPA receptor. Part **d** is adapted from 2-D models provided by B.Forbush, Yale University, New Haven, Connecticut, USA.

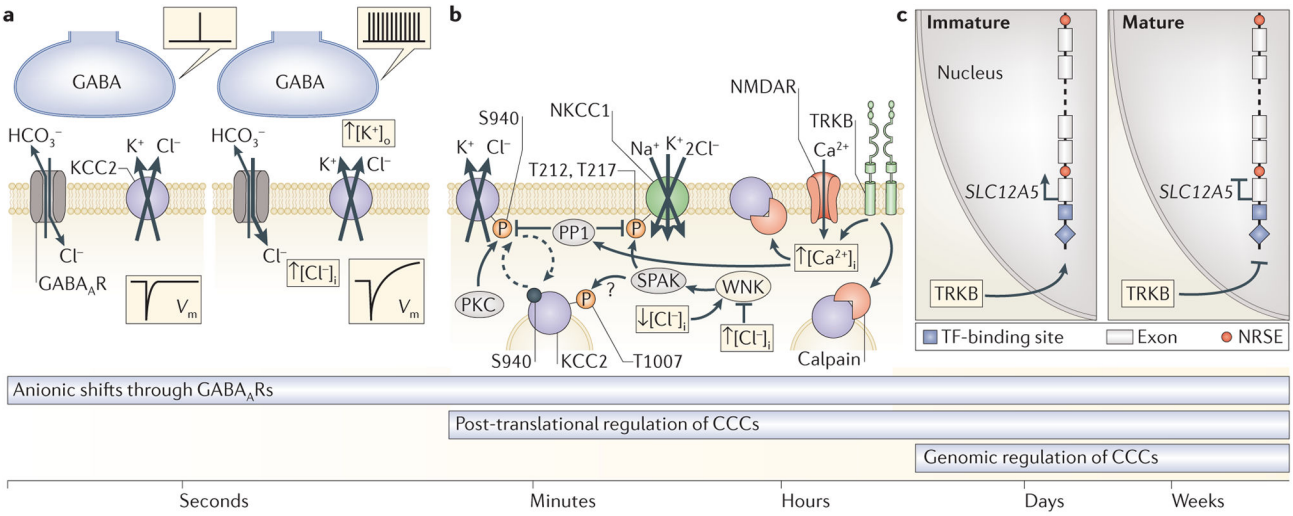

Figure 2. Mechanisms of ionic plasticity and their temporal domains

Cation-chloride cotransporters (CCCs) control ionic plasticity across various overlapping time scales that range from seconds to weeks, and beyond. **a** | Short-term ionic plasticity of GABAergic signalling is based on fast, activity-dependent transmembrane movements of Cl^- and HCO_3^-, which alter the driving force and polarity of GABA-induced currents and thereby cause changes in the postsynaptic membrane potential (V_m). On the left, a single presynaptic event leads to a hyperpolarization of V_m. On the right, repetitive activation of GABAergic terminals evokes a biphasic V_m response. In biphasic V_m responses, the depolarizing HCO_3^- current leads to an increase in $GABA_A$ receptor ($GABA_AR$)-mediated uptake of Cl^- and an increase in intracellular Cl^- concentration ($[Cl^-]_i$). The consequent K^+–Cl^- cotransporter 2 (KCC2)-mediated extrusion of K^+ increases the extracellular K^+ concentration ($[K^+]_o$) and has a depolarizing or even functionally excitatory action on V_m. **b** | Fast functional regulation of CCCs on a time scale of minutes to hours is mediated by post-translational mechanisms, including (de)phosphorylation of key residues on the intracellular domains of KCC2 and Na^+–K^+–$2Cl^-$ cotransporter 1 (NKCC1) (see also FIG. 1d) and calpain-mediated cleavage of KCC2. Constitutive membrane recycling (dashed arrows) of KCC2 is regulated by the phosphorylation state of the C-terminal serine residue S940. Phosphorylation of S940 by protein kinase C (PKC) limits clathrin-mediated endocytosis of KCC2. By contrast, protein phosphatase 1 (PP1)-dependent dephosphorylation of S940 leads to internalization of KCC2 and a reduction in neuronal Cl^- extrusion capacity following intense activation of NMDA receptors (NMDARs) and an increase in $[Ca^{2+}]$. Under such conditions, KCC2 is also C-terminally cleaved by the Ca^{2+}- and brain-derived neurotrophic factor (BDNF)-activated protease calpain, which results in irreversible inactivation of KCC2 (BOX 3). NKCC1 is kinetically regulated through a Cl^--sensing cascade involving WNK (with no lysine kinase) and the STE20-related kinases SPAK and OSR1 (oxidative stress responsive kinase 1; not shown). WNKs are allosterically modulated by Cl^-, with a decrease in $[Cl^-]_i$ leading to activation of SPAK and OSR1 by WNKs. Consequent SPAK-mediated phosphorylation of key N-terminal threonine residues (for example, T212 and T217; see also FIG. 1d) of NKCC1 results in its activation and Cl^- accumulation. By contrast, dephosphorylation of these residues by PP1 inactivates NKCC1.

