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Review

Cation-Chloride Cotransporters and Neuronal Function

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Recent years have witnessed a steep increase in studies on the diverse roles of neuronal cation-chloride cotransporters (CCCs). The versatility of CCC gene transcription, posttranslational modification, and trafficking are on par with what is known about ion channels. The cell-specific and subcellular expression patterns of different CCC isoforms have a key role in modifying a neuron's electrophysiological phenotype during development, synaptic plasticity, and disease. While having a major role in controlling responses mediated by $GABA_A$ and glycine receptors, CCCs also show close interactions with glutamatergic signaling. A cross-talk among CCCs and trophic factors is important in short-term and long-term modification of neuronal properties. CCCs appear to be multifunctional proteins that are also involved in shaping neuronal structure at various stages of development, from stem cells to synaptogenesis. The rapidly expanding work on CCCs promotes our understanding of fundamental mechanisms that control brain development and functions under normal and pathophysiological conditions.

Introduction

Ion trafficking across biological membranes is mediated by channels and transporters, and these two types of molecules form the basis of electrical signaling in the nervous system. However, the volume of research on voltage- and ligand-gated channels is orders of magnitude higher than what has been devoted to the study of neuronal ion transporters. This bias has no rational foundation: it does not reflect a difference in the impact or versatility of their functions. What is often ignored is that ion transporters are as important as channels in the generation of electrical signals.

During the recent years, there has been a sharp increase in the interest of neurobiologists in plasmalemmal ion-transport mechanisms, especially in cation chloride cotransporters (CCCs). Initially, CCCs were studied in nonneuronal cells in order to elucidate their role in volume regulation (Payne et al., 2003). More recently, it has turned out that these transporters are crucial in the control of the electrochemical Cl^- gradient that is required for ''classical'' hyperpolarizing postsynaptic inhibition mediated by $GABA_A$ receptors ($GABA_A$Rs) and glycine receptors (Figure 1). The rapidly expanding research within this field has shown that CCCs play a fundamental role in neuronal proliferation, differentiation, disease, damage, and recovery, as well as in a variety of phenomena traditionally associated with plasmalemmal receptor- and channel-mediated effects, such as those involved in synaptic plasticity. Different CCCs show distinct cellular and subcellular expression patterns, and their functions are modulated via short-term and long-term changes at the level of transcription, translation, posttranslational modifications, and trafficking. Furthermore, CCCs are involved in intricate signaling cascades with growth factors and hormones. In view of their functional versatility, CCCs are likely to gain importance in drug research.

The milieu intérieur of the central nervous system (CNS) is tightly regulated by the blood-brain barrier and other epithelial structures, such as the choroid plexus, which involves a wide spectrum of ion transporters, including CCCs (Pedersen et al., 2006; Praetorius, 2007). These topics will not be discussed in the present review, and, with our main focus on neuronal CCCs, only some basic aspects of the ion-regulatory functions of glial cells will be touched upon. Outside the nervous system, CCCs are expressed in all organ systems, hence playing a key role in, for instance, salt transport and the control of blood pressure (Pedersen et al., 2006; Russell, 2000; Flatman, 2008). It is important to recognize the wide spectrum of CCC functions in experiments on transgenic animals and on drugs that affect these ion transporters.

Structure and Molecular Diversity

The CCC family in mammals consists of nine members encoded by the genes *Slc12a1-9*. The CCC proteins are glycoproteins with apparent molecular weights of 120–200 kDa. Seven out of the nine CCCs described so far are plasmalemmal ion transporters (Gamba, 2005; Mercado et al., 2004; Payne et al., 2003), and in terms of function they fall into three categories: two members are Na-K-2Cl cotransporters (NKCCs; isoforms NKCC1 and NKCC2), one is an Na-Cl cotransporter (NCC), and four are K-Cl cotransporters (KCCs; isoforms KCC1-4). The physiological roles of the remaining two CCCs (CIP1 or *Slc12a8*, and CCC9 or *Slc12a9*) are yet unknown.

The predicted secondary structure of CCCs, confirmed only for NKCC1 so far (Gerelsaikhan and Turner, 2000), consists of 12 membrane spanning segments that are flanked by intracellular termini that constitute about half of the protein. Hetero- and homo-oligomers of the different CCCs have been described for nearly all CCCs (Blaesse et al., 2006; Pedersen et al., 2008;

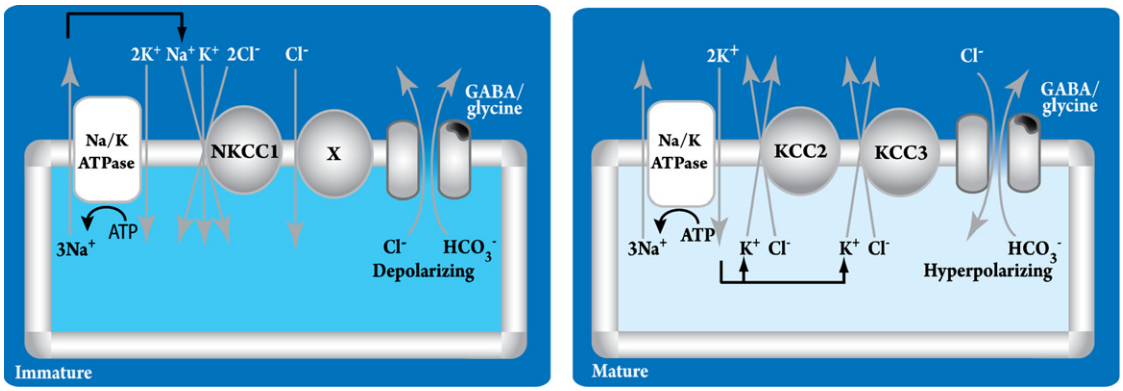

Figure 1. Ion-Transport Mechanisms Underlying $GABA_A$ and Glycine Receptor-Mediated Responses in Immature and Mature Neurons
(Left) NKCC1 mediates Cl^- uptake in immature hippocampal and cortical neurons, while an as yet unidentified Cl^- transporter denoted by X operates in some neurons either in parallel with NKCC1 or in its absence. (Right) In mature CNS neurons, KCC2 is the principal K-Cl cotransporter, and it is often coexpressed with KCC3. Cl^- transport by all CCCs is fuelled by the Na^+ and K^+ gradients generated by the Na-K ATPase. Because the intracellular concentration of Cl^- is high in immature neurons (indicated by the shade of blue), E_{GABA} and E_{Gly} are not directly affected by the intracellular HCO_3^- concentration, while in mature neurons, the channel-mediated net efflux of HCO_3^- can have a major effect on the reversal potentials. For further details, see text.

Simard et al., 2007; and references cited therein). However, the available data are not conclusive regarding the mechanisms whereby oligomerization affects CCC function.

With the exception of NKCC2 and NCC, which are predominantly found in kidney, all CCCs are expressed in neurons or glial cells—or both—at least at some stage of CNS development. KCC1 is considered a "household" isoform in various cell types, including glial cells, but present data suggest little or no expression in central neurons (Kanaka et al., 2001; Li et al., 2002; Payne et al., 1996; Rivera et al., 1999; Rust et al., 2007). In contrast, KCC2 is an exceptional CCC, as it is exclusively expressed in CNS neurons (Karadsheh and Delpire, 2001; Payne et al., 1996; Uvarov et al., 2005; Williams et al., 1999). There has been surprisingly little work on KCC3 despite its presence in various brain regions (Becker et al., 2003; Boettger et al., 2003; Le Rouzic et al., 2006; Pearson et al., 2001). Interestingly, KCC3 protein levels show a developmental increase (Boettger et al., 2003; Pearson et al., 2001) that is similar to the extensively studied upregulation of KCC2, a topic to be discussed in detail below. Even less is known about the possible role of KCC4 in the nervous system, an isoform that is expressed at a high level in the embryonic brain (Li et al., 2002). Another CCC that has attracted considerable interest among neurobiologists is NKCC1 (Gamba, 2005; Payne et al., 2003).

Evolution of CCCs

Transporter proteins, such as CCCs, may have arisen by duplication of genes encoding channel-type protein subunits with a smaller number of transmembrane segments (Saier, 2003). The existence of putative CCC homologs in *C. elegans* and in a cyanobacterium points to an early evolutionary origin of this protein family (Xu et al., 1994). Consistent with this, homologs of CCCs are present in diverse organisms. Many plant species have a CCC gene that seems to encode for a functional bumetanide-sensitive Na-K-2Cl cotransporter (Colmenero-Flores et al., 2007). Given that *Drosophila* apparently has only one KCC gene, and that its expression is neuron specific (Hekmat-Scafe et al., 2006), it is tempting to speculate that the first KCC evolved in animals with a nervous system to serve inhibitory neurotransmission.

Alternative Splicing

Alternative splicing greatly increases the diversity of the proteome. Alternative transcripts have often distinct expression patterns, and they can be translated into proteins with different structures, phosphorylation sites, or subcellular localizations. With regard to CCCs expressed in the CNS, splice variants are known for KCC1, KCC2, KCC3, and NKCC1 (Gamba, 2005; Mercado et al., 2004; Uvarov et al., 2007). KCC3a is expressed in the brain, whereas KCC3b is restricted to organs outside the CNS. In the adult rat brain, alternative splicing of KCC3a has been reported to create a neuron-specific KCC3c form, whereas the native KCC3a is restricted to glia (Le Rouzic et al., 2006). Two functional isoforms with distinct expression patterns were described for NKCC1 (Randall et al., 1997; Vibat et al., 2001). NKCC1a and NKCC1b are both expressed ubiquitously, but the relative expression level for NKCC1b is higher in the adult brain. The difference between NKCC1a and NKCC1b is an NKCC1a-specific 16 amino acid peptide encoded by exon-21 that contains a putative PKA phosphorylation site. In three out of four NKCC1 mutant mice described so far, both isoforms are disrupted (Table 1). However, the mouse characterized by Dixon et al. (1999) is an NKCC1a-specific knockout.

