Generation and Characterization of the Cation-Chloride Cotransporter KCC2 Hypomorphic Mouse

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ABBREVIATIONS

BDNF	brain-derived neurotrophic factor
CCC	cation-Cl^- cotransporter
CDR	coding region
CIP	cotransporter-interacting-protein
CNS	central nervous system
CP	cortical plate
E	embryonic day
E_m	electrochemical equilibrium potential (or reversal potential)
EST	expressed sequence tag
GABA	γ-aminobutyric acid
GAD	glutamic acid decarboxylase
GAT	GABA transporter
IHC	immunohistochemistry
KCC	K^+-Cl^- cotransport
KO	knock-out
KI	knock-in
LORR	loss of righting reflex
mRNA	messenger RNA
Neo	neomycin resistance gene
NKCC	Na^+-K^+-Cl^- cotransport
NCC	Na^+-Cl^- cotransport
Mo	mouse
P	postnatal day
PPI	prepulse inhibition
PNS	peripheral nervous system
PTZ	pentylenetetrazole
Rb	rabbit
SLC	solute carrier
SLC12A(1-9)	solute carrier family 12 member 1-9
TM	transmembrane
UTR	untranslated region
VZ	ventricular zone
WB	Western blot

ORIGINAL PUBLICATIONS

This thesis is based on the following publications, herein referred to by their Roman numerals (**I-IV**), and on some unpublished results:

I Li H*, **Tornberg J***, Kaila K, Airaksinen MS, and Rivera C. Patterns of cation-chloride cotransporter expression during embryonic rodent CNS development. *Eur J Neurosci*. 2002 12: 2358-70.
*equal contribution

II Vilen H, Eerikainen S, **Tornberg J**, Airaksinen MS, and Savilahti H. Construction of gene-targeting vectors: a rapid Mu in vitro DNA transposition-based strategy generating null, potentially hypomorphic, and conditional alleles. *Transgenic Res* 2001 10: 69-80.

III **Tornberg J**, Voikar V, Savilahti H, Rauvala H, and Airaksinen MS. Behavioural phenotypes of hypomorphic KCC2-deficient mice. *Eur J Neurosci*. 2005 5: 1327-37.

IV **Tornberg J**, Segerstråle M, Kulesskaya N, Voikar V, Taira T, and Airaksinen MS. KCC2-deficient mice show reduced sensitivity to diazepam, but normal alcohol-induced motor impairment, gaboxadol-induced sedation and neurosteroid hypnosis. *Neuropsychopharmacology*. 2006 Aug 16; [Epub ahead of print]

ABSTRACT

The cation-Cl^- cotransporter (CCC) family comprises seven well-characterized molecules: a thiazide-sensitive Na^+-Cl^- cotransporter (NCC), two loop diuretic-sensitive Na^+-K^+-$2Cl^-$ cotransporters (NKCC1-2), and four K^+-Cl^- cotransporters (KCC1-4). These membrane proteins are involved in several physiological activities including transepithelial ion absorption and secretion, and cell volume regulation. In neuronal tissues, NKCC1 and KCC2 are especially important in determining the intracellular Cl^- levels and hence the neuronal responses to inhibitory neurotransmitters GABA and glycine. Loss-of-function mutations to three members of the CCC family have been identified as the cause for two inherited kidney diseases, Bartter's and Gitelman's diseases, and for one neurological disease, Anderman's disease.

One aim of the work was to elucidate the possible roles for CCC isoforms in the control of nervous system development. For this purpose we conducted a comprehensive analysis of the distribution patterns of KCC1-4 and NKCC1 in rodent central nervous system (CNS) during embryonic development. Characterization of the CCC isoform distribution in the embryonic rodent brain revealed that KCC2 mRNA is developmentally up-regulated and follows neuronal maturation, and that NKCC1 and KCC4 transcripts are highly expressed in the proliferative zones of subcortical regions around the peak of neurogenesis. KCC1 and KCC3 mRNA were shown to display generally low expression throughout the embryogenesis. These expression profiles suggest a role for CCC isoforms in maturation of synaptic responses and in the regulation of neuronal proliferation during embryogenesis.

The major aim of this work was to study the biological consequences of the deficiency of a neuronal-specific KCC2 isoform in the adult CNS by generating transgenic mice that retain 15-20% of normal KCC2 levels. In addition, by using these mice as a tool for *in vivo* pharmacological analysis, we have investigated the requirements for KCC2 in tonic versus phasic $GABA_A$ receptor-mediated inhibition. In contrast to the KCC2 null mice that die at birth, KCC2-deficient mice display normal reproduction and life span but have reduced body weight (-20%). The mice show several behavioral abnormalities, including increased anxiety-like behavior, impaired performance in a water maze, alterations in nociceptive processing, and increased susceptibility for chemically-induced seizures. In contrast, the mice displayed apparently normal spontaneous locomotor activity and motor coordination.