Reciprocal regulation of transport activities of NKCC1 and KCC2 by the WNK SPAK and WNK OSR1 cascade has been demonstrated in heterologous expression systems and may also take place in neurons. At least one SPAK and OSR1 phosphorylation site (T1007) is found in KCC2b, the principal KCC2 splice variant found in neurons. However, it is not clear how phosphorylation of KCC2 by SPAK and OSR1 affects neuronal Cl^- extrusion capacity. **c** | Neuron-specific expression of KCC2 is ensured via multiple transcriptional mechanisms, including the actions of neuron-restrictive elements (NRSEs; also known as RE1), which silence *SLC12A5* (encoding KCC2) in non-neuronal cells and neuron-enriched transcription factors (TFs). Neuron-enriched TFs, for example, members of the early growth response (EGR; not shown) family, are sensitive to signalling by neurotrophic factors, such as BDNF and its receptor tropomyosin-related kinase B (TRKB), which exert qualitatively different effects on *SLC12A5* transcript expression in immature and mature neurons. The TRKB-mediated cascades may reverse during neuronal trauma, resulting in recapitulation of immature-like Cl^- homeostasis in diseased neurons (BOX 3). Long-term consolidation of changes in KCC2 following trauma or during epileptogenesis is likely to be mediated to an extent by the above transcriptional mechanisms.