The mammalian KCC2 (*Slc12a5*) gene generates two isoforms, KCC2a and KCC2b, with different N termini (Uvarov et al., 2007). The mRNA levels of the two isoforms are similar in the neonatal mouse. While the overall expression of KCC2a remains relatively constant during postnatal development, the expression of KCC2b is strongly upregulated, especially in the cortex (Uvarov et al., 2007). This indicates that KCC2b is responsible for the extensively studied "developmental shift" from depolarizing to hyperpolarizing GABAergic responses, a conclusion supported by the finding that GABAergic responses remain depolarizing in cortical cultures from knockout mice (Zhu et al., 2005), which are now known to lack KCC2b only (Uvarov et al., 2007).

Table 1. Major Phenotypes of Mice with Disruptions of Genes Encoding CCCs

	Affected Isoforms	Mice Described by	Major Characteristics of Phenotype
KCC1	KCC1 knockout	Rust et al., 2007	no obvious phenotype
KCC2	KCC2a and KCC2b knockout	Hübner et al., 2001b	die after birth due to respiratory failure
	KCC2b knockout	Woo et al., 2002	survival up to P17; generalized seizures; low body weight
	hypomorphic mice; ~17% of normal KCC2 (KCC2a and KCC2b)	Tornberg et al., 2005	increased anxiety-like behavior; increased seizure susceptibility; impaired spatial learning and memory; reduced sensitivity to thermal and mechanical stimuli
KCC3	KCC3a-c knockout	Howard et al., 2002; Boettger et al., 2003	peripheral neuropathy; deafness (inner ear defects); neurodegeneration; reduced seizure threshold; hypertension (neurogenic mechanism; Rust et al., 2006)
KCC4	KCC4 knockout	Boettger et al., 2002	deafness (inner ear defects); renal tubular acidosis
NKCC1	NKCC1a and NKCC1b knockout	Delpire et al., 1999; Flagella et al., 1999; Pace et al., 2000	deafness and imbalance (inner ear defects); impaired blood pressure; growth retardation; defective spermatogenesis; alterations in locomotion; increased threshold to thermal stimulation
	NKCC1a knockout	Dixon et al., 1999	deafness and imbalance (inner ear defects)

Analyzing the phenotypes of knockout mice has shed some light on the physiological functions of the two KCC2 isoforms, which have distinct functions in the brain (Uvarov et al., 2007). Mice that completely lack KCC2 expression die immediately after birth due to severe motor defects, including respiratory failure (Hübner et al., 2001b). In contrast, KCC2b-specific knockout mice are viable, but show abnormal posture and gait and frequent seizures within a couple of days after birth and die at around 2 postnatal weeks, perhaps because of the recurrent seizures (Table 1; Woo et al., 2002). KCC2a is obviously important for some basic functions of lower brain structures, and it is sufficient to promote survival for up to 3 postnatal weeks, but the absence of KCC2b leads to seizures during this period.

Most published data on the spatial and temporal expression patterns of KCC2 reflect the expression of both KCC2a and KCC2b, because the mRNA probes and antibodies used in these studies detect both isoforms. Hence, in most of what is discussed below, "KCC2" will refer to both splice variants.

Expression Patterns of CCCs

A wealth of data indicate that one of the major factors underlying phenotypic variation among distinct types of neurons is based on the cell-specific as well as subcellular patterns of CCC expression.

Neuron-Specific Expression of KCC2

The expression of only a few genes is as tightly restricted to central neurons as *Slc12a5* (encoding KCC2). KCC2 mRNA is abundant in most neurons of the mature CNS but shows negligible expression in nonneuronal cell types, neuronal progenitors, or peripheral neurons. The CNS neuron-specific expression of KCC2 seems, at least in mammals, to be ensured by multiple, redundant mechanisms, including binding sites for neuron-enriched transcription factors such as Egr4 and neuron-restrictive silencing elements (Uvarov et al., 2005, 2006) (Figure 2).

KCC2 is located in the plasma membrane of somata and dendrites in various brain regions, e.g., cerebellar granule cells (Williams et al., 1999), thalamic relay cells (Bartho et al., 2004), auditory brainstem neurons (Blaesse et al., 2006), and cortical neurons (Gulyas et al., 2001; Szabadics et al., 2006). Freund and coworkers demonstrated high levels of KCC2 in the dendritic spines of rat cortical pyramidal neurons and interneurons (Gulyas et al., 2001), a puzzling observation in light of the preferential localization of glutamatergic synapses to spines and GABAergic synapses to somata and dendritic shafts. However, this paradox is at least partly resolved by a recent study that demonstrated a morphogenic role of KCC2 in spine formation independent of its role in ion transport (Li et al., 2007; see below).

In immature neurons in the auditory brainstem, KCC2 is initially expressed at a high level in a functionally inactive form, and the neurons generate depolarizing $GABA_A$ and glycine responses due to the presence of an unidentified Cl^- uptake transporter (denoted as "X" in Figure 1; Balakrishnan et al., 2003; Blaesse et al., 2006). Functionally inactive KCC2 has also been observed in primary cortical cultures during the first few days in vitro (Khirug et al., 2005). These results imply that the available data supporting a key role of NKCC1 in Cl^- uptake in immature neocortical and hippocampal neurons cannot be directly extrapolated to other types of neurons; and further, the mere presence of KCC2 protein in a neuron does not necessarily imply that it is functionally active.

Expression Patterns of NKCC1

Because of inconsistent and contradictory results, general statements about developmental changes or about the cellular and subcellular distribution of NKCC1 in CNS neurons are hardly possible. Even the widespread idea that neuronal NKCC1 expression decreases during postnatal rodent development (Hübner et al., 2001a; Yamada et al., 2004) is challenged by data that show a developmental increase in the NKCC1 mRNA and protein levels (Clayton et al., 1998; Mikawa et al., 2002; Wang et al., 2002; Yan et al., 2001a). A paper by Delpire and coworkers (Plotkin et al., 1997), often cited as evidence for a postnatal downregulation of NKCC1 in the rat forebrain, actually demonstrates roughly similar levels at P0 and P21. As described below, NKCC1 is functional in a wide variety of both juvenile and mature central neurons.