Pharmacological analysis of KCC2-deficient mice revealed reduced sensititivity to diazepam, but normal gaboxadol-induced sedation, neurosteroid hypnosis and alcohol-induced motor impairment. Electrophysiological recordings from CA1-CA3 subregions of the hippocampus showed that KCC2 deficiency affected the reversal potentials of both the phasic and tonic GABA currents, and that the tonic conductance was not affected. The results suggest that requirement for KCC2 in GABAergic neurotransmission may differ among several functional systems in the CNS, which is possibly due to the more critical role of KCC2 activity in phasic compared to tonic GABAergic inhibition.

1. REVIEW OF THE LITERATURE

1.1 Introduction to cation-Cl^- cotransporters

In all cells and organelles, the uptake and efflux of crucial compounds such as sugars, amino acids, nucleotides, inorganic ions, and drugs is controlled by membrane transporter proteins. Approximately 5% (>2000) of all human genes are transporter-related (Hediger et al., 2004), consistent with the critical role of transporters in cell homeostasis. One class of membrane transporters consists of families of the solute carrier (SLC) gene series, which encode uniporters (passive transporters), cotransporters (coupled transporters), and antiporters (exchangers). In the human genome, 43 SLC families including a total of 319 transporter genes have been identified (Hediger et al., 2004). One of the identified SLC families is a cation-Cl^- cotransporter (solute carrier family 12, SLC12) gene family, which contains all genes that encode electroneutral cation-Cl^- coupled cotransporters (CCCs) (Gamba, 2005). Nine members of the CCC family have been identified in humans (Table 1). The phylogenetic tree of CCCs (Fig. 1) reveals two major subdivisions. One branch comprises cotransporters that exhibit ~70% identity and transport K^+ coupled with Cl^-. The other branch includes carriers that display ~50% identity and transport Na^+ (with or without K^+) coupled with Cl^-. The members of the gene family share a common basic topology, as assessed by hydropathy profiles of predicted proteins. The CCCs contain hydrophilic C- and N-terminal regions flanking a central hydrophobic domain containing 12 putative transmembrane (TM) domains. The central domain is the protein section that possesses the highest conservation, whereas the N-terminal domain is the most variable segment of these proteins (Mercado et al., 2004).

Table 1. *SLC12A(1-9)*, the electroneutral cation-Cl^- coupled cotransporter family

Human gene name	Protein name	Human gene locus	Number of residues, aa	Molecular Mass, kDa	Reference
SLC12A1	NKCC2	15q15-q21.1	1099	121	(Simon et al., 1996a)
SLC12A2	NKCC1	5q23.3	1212	132	(Payne et al., 1995)
SLC12A3	NCC	16q13	1030	114	(Simon et al., 1996b)
SLC12A4	KCC1	16q22.1	1011-1085	118-120	(Gillen et al., 1996) (Gamba, 2005)
SLC12A5	KCC2	20q12-q13.1	1116	123.5	(Sallinen et al., 2001) (Song et al., 2002)
SLC12A6	KCC3a KCC3b	15q14	1150 1099	128 122	(Howard et al., 2002)
SLC12A7	KCC4	5p15.3	1083	119	(Mount et al., 1999)
SLC12A8	SLC12A8	3q21	714	NA	(Hewett et al., 2002)
SLC12A9	CIP1	7q22	914	96	(Caron et al., 2000)

NA, information not available

There is one structural feature that sets the two major CCC subdivisions apart. The K^+-Cl^- cotransporters and CIP1 (for cotransporter-interacting-protein 1) have a large extracellular loop between TM5 and TM6 that contains several consensus *N*-linked glycosylation sites, whereas the Na^+ coupled cotransporters and SLC12A8 have a similar loop between TM7 and TM8. Besides their structures and ligands, the CCCs are classified according to their inhibitors. The Na^+-Cl^- cotransporter (NCC) is specifically inhibited by the benzothiadiazine (thiazide) class of diuretic agents (Stokes, 1984), and the Na^+-K^+-$2Cl^-$ cotransporter (NKCC) is specifically sensitive to 'loop'-type diuretics of the sulfamoyl-benzoic acid class such as bumetanide and furosemide (O'Grady et al., 1987). The K^+-Cl^- cotransporter (KCC) is sensitive to both furosemide and bumetanide. Furosemide has about equal potency for both NKCC and KCC, wheras bumetanide is significantly more efficient (~500-fold) inhibitor of NKCC than KCC (Payne et al., 2003).

The principal function of these proteins is to translocate Cl^- accompanied with cations across the plasma membrane. This occurs by Na^+-Cl^-, Na^+-K^+-$2Cl^-$, or K^+-Cl^- stoichiometry and is thus an electroneutral process (produces no net charge movement across the membrane). The CCCs are secondarily active transporters that derive energy for the transport from the electrochemical ion gradients across the plasma membrane, generated and maintained by primary-active transporters (Na^+-K^+-ATPase). The plasma membrane cation gradients also determine the direction of CCC transport. NCC and NKCC utilize the driving force of the Na^+ gradient and thus translocate ions inside the cell, whereas KCC exploits the driving force of the K^+ gradient and extrudes ions from the cell. Because Na^+-K^+-ATPase quickly re-establishes the physiological Na^+ and K^+ concentrations across the plasma membrane, the net effect of CCC activity is Cl^- movement into or out of the cell.