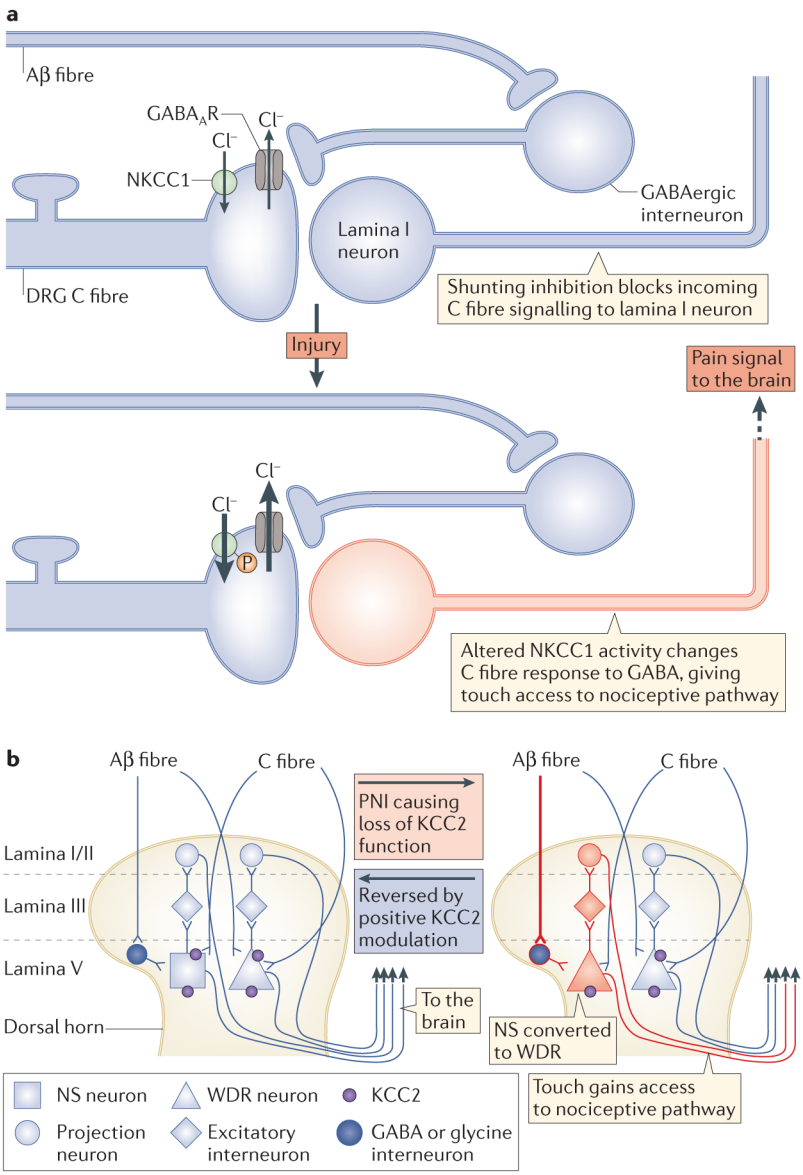

Figure 3. Cation-chloride cotransporters in pain

There are two major theories concerning the role of cation-chloride cotransporters (CCCs) in chronic pain, and more specifically in touch-evoked pain (allodynia). Under normal conditions (part **a**), the activation of Aβ fibres, the myelinated fibres responsible for light touch sensation, leads to primary afferent depolarization in C fibres and consequent presynaptic shunting inhibition of pain-conducting C fibres by dorsal horn GABAergic interneurons. This requires the expression of Na^+–K^+–$2Cl^-$ cotransporter 1 (NKCC1) in dorsal root ganglion (DRG) C fibre neurons. The inhibition blocks the signalling of the C fibres to lamina I projection neurons. Following injury, signalling cascades lead to phosphorylation and thereby kinetic activation of NKCC1 (FIG. 1d) in C fibre terminals. This may be linked to an increase in $[Cl^-]_i$, potentially converting Aβ fibre-mediated inhibition into frank excitation of C fibres and leading to activation of pain signalling via lamina I projection neurons. This provides a neurophysiological explanation for allodynia.

Very recent evidence suggests that nociceptive-specific (NS) neurons in the deep laminae of the dorsal horn (lamina V) lose KCC2 expression following peripheral nerve injury (PNI), thereby altering the response of these neurons to GABA and unmasking an Aβ fibre-mediated input to NS neurons, effectively converting them to wide dynamic range (WDR) neurons (part **b**). As in the scenario outlined in part **a**, this gives the Aβ fibre pathway access to pain signalling through feedforward activation of projection neurons in lamina I/II. Importantly, conversion of NS neurons to WDR neurons following PNI is reversed by positive modulation of KCC2 and this treatment also reduces touch-evoked pain in behavioural assays. $GABA_AR$, $GABA_A$ receptor.