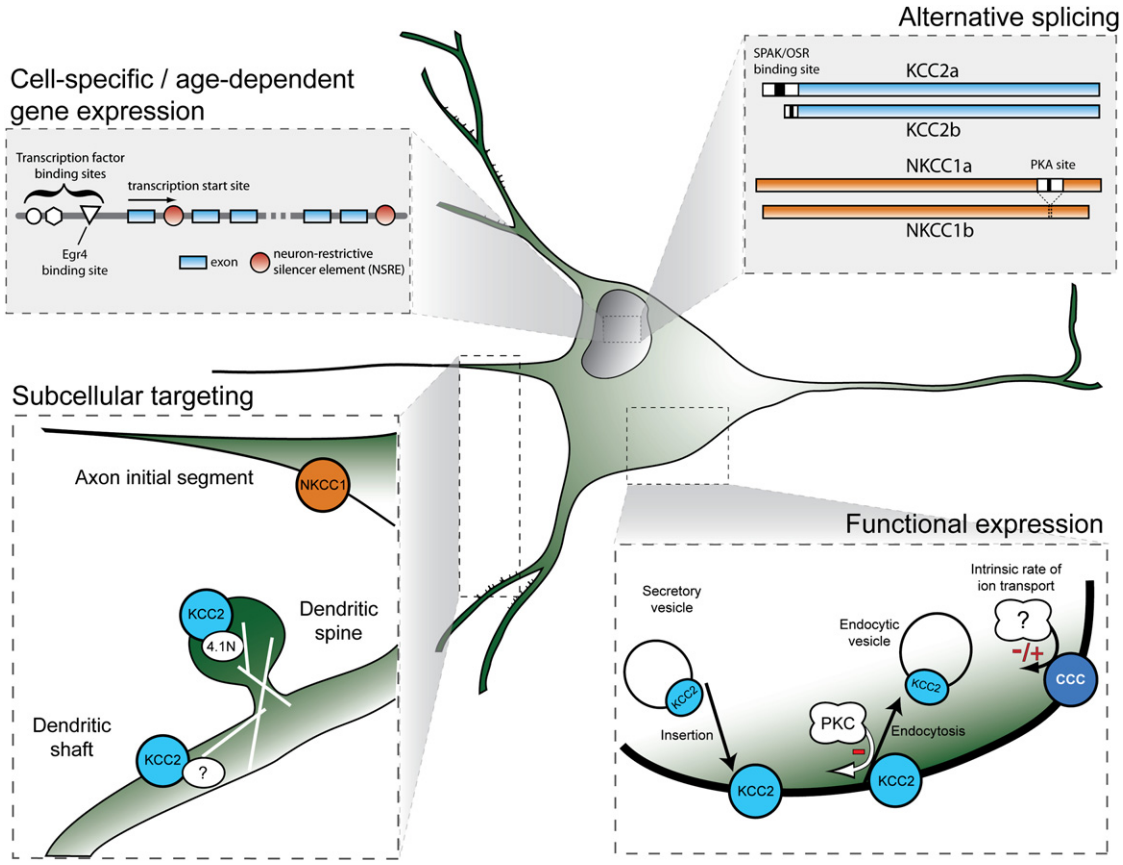

Figure 2. Regulation of CCC Functionality

CCC functions are regulated by transcriptional control, alternative splicing, subcellular targeting, and posttranslational modifications that lead to cell-specific as well as discrete subcellular expression patterns of the distinct CCC proteins. (Upper left) An example of transcriptional control for KCC2. Neuron-enriched transcription factors (e.g., Egr4) and NRSEs are responsible for the neuron-specific expression of KCC2 and regulate the expression level (Uvarov et al., 2005, 2006). (Upper right panel) Alternative splicing of KCC2 and NKCC1 where the various splice variants have distinct binding sites for kinases such as SPAK/OSR and PKA (Randall et al., 1997; Uvarov et al., 2007). (Lower left) An example of subcellular targeting of NKCC1 into the axon (axon initial segment; Khirug et al., 2008) and of KCC2 into the dendritic shafts and spines. The mechanisms whereby various CCCs are targeted into axons versus dendrites and somata are not known. While protein 4.1N is involved in the anchoring of spine KCC2 (Li et al., 2007), it is not clear whether this is true for KCC2 located in dendritic shafts and neuronal somata (indicated by a question mark). (Lower right) Expression levels of plasmalemmal functional CCCs are influenced, among other things, by the rate of insertion from secretory (exocytic) vesicles and removal by endocytic vesicles. The illustration shows how kinases can modify CCC function by influencing the relative rates of endo- and exocytosis. In this specific example, the KCC2 endocytosis is suppressed by a PKC-mediated phosphorylation of KCC2 (Lee et al., 2007). The scheme also highlights the lack of evidence (indicated by a question mark) for direct modulation of the intrinsic rate of ion transport by neuronal CCCs.

An essential role of NKCC1 in cell proliferation has been proposed in many studies (for review, see Russell, 2000). The high expression of NKCC1 in embryonic ventricular zones (Hübner et al., 2001a; Li et al., 2002; but see Clayton et al., 1998) points to a role in neuronal proliferation. NKCC1 is also expressed in the inner ear, where a lack of this protein leads to a defect in the production of endolymph and hence to deafness that is not neuronal in origin (Delpire et al., 1999; Dixon et al., 1999). Similar effects have been observed in KCC3 and KCC4 knockout mice (Boettger et al., 2002, 2003). NKCC1 is also expressed in dorsal root ganglion (DRG) cells (Rocha-Gonzalez et al., 2008) and in olfactory receptor neurons (Kaneko et al., 2004; Reisert et al., 2005).

Much of the published data on NKCC1 protein expression in CNS neurons has been obtained using a monoclonal NKCC antibody, T4 (Lytle et al., 1995). While T4 is a useful tool in immunoblots (Lytle et al., 1995; Zhang et al., 2006), interpretation of these data are complicated by NKCC1 expression in both neurons and glia (Kanaka et al., 2001; Hübner et al., 2001a). Further, perhaps because of differences in the methods of epitope retrieval, there are conflicting data on T4's capability to reliably detect NKCC1 immunohistochemically (Chen et al., 2005; Dixon et al., 1999; Li et al., 2008; Zhang et al., 2006, 2007). An antibody generated by Forbush and coworkers detects the phosphorylated NKCC1, thereby providing data on both the presence and functional state of NKCC1 (Flemmer et al., 2002). In the absence of reliable immunohistochemical techniques, other methods, including specific pharmacological block with bumetanide and analysis of NKCC1 knockout mice, have been used to examine the expression patterns of functional NKCC1 (Khirug et al., 2008).

Coexpression of KCC2 and NKCC1

There are no data that would suggest K-Cl cotransport in axons and no evidence for the presence of axonal KCC2 protein (e.g.,

Hübner et al., 2001b; Szabadics et al., 2006). The terminals of retinal ON bipolar neurons have been reported to contain KCC2 (Vardi et al., 2000), but it is perhaps not appropriate to call these neurites "axons," as they do not generate action potentials. In contrast to this, functional evidence indicates that NKCC1 is expressed in axons (e.g., Jang et al., 2001; Khirug et al., 2008; see below). The mechanisms that underlie the differential subcellular targeting of distinct CCCs to the axonal and somatodendritic compartments are not known, but recent evidence from work on NKCC1 in epithelial cells suggests that alternative splicing can play a major role (Carmosino et al., 2008). The coexpression of NKCC1 and KCC2 in specific neurons that is evident on the basis of structural and functional data (e.g., Duebel et al., 2006; Gavrikov et al., 2003; Khirug et al., 2008; Martina et al., 2001; Marty and Llano, 2005; Romo-Parra et al., 2008; Szabadics et al., 2006; Trevino and Gutierrez, 2005; Vardi et al., 2000) indicates a tight control of $[Cl^-]_i$ that can result in the generation of intraneuronal Cl^- gradients (Figure 2; and below).

KCC3 and KCC4 Expression Patterns

There is not much data on the neuronal expression patterns of KCC3 and KCC4, but what is available clearly indicates that further work on these two isoforms is highly motivated. In immunoblots, KCC3 was detected in all adult CNS regions, and KCC3 immunoreactivity was mainly located in myelinated tracts of the spinal cord and brain, although cells in the choroid plexus, pyramidal neurons in the hippocampus and cortex, and cerebellar Purkinje cells were also labeled (Pearson et al., 2001). In contrast, two other studies failed to observe significant KCC3 expression in the CNS white matter in adult mice (Boettger et al., 2003; Howard et al., 2002). KCC3 seems, however, to be expressed by the Schwann cells in peripheral nerves of juvenile mice (Byun and Delpire, 2007). KCC4 mRNA is highly expressed in the ventricular zone during embryonic development (Li et al., 2002), and in adult brain tissue it has been detected in the suprachiasmatic nucleus (Le Rouzic et al., 2006).

Basic Properties and Modes of Operation of CCCs

With regard to their source of energy, ion transporters fall into two categories: primary active transporters are directly fueled by ATP, while secondary active transporters take the energy for transport of the *driven ion* from the electrochemical gradient of another ion species. The ubiquitous primary active transporter, the Na-K ATPase, generates plasmalemmal K^+ and Na^+ gradients that provide the main source of energy for most secondary active transporters, including all CCCs (Figure 1). Extrusion of Cl^- via KCC-mediated K-Cl cotransport is driven by the K^+ gradient, while uptake of Cl^- in many (but not all; e.g., Balakrishnan et al., 2003; Zhang et al., 2007) neurons is driven by NKCC1 that exploits the plasmalemmal Na^+ gradient as its energy source for Na-K-2Cl cotransport. The KCC2 cotransporter operates at near-equilibrium (Payne et al., 2003), which permits maximization of the efficiency of harvesting energy from the K^+ gradient to fuel the extrusion of Cl^-. The transport stoichiometry of CCCs renders them electrically silent, thus they do not directly influence the neuronal membrane potential. Yet it should be noted that electroneutrality as such does not provide any information on the possible presence or absence of effects of membrane voltage on the transport process (see Kaila, 1994).

The electroneutrality of CCCs is an obvious obstacle in electrophysiological work on CCCs, precluding direct monitoring of their function. Therefore, electrophysiological experiments on CCCs exploit anion-permeable channels (mainly $GABA_A$ and glycine receptors) as "read-out devices," either by monitoring the reversal potential of the channel-mediated currents or using more sophisticated Cl^- loading techniques that will be described below. Additional techniques for analyzing ion transport by CCCs are based on assays of ^{86}Rb fluxes in cultured cells (Payne, 1997) and on the use of Cl^--sensitive indicators (Berglund et al., 2006; Verkman, 1990).

NKCC1 can be selectively blocked by a diuretic compound, bumetanide (1–10 μM), which has an up to 500-fold higher affinity for NKCC1 (K_i ~0.1 μM) than for KCC2 (Payne et al., 2003). Furosemide, another diuretic, blocks both NKCC1 and KCCs with an equal potency, and it is often used at 100 μM or higher to inhibit KCC2 in experiments on neuronal cultures and slices. 100 μM does not provide a full block, and an obvious confounding factor is the simultaneous effect on NKCC1. In addition, furosemide has inhibitory effects on *N*-methyl *D*-aspartate (NMDA) and $GABA_A$ receptors (Staley, 2002). Dihydroindenyl-oxy-alkanoic acid (DIOA) is yet another nonspecific drug that has been extensively used to block K-Cl cotransport (Payne et al., 2003). The lack of drugs with a specific action on KCC2 (and KCCs in general) has been a major problem in experimental work on neuronal CCCs. However, in a recent high-throughput screening study based on 234,000 compounds, a number of molecules that inhibit KCC2 with little effect on NKCC1 were identified (Delpire et al., 2009). Notably, an effective block of KCCs is achieved (sometimes on purpose but often unintentionally) in electrophysiological experiments based on whole-cell or gramicidin perforated-patch clamp recordings by replacing intracellular K^+ with Cs^+, a standard procedure to obtain a better space clamp. Cs^+ blocks KCCs because the cation selectivity sequence of K-Cl cotransport is $K^+ \approx Rb^+ \approx NH_4^+ >> Cs^+$ (Payne et al., 2003; Williams and Payne, 2004).