Table 1

Effects of manipulation of CCC expression on neuronal chloride homeostasis

CCC	Model	Age	Effect	Refs
KCC2	Knockdown of gene encoding KCC2 in CA1 pyramidal neurons in rat organotypic HC slices	P11–13 (DIV 2–5)	Loss of hyperpolarizing DF_{GABA}	2
	KCC2-null mouse spinal motor neurons	E18.5	Excitatory responses to GABA and glycine in knockout but not wild-type neurons	73
	KCC2b-null mouse spinal motor neurons	P5–8	E_{IPSP} more positive than in wild-type neurons	178
	KCC2b-null mouse cultured cortical neurons	DIV 18–21	Lack of hyperpolarizing responses to GABA	4
	CA1 pyramidal neurons of compound-heterozygous KCC2 mice with lack of one allele and reduced expression of the second allele (mice retain ~20% of normal KCC2 brain expression)	P30	E_{GABA} more positive than in wild-type neurons	177
	KCC2 overexpression in cultured rat HC neurons	DIV 5–7	Hyperpolarizing shift in E_{GABA} to values observed in mature neurons; decrease in GABA-elicited Ca^{2+} responses	148,180
	KCC2 overexpression via *in utero* electroporation of rat or mouse layer 2/3 pyramidal neurons	P1–7	Hyperpolarizing shift in E_{GABA} to values observed in mature neurons; enhancement of Cl^- extrusion capacity	31,140, 181
	Knockdown of gene encoding KCC2 in cultured rat HC neurons	DIV 23–24	Loss of Cl^- extrusion capacity	29,151
	Cell-specific KCC2-null, Cre–*lox* mouse cerebellar Purkinje neurons	P25–64	~60% decrease in hyperpolarizing DF_{GABA}	5
	Cell-specific KCC2-null, Cre–*lox* mouse cerebellar granule cells	P30–62	Depolarizing shift in both V_m and E_{GABA}	5
	KCC2 overexpression in embryonic zebrafish spinal cord neurons	26–32 HPF	Shift from depolarizing to hyperpolarizing DF_{Gly}	182
	Knockdown of gene encoding KCC2 in zebrafish retinal ganglion cells	2.5–6 DPF	Loss of somatodendritic and inter-dendritic E_{GABA} gradients	179
KCC3	Cell-specific KCC3-null, Cre–*lox* mouse cerebellar Purkinje neurons	P25–64	No change in E_{GABA} or DF_{GABA}	5
	KCC3-null mouse cerebellar Purkinje neurons	P12–14	E_{GABA} more positive than in wild-type neurons	116
	KCC3-null mouse DRG sensory neurons	Adult	Increase in $[Cl^-]_i$ compared to wild-type neurons	51
NKCC1	Knockdown of gene encoding NKCC1 in newborn DGCs of adult mouse	P49–56	Loss of depolarizing DF_{GABA}	296
	NKCC1-null mouse CA1 pyramidal neurons	P1	Decrease in depolarizing DF_{GABA} ; decrease in GABA-elicited Ca^{2+} responses	94
	NKCC1-null mouse CA1 pyramidal neurons	P3–4	Loss of depolarizing DF_{GABA}	10
	NKCC1-null mouse CA3 pyramidal neurons	P6–7	Loss of depolarizing DF_{GABA}	97
	NKCC1-null mouse DGCs	P16–20	Loss of axosomatic E_{GABA} gradient	185

CCC, cation-chloride cotransporter; $[Cl^-]_i$, intracellular Cl^- concentration; DF_{GABA}, the driving force of the $GABA_A$ receptor ($GABA_AR$)-mediated current; DGC, dentate granule cell; DIV, day *in vitro*; DPF, days post-fertilization; DRG, dorsal root ganglion; E, embryonic day; E_{GABA}, the reversal potential of $GABA_AR$-mediated responses; E_{IPSP}, reversal potential of inhibitory postsynaptic potentials (IPSPs); HC,

hippocampal; HPF, hours post-fertilization; KCC, K^+–Cl^- cotransporter; NKCC, Na^+–K^+–$2Cl^-$ cotransporter; P, postnatal day; V_m, membrane potential.