Neuronal Volume Regulation

In the brain, cell swelling and the concomitant decrease in the interstitial space can lead to pathophysiological activity as well as to ischemia due to the compression of blood vessels. While cellular swelling induced by hypotonia (as often used in experiments on nonneuronal cells) causes a net influx of water and thereby leads to a fall in $[Cl^-]_i$, neuronal swelling is usually brought about by intense synaptic activity and a concomitant massive channel-mediated net influx of cations and Cl^- that drives an osmotically obliged net influx of water. Hence, the mechanisms of swelling and KCC-mediated recovery are radically distinct under these two conditions. In case of neuronal swelling, recovery can be based on two distinct mechanisms: a genuine volume sensor that is able to kinetically activate K-Cl cotransport, and/or an enhanced driving force for K-Cl cotransport due to elevated intraneuronal Cl^-. Interestingly, neurons are thought to be devoid of water channels (aquaporins; Amiry-Moghaddam and Ottersen, 2003), which lowers their sensitivity to osmotic challenges. Consistent with a role for KCC3 in neuronal volume regulation, recovery of cell volume after hypotonic

swelling is compromised in cortical neurons from KCC3 knockout mice (Boettger et al., 2003). In contrast, KCC2's role in neuronal volume regulation is probably secondary to the steep dependence of its net Cl^- extrusion on the momentary intracellular chloride load. Nevertheless, this does not exclude an additional regulatory role for KCC2 in response to an osmotic challenge (Bergeron et al., 2006).

Setting E_{Cl} and E_{GABA-A}

One of the main tasks of CCCs is to maintain a constant $[Cl^-]_i$ in the face of channel-mediated fluxes that tend to dissipate the transmembrane electrochemical Cl^- gradient, i.e., the driving force of $GABA_A$R-mediated Cl^- currents. $[Cl^-]_i$ is also affected by chloride-dependent secondary active ion transporters involved in neuronal HCO_3^-/pH regulation (Chesler, 2003; Hentschke et al., 2006; Romero et al., 2004). The sodium-independent Cl/HCO_3 exchanger AE3 may act as a significant Cl^- uptake mechanism in neurons (Gonzalez-Islas et al., 2008), and in some neurons might represent transporter X in Figure 1. In addition to its role in neuronal Cl^- homeostasis, HCO_3^- acts as a significant carrier of depolarizing current across $GABA_A$Rs (Kaila and Voipio, 1987). This is because neuronal pH regulation leads to a much higher intracellular level of HCO_3^- than would be expected on the basis of a passive distribution. Hence, the reversal potential of $GABA_A$ receptor-mediated responses (E_{GABA-A}) is not identical to the equilibrium potential of Cl^- (E_{Cl}), but shows a deviation to more positive values (Kaila and Voipio, 1987). The bicarbonate-mediated current component can be larger than the one mediated by Cl^- in adult mammalian neurons (Gulledge and Stuart, 2003; Kaila et al., 1993). Notably, the reversal potential of the potassium-selective channels activated by $GABA_B$ receptors (E_{GABA-B}) would be identical to E_{GABA-A} if (1) K-Cl cotransport were at equilibrium (see below) and (2) $GABA_A$ receptors were not permeable to bicarbonate. This is clearly not the case, and E_{GABA-B} attains values that are significantly more negative than E_{GABA-A} (Farrant and Kaila, 2007; Kaila, 1994).

Functional Regulation of CCCs

Thermodynamics versus Kinetics

Thermodynamics can be used when examining the energetic conditions that are a prerequisite for a change in a system from state A to state B, but the physical mechanisms and the time course of this change play no role in this context. The energetic conditions required for transmembrane ion movements to occur can be derived from thermodynamics, which allows, for example, calculating Nernstian *ionic equilibrium potentials* (in the case of channel-mediated movements) as well as *the equilibrium conditions for a given ion transporter*. It should be emphasized that a thermodynamic analysis does not tell anything about *transport kinetics* (the temporal, dynamic aspects of ion movements). Hence, even a steep, energetically favorable ion gradient does not "activate" an ion transporter. In a manner analogous to a closed ion channel, an ion transporter may be fully inactive despite the presence of a strong energetic driving force of its substrate ions. The kinetics of transport is determined by the number of functional plasmalemmal transporters as well as their intrinsic transport rates.

Equilibrium Conditions for KCCs and NKCCs

The reversal potential of a current mediated by an ion channel defines the electrochemical equilibrium for the current: despite a free pathway provided by open channels for electrodiffusion, no net movement of ions takes place. In an analogous manner, an ion transporter is at thermodynamic equilibrium when no net flux of ions is mediated by the transporter even if it is fully activated. Under equlibrium conditions, ion fluxes do take place, but the net influx and efflux of any given ion species are exactly equal.

For KCCs, the equilibrium is defined by $[K^+]_o[Cl^-]_o = [K^+]_i[Cl^-]_i$. This simple equation has important consequences. First, it implies that $E_K = E_{Cl}$, which means that E_{GABA-A} and E_{GABA-B} would be equal under KCC equilibrium if $GABA_A$Rs and the channels activated by $GABA_B$Rs were ideally selective for Cl^- and K^+, respectively. Second, it shows that when KCCs are active, $[Cl^-]_i$ is highly sensitive to changes in $[K^+]_o$, and a modest increase in $[K^+]_o$ will lead to net uptake of Cl^- by KCC2. Finally, it also implies that the driving force for extrusion of Cl^- by KCCs is enhanced during an elevation of $[Cl^-]_i$, and therefore KCCs may also *contribute* to extracellular K^+ transients (Voipio and Kaila, 2000). While it is worth re-emphasizing that thermodynamic considerations as such are not sufficient to make predictions about changes in the magnitude of ion transport in response to an increase in the deviation from equilibrium (i.e., the driving force acting on the transporter), we used the somewhat fuzzy term "thermodynamic regulation" (Payne et al., 2003) to define a situation where net fluxes mediated by a close-to-equilibrium transporter such as KCC2 are directly affected by the driving forces of the substrate ions. However, even KCC2 appears not to be constitutively active under all conditions, and an increasing amount of evidence indicates that kinetic regulation plays a crucial role.

The equilibrium of NKCCs is defined by $[Na^+]_o[K^+]_o[Cl^-]_o^2 = [Na^+]_i[K^+]_i[Cl^-]_i^2$. The second power of $[Cl^-]_i$ looks intriguing, and at first sight one might think that slight changes in $[Cl^-]_i$ might exert an effect on NKCC1 via "thermodynamic regulation." However, under physiological conditions, NKCC1 is not close to equilibrium (Russell, 2000), but rather it is controlled by kinetic regulatory mechanisms that include a $[Cl^-]_i$-sensitive step (see below). For instance, in mammalian DRG neurons, kinetic constraints inactivate Cl^- uptake by NKCC1 even in the presence of a substantial thermodynamic driving force (Rocha-Gonzalez et al., 2008).

The Plasmalemmal Na-K ATPase

It is immediately clear from the close functional relationship between the plasmalemmal Na-K ATPase and CCCs (see Figure 1) that *any factor* that has an effect on the Na-K ATPase will have an influence on the operation of CCCs. This means that endogenous ligands (e.g., endogenous ouabains and agrin) that regulate neuronal Na-K ATPase isoforms will also affect CCC functions (Hilgenberg et al., 2006). Moreover, manipulations that lead to altered KCC or NKCC function may in fact reflect a change in Na-K ATPase function. For instance, when examining changes in CCC function following neuronal stimulation, the tight coupling between Na-K ATPases, CCCs, and E_{GABA-A} should be kept in mind. Interestingly, structural interactions have been demonstrated between the Na-K ATPase and KCC2 via coimmunoprecipitation of the $\alpha 2$ subunit and KCC2 in synaptic membrane fractions (Ikeda et al., 2004). Furthermore, the brain-type creatine kinase, an enzyme involved in the

production of ATP, has been shown to activate KCC2 and KCC3 via a protein-protein interaction (Inoue et al., 2006; Salin-Cantegrel et al., 2008).

Kinetic Regulation of CCCs

As pointed out before, the ion-translocating capacity of CCCs cannot be directly monitored using electrophysiological techniques, and there are no data available on the transport capacity of any CCC at the molecular level. Furthermore, except for a few cases, published data on the kinetic activation or inactivation of a given neuronal CCC do not provide information on whether this reflects a change in the number of membrane-located transporters, a change in their intrinsic rates of ion transport, or both (Figure 2). In the following, "kinetics" and "kinetic regulation" are terms that lump together the membrane trafficking of the CCCs as well as the (putative) changes in their unitary rates of ion transport.

Phosphorylation mechanisms play a pivotal role in the kinetic regulation of CCCs. In pioneering studies, it was shown that phosphorylation activates NKCC1 (Lytle and Forbush, 1992). A fall in $[Cl^-]_i$ below a homeostatic "set point" promoted direct phosphorylation and activation of NKCC1, leading to an active uptake of Cl^- (Russell, 2000). In contrast, the conventional view is that phosphorylation inactivates KCCs (Payne et al., 2003). However, there is evidence that tyrosine phosphorylation leads to activation of KCC2 (Vale et al., 2005; Wake et al., 2007; but see Stein et al., 2004) and that KCC2 is also directly phosphorylated and thereby activated by the protein kinase C (PKC; Lee et al., 2007; Banke and Gegelashvili, 2008). The activation by PKC is probably due to increased surface stability of the phosphorylated protein (Lee et al., 2007). In addition to PKC, several other kinases can regulate CCCs (Delpire and Gagnon, 2008; Kahle et al., 2008b). The serine-threonine kinases Wnks and SPAK/OSR1 are of particular interest in that they seem to be coregulators of NKCCs and KCCs. Studies with NKCCs in nonneuronal cells suggest that chloride-sensitive Wnks phosphorylate and activate the SPAK/OSR1, which in turn phosphorylates and activates NKCCs. While KCC2a and KCC3a contain a SPAK-binding motif, KCC2b and KCC3b do not, and the biological significance of this difference remains unknown. Most of the work above has been done in nonneuronal cells, and for neurobiologists, a crucial question is which of these mechanisms are relevant for CNS function.

CCCs and the Polarity of $GABA_A$ Receptor-Mediated Responses

Chloride Extrusion and Hyperpolarizing GABA Responses

The pioneering work on the role of K-Cl cotransport in conventional hyperpolarizing GABAergic transmission was done in crayfish preparations (Aickin et al., 1982) and mammalian cortical neurons (Misgeld et al., 1986; Thompson and Gähwiler, 1989). In neurons, E_K is more negative than the resting V_m, which allows K-Cl cotransport to support conventional hyperpolarizing IPSPs (recall that when KCC is close to equilibrium, E_{Cl} is close to E_K). However, the HCO_3^- permeability of $GABA_A$Rs implies that $E_{GABA\text{-}A} > E_{Cl}$, and hence active Cl^- extrusion is a *necessary* but not a *sufficient* condition for hyperpolarizing $GABA_A$ responses (Kaila et al., 1993; Kaila, 1994; Rivera et al., 1999). In most central neurons studied so far, the main chloride extruder is KCC2, but in some neurons KCC3 appears to have a significant role in setting $E_{GABA\text{-}A}$ (Boettger et al., 2003).

Chloride Uptake and Depolarizing GABA Responses

NKCC1 appears to be the main Cl^- uptake mechanism responsible for GABA-mediated depolarizing currents mediated by postsynaptic and extrasynaptic receptors in developing hippocampal and neocortical neurons (Achilles et al., 2007; Sipilä et al., 2006; Yamada et al., 2004). A prominent exception from the conventional hyperpolarizing or slightly depolarizing responses evoked by GABA in mature cortical neurons was reported by Szabadics et al. (2006; but see Glickfeld et al., 2009). They showed that the GABA-mediated postsynaptic potentials evoked by chandelier cells (a class of interneurons that exclusively target the axon initial segment of principal neurons) had a depolarizing driving force of 15 mV. The underlying Cl^- uptake mechanism was identified as NKCC1 in subsequent work, which showed that the GABA-mediated depolarization in the axon initial segment was abolished by bumetanide and in NKCC1 knockout mice (Khirug et al., 2008). The role of NKCC1 in neuronal Cl^- uptake is not restricted to cortical structures. For instance, in olfactory receptor neurons, a drastic reduction in the driving force of the depolarizing odor-activated Cl^- current occurs in the presence of bumetanide and in NKCC1 knockout mice (Reisert et al., 2005; see also Kaneko et al., 2004). In contrast, the bumetanide-insensitive transporter(s) accounting for depolarizing GABA and glycine actions in the immature auditory brainstem and retinal ganglion and amacrine cells have not been identified (Balakrishnan et al., 2003; Zhang et al., 2007).

Depolarizing actions by $GABA_A$ and glycine receptors have also been observed in presynaptic terminals. A classical example is the primary afferent depolarization (PAD) in DRG neurons (Rudomin and Schmidt, 1999; Russell, 2000), which seems to be largely but not fully attributable to Cl^- uptake mediated by NKCC1 (Rocha-Gonzalez et al., 2008). However, all of the available data are based on measurements carried out in the cell body, which may not be representative of other cellular compartments, and it will be critical to obtain an estimate of $[Cl^-]_i$ in the central terminals of primary afferent fibers. Within the CNS, Cl^- uptake has been shown to produce depolarizing presynaptic GABA or glycine responses with a consequent increase in glutamate release in the ventromedial hypothalamus (Jang et al., 2001), the calyx of Held (Price and Trussell, 2006), terminals on isolated hippocampal CA3 neurons (Jang et al., 2006), and parallel fibers in the cerebellum (Stell et al., 2007). In these studies, presynaptic NKCC1-mediated Cl^- accumulation was evident in the hypothalamus, but not in the calyx of Held.

Intraneuronal Cl^- Gradients

NKCC1 and KCCs are coexpressed in certain types of mature neurons, and spatially distinct expression patterns can result in steady-state chloride gradients and compartmentalization of $E_{GABA\text{-}A}$ within an individual neuron (e.g., Duebel et al., 2006; Gavrikov et al., 2003; Khirug et al., 2008; Price and Trussell, 2006; Szabadics et al., 2006). The steady-state $E_{GABA\text{-}A}$ gradients can be surprisingly large; in cortical principal neurons, the axo-somato-dendritic Cl^- gradient attains a value up to 15–20 mV (Khirug et al., 2008; Szabadics et al., 2006). A similar gradient

is seen between the presynaptic terminals and the soma of globular bushy cells in the auditory brainstem (Price and Trussell, 2006). Given that distinct GABAergic interneurons in various brain structures target anatomically distinct sites in the postsynaptic neurons (Freund and Buzsaki, 1996), it is not correct to assign a singular E_{GABA-A} value to a given neuron—a more appropriate approach is to *specify the E_{GABA-A} level of a given GABAergic input in the postsynaptic neuron*.

Assessing the Efficacy of Neuronal Cl^- Extrusion

The gramicidin-perforated patch clamp technique is often thought to be an ideal electrophysiological technique to study the presence and efficacy of neuronal Cl^- extrusion. Because the gramicidin-induced pores are not permeable to Cl^-, there is no flux of Cl^- between the recording pipette and the intracellular compartment (Kyrozis and Reichling, 1995). A novel approach where the cell membrane is kept intact relies on measuring E_{GABA-A} on the basis of the reversal potentials of single $GABA_A$ and NMDA receptor channels (Tyzio et al., 2006, 2008). However, even if an ideal technique were available, measuring E_{GABA-A} in a resting neuron can at best verify the presence of Cl^- extrusion, but this provides no information on the efficacy of Cl^- transport. Even a very inefficient Cl^- extrusion mechanism will be able to maintain a hyperpolarizing E_{GABA-A} if the net Cl^- influx into a neuron (i.e., the cellular Cl^- load) is small. Momentary changes in a target neuron's ionic load are among the most important context-dependent variables that influence GABAergic signaling (Buzsaki et al., 2007; Farrant and Kaila, 2007). Hence, in order to gain information that is physiologically more relevant than an estimate of the steady-state E_{GABA-A} in a resting neuron, it is important to design experiments to enable an assessment of the efficacy of Cl^- extrusion. Quantitative assessments of the efficacy of Cl^- extrusion are based on a procedure where a defined Cl^- load is imposed on a cell, and the cell's capability to maintain the $[Cl^-]_i$ provides a valid estimate of the efficacy of extrusion (e.g., Jarolimek et al., 1999; Khirug et al., 2005; Rivera et al., 2002). Ion-loading techniques have been routinely used in the study of neuronal pH regulation (Chesler, 2003), but the resting steady-state value of intraneuronal pH does not provide an idea of the efficacy of acid extrusion. By analogy, E_{GABA-A} is not a reliable indicator of a neuron's capacity to regulate its $[Cl^-]_i$, as was nicely illustrated in a recent study on chronically injured cortex, which showed that a marked decrease in KCC2 function was not associated with a detectable change in E_{GABA-A} (Jin et al., 2005).

The "Developmental Shift" in $GABA_A$ Receptor-Mediated Signaling

It is frequently stated in both reviews and primary research papers that "during the neonatal period," KCC2 is upregulated, leading to a negative shift in E_{GABA-A} from depolarizing to hyperpolarizing. This is a misleading generalization from work on rodent hippocampal and cortical pyramidal neurons, and there are pronounced *species-specific differences* in the temporal expression patterns of KCC2 within a given neuronal population. At the time of birth, "altricial" species such as rats show delayed KCC2 expression in the hippocampus and neocortex, while in the guinea pig (a species with precocious neonates) KCC2 expression has already reached a high level (Rivera et al., 1999). This is also true for the perinatal human hippocampus and neocortex (Vanhatalo et al., 2005; but see Dzhala et al., 2005), and a very early onset of KCC2 expression is seen in the cortical subplate (Bayatti et al., 2008). On the other hand, *within a single species*, high levels of KCC2 expression are first seen in the spinal cord and subcortical brain regions, and thereafter in the cortex (Li et al., 2002; Rivera et al., 1999; Vinay and Jean-Xavier, 2008; see also Blaesse et al., 2006). The novel data on KCC2 isoforms (Uvarov et al., 2007) indicate that KCC2b is responsible for the developmental increase in neuronal Cl^- extrusion, at least in the cortex and in the auditory brainstem (Balakrishnan et al., 2003; Zhu et al., 2005). Interestingly, the shift from $GABA_A$ receptor depolarization to hyperpolarization of gonadotropin-releasing hormone neurons is delayed until the time of puberty (Clarkson and Herbison, 2006).

A relatively high $[Cl^-]_i$ is the "default" state of a wide variety of fully differentiated nonneuronal mammalian cells (Pedersen et al., 2006; Russell, 2000). Neurobiologists tend to regard immature neurons with their high $[Cl^-]_i$ and rather depolarized E_{GABA-A} as something exceptional while, in fact, it is the mature central neurons with their low $[Cl^-]_i$ that are the "aberrant" ones. $[Cl^-]_i$ in immature CNS neurons is typically in the range of 25–40 mM (e.g., Achilles et al., 2007; Balakrishnan et al., 2003; Kakazu et al., 1999; Kilb et al., 2002; Rohrbough and Spitzer, 1996; Sipilä et al., 2006; Yamada et al., 2004) and values typically of ~5 mM are seen in neuronal somata or dendrites after maturation (e.g., Khirug et al., 2008; Tyzio et al., 2008).

Inhibitory and Excitatory Actions of GABA

Unfortunately, the current literature on the developmental shift of E_{GABA-A} and its consequences is burdened by a major misconception: it is often assumed that depolarizing GABA actions imply excitation and that a necessary and sufficient condition for a genuinely inhibitory GABA action is an E_{GABA-A} that is more negative than resting V_m. This idea is a misleading simplification, which emphasizes the role of *voltage inhibition* (the hyperpolarizing response associated to an inhibitory postsynaptic potential) and ignores the crucial role played by *shunting inhibition* (based on the conductance of postsynaptic $GABA_A$Rs). Shunting inhibition acts by decreasing the temporal and spatial summation of excitatory inputs. Hence, several points should be kept in mind when considering the relationships between the steady-state values of E_{GABA-A} and the efficacy of inhibition. The opening of $GABA_A$Rs will have a shunting inhibitory action regardless of the value of E_{GABA-A}. Moderately depolarizing GABA actions can be functionally inhibitory and sometimes even more effective than hyperpolarizing responses because of the intrinsic outward rectification of $GABA_A$ currents and because depolarization leads to the inactivation of Na^+ channels and activation of K^+ channels. Finally, GABAergic transmission is not necessarily excitatory even if the postsynaptic response would be more positive than spiking threshold. This is because the spike voltage threshold is not a fixed parameter, but depends on the rate of change of the membrane potential, and also on the background conductance (e.g., Katz, 1966).

In conclusion, the steady-state levels of E_{GABA-A} do not give a reliable estimate of the physiologically relevant efficacy of postsynaptic inhibition. This does not, of course, imply that Cl^- transport is irrelevant here. It is important to recognize that

neurons in vivo undergo continuous fluctuations in their membrane potential, and fast changes take place in the driving force of the $GABA_AR$ current as well as in the momentary Cl^- load a neuron experiences. *Hence, functional inhibition is largely determined by the efficacy of Cl^- extrusion,* which has to be high enough to maintain a level of E_{GABA-A} sufficiently negative (but not necessarily hyperpolarizing) to prevent the cell from firing.

In view of the large number of variables described above, it is not surprising that GABA can have "dual" actions in developing neurons (Khalilov et al., 1999; Lamsa et al., 2000). The duration of the depolarizing postsynaptic potential outlasts the local conductance increase, i.e., the "shunt." Hence, depolarizing GABAergic responses can facilitate the triggering of action potentials by glutamatergic neurons (Gao et al., 1998). This mode of action is consistent with a facilitatory or "permissive" role for depolarizing GABAergic transmission in the generation of early network events (see Sipilä et al., 2005; Zheng et al., 2006; and below). In an elegant study on neonatal rodent motoneurons, Jean-Xavier et al. (2007) first showed that depolarizing IPSPs (dIPSPs) were able to facilitate spike triggering by subthreshold excitatory events in their late phase. Notably, the window of facilitation became wider as E_{Cl} was more depolarized, and started earlier when the site of dIPSP generation was moved away from the excitatory input. Therefore, in addition to E_{GABA-A}/E_{Gly}, the neuronal and synaptic architecture as well as the temporal relationships among GABA/glycinergic and glutamatergic postsynaptic events are critical factors that control the qualitative postsynaptic effects of depolarizing GABA/glycinergic transmission. In summary, the widely spread idea that a dichotomous "depolarizing-to-hyperpolarizing switch" would control the inhibitory or excitatory nature of GABA/glycinergic transmission in developing circuits is a profound oversimplification.

The Developmental Shift in E_{GABA-A}

A large shift of E_{GABA-A} from depolarizing to more negative and often hyperpolarizing values appears to be a ubiquitous feature of central neurons. A pioneering electrophysiological study on this topic was carried out by Obata et al. (1978) in chick spinal motor neurons, and the first observations related to the molecular mechanisms of the shift were made by Rivera et al. (1999), who showed that upregulation of KCC2 renders GABA hyperpolarizing in rat CA1 hippocampal neurons. The key observation was an increase in KCC2 expression during the first 2 postnatal weeks, a time window that was known to be associated with a negative shift in E_{GABA-A}. Because there are no selective drugs to block CCCs in an isoform-specific manner, gene knockdown experiments were done to confirm the role of KCC2.

In various regions of the brain, the appearance of hyperpolarizing $GABA_AR$ signaling occurs earlier in the female and has been attributed to higher KCC2 and/or lower NKCC1 activity (Galanopoulou, 2008; Perrot-Sinal et al., 2007). The sex-specific GABAergic responses appear to promote sexual differentiation of the brain (Auger et al., 2001; Galanopoulou, 2008). In a recent study on rat pup hippocampi, Tyzio et al. (2006) reported that maternal oxytocin induces a perinatal hyperpolarizing shift in E_{GABA-A} to astonishingly negative values (−100 mV and beyond). The authors suggested that oxytocin blocks NKCC1, thereby protecting the newborn brain from anoxic-ischemic damage, a condition that is a major cause of neurological dysfunctions in humans. However, the implications of these findings for the human condition call for further studies.

Neuronal Maturation and Emerging Networks

Depolarizing GABA and the Generation of Endogenous Activity in Developing Networks

There is a wide consensus that endogenous activity generated prior to the maturation of sensory inputs plays a crucial role in the development of neuronal networks (Ben-Ari et al., 2007; Katz and Shatz, 1996; O'Donovan, 1999; Spitzer, 2006). According to a rather widespread and simplistic view, this kind of early activity is directly driven by depolarizing GABAergic transmission, and the "depolarization-hyperpolarization switch" puts an end to it. Notably, Minlebaev et al. (2007) have shown that in the neonatal neocortex in vivo, blocking GABAergic transmission has little effect on the basic patterns of the generation of spontaneous events known as "spindle bursts" (Khazipov et al., 2004). In contrast, the dominating pattern of neocortical spontaneous activity in vitro, which is reminiscent of hippocampal sharp potentials, is sensitive to $GABA_A$ receptor antagonists (Rheims et al., 2008). This type of activity, originally termed "giant depolarizing potentials" (GDPs), has been studied in detail in the developing hippocampus, and there are two hypotheses regarding the roles of depolarizing GABA in their generation. In the scenario proposed by Ben-Ari and coworkers (Ben-Ari et al., 2007), GABAergic interneurons are mainly responsible for pacing GDPs in a phasic manner (Ben-Ari et al., 1989). While all hippocampal regions are able to produce GDP-like activity, the CA3 region shows the highest propensity for GDP generation. Concerning the role of depolarizing GABA, a more recent view is that the GDPs are primarily paced by the CA3 glutamatergic pyramidal neurons, and depolarizing GABAergic signaling (both synaptic and tonic) has a facilitatory role in their generation (Sipilä et al., 2005). In other words, depolarizing GABAergic transmission is temporally nonpatterned and has a permissive or facilitatory role, while the intrinsic bursting properties of the CA3 glutamatergic neurons are responsible for the temporal patterning of GDPs. An analogous mechanism has recently been proposed for starburst cells in the developing retina (Zheng et al., 2006). Notably, GDPs are blocked by bumetanide both in vitro and in vivo, pointing to a crucial role for NKCC1 (Dzhala et al., 2005; Sipilä et al., 2006).

Crosstalk among Neurotrophic Mechanisms, CCCs, and GABAergic Signaling

The concerted actions of CCCs and GABAergic signaling in brain development comprise an extremely wide spectrum of phenomena, and these will of course manifest themselves in neuronal network function and behavior. In fact, GABA's role in the formation of neuronal connectivity starts already during the early stages of neurogenesis (Owens and Kriegstein, 2002). Moreover, GABAergic transmission plays a key role during "critical" periods, when the nervous system is particularly prone to both normal and aberrant types of input (Hensch, 2005; Kanold and Shatz, 2006).

$GABA_ARs$ are functional in neuronal precursors and immature neurons, long before the formation of GABAergic synapses (Demarque et al., 2002; LoTurco et al., 1995; Owens et al., 1999).

During neuronal development, depolarizing extrasynaptic and synaptic GABAergic signaling promotes action potential activity, opening of voltage-gated Ca^{2+} channels, and activation of NMDA receptors (Ben-Ari et al., 2007; Fukuda et al., 1998; Hennou et al., 2002; Kandler and Gillespie, 2005; Serafini et al., 1995). These responses lead to transient elevations of intracellular Ca^{2+} levels and activation of downstream intracellular signaling cascades, which are central in mediating the trophic effects of GABA during development (Ben-Ari et al., 2007; Owens and Kriegstein, 2002). Trophic effects of GABA have been observed in vitro at various levels of neuronal and network development, including DNA synthesis, migration, morphological maturation of individual neurons, and synaptogenesis (Akerman and Cline, 2006; Liu et al., 2005, 2006; LoTurco et al., 1995; Marty et al., 1996, 2000; Owens and Kriegstein, 2002; Represa and Ben Ari, 2005; Reynolds et al., 2008; Simat et al., 2007; Wang and Kriegstein, 2008; see also Andang et al., 2008). Interestingly, the developmental patterns of GABAergic signaling are repeated during adult neurogenesis in the dentate gyrus in vivo (Esposito et al., 2005; Ge et al., 2006; Laplagne et al., 2007; Tozuka et al., 2005; see also Toni et al., 2008). BDNF has been ascribed a key role in a number of the studies cited above. It is a well-known fact that the release of trophic factors is enhanced during synchronous neuronal activity (Lessmann et al., 2003), and one of the functions of the early spontaneous network events described above may be to promote the release of these substances.

Despite the above findings related to depolarizing GABA actions, their significance in normal neuronal development in vivo is not clear (Wang and Kriegstein, 2009). Somewhat surprisingly, synaptogenesis and early brain development are hardly affected in knockout mice where GABA synthesis, vesicular transport, or vesicular release are eliminated (Jin et al., 1999; Varoqueaux et al., 2002; Verhage et al., 2000; Wojcik et al., 2006). On the other hand, using in utero electroporation to overexpress KCC2 in proliferating neurons, Cancedda et al. (2007) found that while neuronal migration was not affected (but see Heck et al., 2007), morphological maturation was markedly impaired in immature neurons that were devoid of depolarizing GABAergic responses.

An intriguing hypothesis suggests that presynaptically released GABA acts as a self-limiting trophic factor in a negative feedback loop where depolarizing postsynaptic $GABA_AR$ responses are required for the developmental upregulation of KCC2 (Ganguly et al., 2001; see also Leitch et al., 2005). However, subsequent studies showed that developmental KCC2 upregulation can take place in hippocampal and midbrain cultures in the complete absence of $GABA_AR$ signaling (Ludwig et al., 2003; Titz et al., 2003). Furthermore, during embryonic development in VIAAT knockout mice, KCC2 mRNA and protein levels are unaffected, despite the absence of GABAergic synaptic transmission (Wojcik et al., 2006).

Role of CCCs in the Formation of Synapses

Depolarizing actions of GABA play at least a permissive role in excitatory and inhibitory synaptogenesis (Akerman and Cline, 2007). In the *Xenopus* tadpole visual system in vivo, early overexpression of KCC2 led to a reduction in neuronal $[Cl^-]_i$ levels, interfering with the maturation of the glutamatergic system and enhancing the development of GABAergic synapses (Akerman and Cline, 2006). Overexpression of KCC2 in early hippocampal cultures selectively increased the number of GABAergic synapses and the frequency of GABAergic miniature postsynaptic currents (Chudotvorova et al., 2005). Consistent with the above work, Riekki et al. (2008) found that miniature IPSC frequency is lower in mice with reduced KCC2 levels (Tornberg et al., 2005).

However, the various roles of CCCs in neuronal development and connectivity may cover a much wider scope than what is caused by their actions on GABAergic and glycinergic responses. Recently, Li et al. (2007) obtained evidence that KCC2 may play a morphogenic role in the formation of excitatory synapses during cortical development. Cultured *KCC2*$^{-/-}$ neurons were found to have long, morphologically aberrant "filopodia-like" spines, but this phenotype could be rescued by transfecting the neurons with an N-terminal deletion construct that is not capable of mediating K-Cl cotransport. This suggested that the role of KCC2 in spine maturation is not based on its transport activity, but might be mediated by interactions with cytoskeletal proteins. KCC2 immunoprecipitation assays from adult mouse brain homogenate suggest an interaction between the KCC2 C-terminal domain and the 4.1N protein, which is known to mediate structural interactions between the cytoskeleton, transmembrane proteins, and adhesion molecules (Denker and Barber, 2002). While it remains to be verified whether KCC2 has a role in spine formation in vivo, neurons from mice with reduced KCC2 protein levels show an increase in the length of dendritic protrusions (Tornberg et al., 2005). Thus, KCC2 may function as a synchronizing factor in the development of inhibitory and excitatory neurotransmission. In this scenario, the progressive developmental increase in the number of functional glutamatergic synapses that target spines will be paralleled by an increase in the KCC2-dependent neuronal Cl^- extrusion capacity, and consequently, by an increase in the efficacy of GABAergic inhibition (Li et al., 2007).

Neuronal Plasticity

Postsynaptic $GABA_A$ signaling has the unique property of "ionic plasticity," which is based on short- and long-term changes in the transmembrane anion gradients. A robust manifestation of short-term GABAergic plasticity is apparent in CA1 pyramidal neurons following high-frequency stimulation of Shaffer collaterals, a classical experimental paradigm to evoke long-term potentiation. Under these conditions, the membrane potential of CA1 pyramidal neurons shows a biphasic response, with a prompt hyperpolarization followed by a large and prolonged depolarization. The depolarizing phase of this GABAergic response is attributable to two mechanisms: an initial and fast positive shift in $E_{GABA\text{-}A}$, caused by a bicarbonate-dependent uptake of Cl^- (see Kaila and Voipio, 1987) that partly overlaps with and is followed by an increase in $[K^+]_o$, which has a direct depolarizing action on the neuronal membrane potential (Fiumelli and Woodin, 2007; Kaila et al., 1997; Voipio and Kaila, 2000). Hence, pronounced short-term changes that affect the efficacy and sometimes even the qualitative (inhibitory versus excitatory) action of GABAergic transmission can take place in an activity-dependent manner without the involvement of changes in the kinetic modulation, trafficking and/or expression of CCCs.

There is evidence that KCC2 has an extremely high rate of turnover (recycling) at the plasmalemma (Lee et al., 2007), which suggests that the transporter is subject to continuous kinetic modulation. Recent work has shown that coincident firing of pre- and postsynaptic neurons or prolonged postsynaptic spiking alone can result in positive shifts in E_{GABA-A} (Woodin et al., 2003; Fiumelli et al., 2005). These shifts are dependent on intracellular Ca^{2+}, and a PKC-dependent mechanism was suggested to mediate the functional downregulation of KCC2 (Fiumelli et al., 2005). This conclusion contradicts Lee et al. (2007), who reported that PKC inhibits the endocytosis of KCC2, resulting in functional upregulation of KCC2, and it has been recently suggested (Brumback and Staley, 2008) that changes in Na-K ATPase activity are responsible for the activity-induced changes in E_{GABA-A} that were observed by Fiumelli and coworkers.

In hippocampal slices exposed to a Mg^{2+}-free solution, the ensuing interictal activity was shown to downregulate KCC2 mRNA and protein in a BDNF-dependent manner, and it was concluded that this pathophysiological plasticity is attributable to transcriptional changes in KCC2 expression levels (Rivera et al., 2004). However, the authors could not exclude the possibility that the decreased efficacy of Cl^- extrusion was caused by changes in posttranscriptional mechanisms. Indeed, there is evidence that BDNF can act on KCC2 function within a time window that is too brief to be mediated by transcriptional effects (Wardle and Poo, 2003). Hence, the overall picture looks rather similar to what is known about the plasticity of glutamatergic transmission: fast changes are mediated by posttranscriptional mechanisms, but at the same time changes in gene expression take place which appear to ''consolidate'' the initial effects.

Disease Mechanisms

Mutations of CCCs

While a large number of genetic defects in ion channels (channelopathies) are known to produce severe dysfunctions of neuronal signaling in diseases such as epilepsy, migraine, and pain (Mulley et al., 2003; Waxman, 2007), little data are available for ion transporters. However, a striking example of a genetic ''transporteropathy'' is provided by hereditary motor and sensory neuropathy with agenesis of the corpus callosum (HMSN/ACC; Salin-Cantegrel et al., 2008), an autosomal recessive disease characterized by progressive sensory neuropathy, mental retardation, and dysmorphic features such as a high arched palate and agenesis of the corpus callosum. All of the four identified mutations that underlie this disease involve C-terminal deletion of KCC3, which renders the transporter nonfunctional (Howard et al., 2002). The mutations disrupt activation of KCC3 by the brain-type creatine kinase, which suggests the first protein-protein interaction involved in HMSN/ACC pathophysiology (Salin-Cantegrel et al., 2008). KCC3 knockout mice have a phenotype that is largely consistent with the human disease (Boettger et al., 2003; Delpire and Mount, 2002; Howard et al., 2002).

A substantial fraction of idiopathic epilepsies are thought to have a polygenic basis (Mulley et al., 2005). Here, it is of interest that *kazachoc* mutations of *Drosophila kcc* that reduce the level of kcc protein are susceptible to epileptic-like seizures (Hekmat-Scafe et al., 2006). However, no mutations or polymorphisms in human CCC genes related to seizure susceptibility have been published so far.

Neuronal Trauma

The brain is highly susceptible to a wide spectrum of acute and chronic pathophysiological conditions, including anoxic-ischemic states, mechanical damage, and epileptic activity. A depolarizing shift in E_{GABA-A} in response to neuronal trauma has been observed following anoxia/ischemia, neurite transection, and osmotic imbalance (Katchman et al., 1994; Pond et al., 2006; van den Pol et al., 1996). The trauma-induced effects on E_{GABA-A} are largely attributable to a downregulation of KCC2 with a consequent increase in $[Cl^-]_i$ (Bonislawski et al., 2007; Fukuda et al., 1998; Nabekura et al., 2002; Papp et al., 2008), and some data point to a concurrent functional upregulation of NKCC1 (Pond et al., 2006; Yan et al., 2001b). There is no doubt that fast changes in CCC gene expression can take place in response to neuronal trauma (De Koninck, 2007; Rivera et al., 2002; Shulga et al., 2008), but the precise roles of transcriptional, posttranscriptional, and posttranslational mechanisms in the pathophysiological effects mediated by CCCs on $[Cl^-]_i$ and E_{GABA-A} remain to be identified (see Figure 2).

Epilepsy

While the large majority (~70%) of epilepsies is idiopathic, the remaining cases are largely attributable to cerebrovascular diseases, traumatic brain injury, and tumors. A downregulation of KCC2 associated with epileptiform activity has been observed in a number of in vivo and in vitro studies in animal models (Jin et al., 2005; Pathak et al., 2007; Rivera et al., 2002, 2004), and this mechanism may act as one of the links between neuronal injury and epilepsy (Pathak et al., 2007). In a pivotal study, Miles and coworkers showed that hippocampal slices obtained from temporal lobe epilepsy patients generated interictal activity that was attributable to depolarizing $GABA_A$-mediated transmission in a subpopulation of principal neurons (Cohen et al., 2002; see also Kohling et al., 1998). A follow-up study showed that the depolarization was related to a downregulation of KCC2 and that, interestingly, the interictal activity was blocked by bumetanide (Huberfeld et al., 2007; see also Munoz et al., 2007). The molecular cascades that lead to KCC2 downregulation in various pathophysiological states include those mediated by BDNF-TrkB signaling (Rivera et al., 2004).

$GABA_A$-mediated transmission is not suppressed in epileptic tissue (Avoli et al., 2005; Kohling et al., 1998), but the Cl^- extrusion capacity seems to play a key role in setting the susceptibility of neurons to epileptiform activity. In transgenic mice, the propensity to epilepsy in vivo and hyperexcitability in vitro are enhanced with an inverse relationship to the level of KCC2 expression (Riekki et al., 2008; Tornberg et al., 2005; Woo et al., 2002; Zhu et al., 2005). GABAergic inhibition can also revert into excitation without any change in the functional expression of KCCs, as mentioned above (Kaila et al., 1997). Indeed, epileptic seizures are associated with large extracellular K^+ transients, which may have a substantial GABAergic component (Avoli et al., 2005; Kaila et al., 1997). A pathophysiological increase in $[K^+]_o$ can lead to a vicious circle that involves a further depolarization of both the membrane potential and E_{GABA-A} (Kakazu et al., 2000; Payne et al., 2003; Thompson and Gähwiler,

1989) and a cell swelling that enhances proepileptic ephaptic signaling (Haglund and Hochman, 2005).

CCC Blockers as Putative Anticonvulsant Drugs

A number of studies have examined the possible utility of diuretic drugs, especially furosemide and bumetanide, in the treatment of epileptic disorders. While some observations indicate an anticonvulsant action (e.g., Haglund and Hochman, 2005; Hochman et al., 1995), the interpretation is complicated by the wide diversity of the drugs' cellular targets within and outside the brain and, in the case of furosemide, because of its lack of specificity at the molecular level (Staley, 2002). The search for novel drugs is particularly important in the case of neonatal seizures, where standard anticonvulsants have poor efficacy (Rennie and Boylan, 2007). At the moment, bumetanide has attracted much attention as a putative drug to suppress seizures in human neonates (Dzhala et al., 2005, 2008; Kahle et al., 2008a), an idea that is largely based on the assumption that seizure susceptibility is linked to depolarizing actions of GABA in the neonate brain (e.g., Ben Ari and Holmes, 2006). However, the expression patterns of KCC2 and the parallel developmental characteristics of the human EEG do not suggest that GABA would be overtly depolarizing in fullterm newborn cortex (Vanhatalo et al., 2005), but the situation is probably different for preterm neonates (Dzhala et al., 2005; Vanhatalo et al., 2005). In addition, work in vitro has resulted in conflicting data regarding the anticonvulsant properties of bumetanide (Kilb et al., 2007). Clearly, much more research is needed to test the hypothesis of a connection from nonpathophysiological depolarizing actions of GABA to seizure generation in neonates and its possible clinical implications.

Neuropathic Pain

Neuropathic pain is a common and highly debilitating condition resulting from damage or dysfunction of peripheral nerves. This type of pain is often refractory to standard analgesic agents, and opioid treatment can result in unacceptable side effects. Symptomatic management of neuropathic pain by suppressing neuronal activity with antiepileptic drugs can also have undesirable side effects (Blackburn-Munro and Erichsen, 2005; Jensen, 2002; Rogawski and Loscher, 2004). Hence, neuropathic pain represents a major unmet medical need. GABA and glycinergic inhibition play a pivotal role via modulatation of the afferent input into nociceptive neurons in the superficial dorsal horn and control of their output to higher areas of the central nervous system (Price et al., 2005). Loss of inhibitory neurotransmission may underlie several forms of chronic pain. De Koninck and coworkers have proposed that suppression of inhibition by a collapse of the chloride gradient in dorsal horn lamina I neurons is a cellular substrate of neuropathic pain (Coull et al., 2003; De Koninck, 2007). They reported that peripheral nerve injury decreased KCC2 expression and produced a depolarizing shift in E_{GABA-A} in a subpopulation of lamina I neurons in rats. This loss of inhibition appeared to be mediated via BDNF released from activated spinal microglia (Coull et al., 2005).

Pathogenic versus Protective Mechanisms

At first sight, downregulation of neuronal Cl^- extrusion and a consequent decrease in the efficacy of inhibition appears to be maladaptive for the survival of diseased neuronal tissue. Here, one should note that the responses of neurons to trauma can be causally related to the pathophysiological sequelae per se, or to intrinsic protective mechanisms that promote neuronal survival and re-establishment of functional connectivity (Payne et al., 2003). It has been proposed that the posttraumatic downregulation of neuronal Cl^- extrusion and the consequent depolarizing shift in E_{GABA-A} are aspects of dedifferentiation of neurons and neuronal networks that are permissive for the "recapitulation" of developmental programs for the rewiring of surviving neurons (Payne et al., 2003; Pieraut et al., 2007; Shulga et al., 2008). Furthermore, in view of the tight link between the Na-K ATPase and CCC functions, it is interesting that seizure activity and neuronal damage have been shown to lead to a downregulation of the sodium pump (e.g., Pylova et al., 1989; Ross and Soltesz, 2000), which may well be a neuroprotective response to minimize local oxygen and glucose demand during trauma-related hyperexcitability and plasticity.

Conclusions and Topics for Future Work

Neurons typically have exceedingly complex morphological properties, and the data reviewed above suggest that many have steady-state intracellular Cl^- gradients that are set by cell-type-specific, subcellular expression patterns of functional CCCs. An immediate question that arises here is the role of such gradients in neuronal signaling during ongoing activity (e.g., oscillations) in the brain. Spatial "sign changes" (inhibitory versus excitatory) in GABAergic signaling have been identified under steady-state conditions, for instance in mature neurons in the retina (Gavrikov et al., 2003). Hence, it is possible that the spectrum of context-dependent actions of GABA include analogous sign changes that take place within a timescale of perhaps tens of milliseconds, thereby adding a further degree of freedom to the computational properties of neurons. Testing this hypothesis will require fast and reliable monitoring of neuronal membrane potential in parallel with measurements of $[Cl^-]_i$, a task that can be achieved only after further developments of Cl^- imaging techniques.

It is likely that in vivo at least some functional CCCs are homo-oligomers (Blaesse et al., 2006; Casula et al., 2001), and work in vitro shows that monomers of distinct CCC isoforms can combine to hetero-oligomers (Simard et al., 2007). But does hetero-oligomerization also take place in vivo? And if it does, what are the subunit compositions of native CCC hetero-oligomers? Information of this kind is physiologically important, and it will have immediate consequences on work aimed at designing drugs (such as antiepileptic or analgesic compounds) that target CCCs in the living organism. Obtaining insight into CCC molecular structure should help in solving these problems. Future studies on polymorphisms will add to the spectrum of both functional and dysfunctional CCCs, a topic that has much potential in the search for novel disease genes.

Current data also demonstrate that the CCCs are not only working as Cl^- transporters. This is in line with a number of previous observations showing that plasmalemmal channels and ion transporters are often multifunctional and that they can exert actions at the level of cellular structure (Denker and Barber, 2002). The role that KCC2 has on spine formation may well be a facet in a much broader picture where CCCs act as morphogenic factors. Little is known about the anchoring of CCCs,

and future work is likely to uncover novel protein-protein interactions that influence the functional and structural roles of CCCs. In addition to the search for ion-regulatory metabolons incorporating CCCs, an intriguing question is whether "plasticity" of anchoring proteins is a determinant of subcellular CCC expression and functionality.

In addition to the electrophysiologically obvious link between CCCs and anion-permeable channels gated by GABA and glycine, CCCs are involved in intricate, bidirectional interactions with trophic factors as well as intracellular signaling cascades. However, we are still at a loss regarding the precise mechanisms whereby, and to what extent, trophic factors control CCC expression and functionality. Clearly, this kind of knowledge is needed to unravel the roles of CCCs in brain development, plasticity, and disease.

While research on the diverse functions on CCCs in the CNS is still in its infancy, the overall picture is exciting and holds promise for major breakthroughs in our understanding of the most fundamental mechanisms underlying the workings of the nervous system. The wide and rapidly increasing research investments employing a wide spectrum of powerful molecular, electrophysiological, and imaging techniques is likely to lead to a fast narrowing of the gap between the level of our knowledge of ion transporters and ion channels.

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