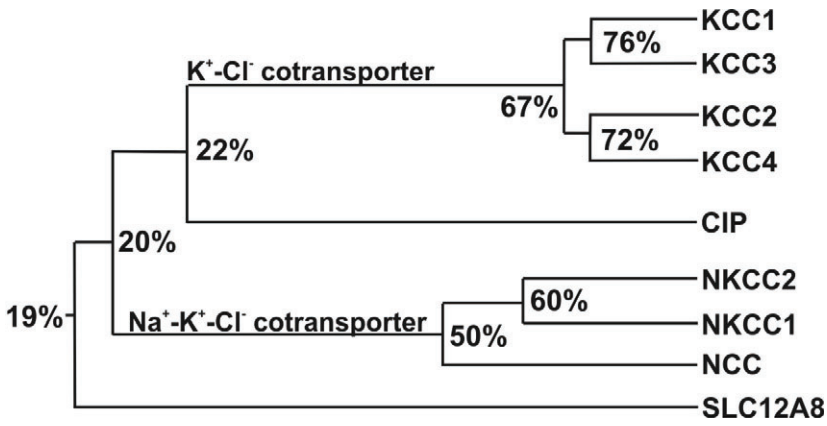


Fig. 1. Phylogenetic tree of human cation-chloride cotransporters (solute carrier family 12, SLC12). The percentage of identical residues between aligned protein sequences is shown at branch points (modified from Gamba, 2005).

The CCCs are necessary for several fundamental physiological processes, including regulation of cell volume in both epithelial and nonepithelial cells, intracellular Cl^- activity and thus the neuronal response to γ-aminobutyric acid (GABA) and glycine, and transepithelial ion absorption and secretion (Gamba, 2005).

1.2 Isoforms of the cation-Cl^- cotransporter gene family

1.2.1 Na^+-K^+-Cl^- and Na^+-Cl^- cotransporters

Evidence for a loop diuretic-sensitive Na^+-K^+-Cl^- cotransport (NKCC) mechanism was first presented in 1980 (Geck et al., 1980). Two isoforms have been identified and form the NKCC subfamily of cation-Cl^- cotransporters in vertebrates. These NKCC carriers are present in many different tissues from a wide variety of animal species. Transport by NKCC serves to maintain high intracellular Cl^- ($[Cl^-]_i$), which is used by epithelial tissues to promote net salt transport and by neural cells to regulate chloride homeostasis. The transport is inhibited by the 5-sulfamoylbenzoic acid loop diuretics furosemide, bumetanide and bentzmetanide. In most cell types, NKCC transport is activated by cell shrinkage as a part of cellular regulatory volume increase mechanisms (Haas and Forbush, III, 2000;Russell, 2000).

NKCC1 is expressed in a wide variety of secretory epithelial and non-epithelial cells, and is often referred to as the housekeeping or secretory isoform (Haas and Forbush, III, 2000;Russell, 2000). In epithelial cells, the cotransporter is expressed on the basolateral membrane and plays a critical role in providing cells with Cl^- that is secreted through the apical membrane (Evans et al., 2000;Shillingford et al., 2002;Bachmann et al., 2003). In the nervous system, NKCC1 plays a critical role in the regulation and control of neuronal excitability (Sung et al., 2000;Yamada et al., 2004;Dzhala et al., 2005). In most cell types studied, this carrier appears to be especially important in the maintenance of cellular volume (Russell, 2000).

NKCC1 was first identified at the molecular level from a shark (Xu et al., 1994), and subsequently from several vertebrate species (Delpire et al., 1994;Payne et al., 1995;Yerby et al., 1997;Moore-Hoon and Turner, 1998;Cutler et al., 2000).

No human disease has been linked with inactivating mutations in *SLC12A2*, but physiological roles for the carrier have been revealed from analysis of NKCC1 knock-out (KO) mice. NKCC1 is expressed in all tissues, but displays especially high expression in the inner ear and the salivary gland where it contributes to the formation of endolymph (Delpire et al., 1999) and the production of saliva (Flagella et al., 1999), respectively. Consistent with this, NKCC1 KO mice are deaf and exhibit classic shaker/waltzer behavior, which is indicative of inner ear defects that result from decreased endolymph secretion (Delpire et al., 1999;Flagella et al., 1999). In addition, these mice display severe impairment in the production of saliva (Evans et al., 2000). Consistent with the ubiquitous expression profile of the protein, NKCC1 KO mice show additional phenotypes in a variety of organs, including alterations in locomotion and nociceptive processing (Sung et al., 2000;Pace et al., 2000), defects in gastrointestinal ion transport

(Grubb et al., 2000), male infertility due to defective spermatogenesis (Pace et al., 2000), and reduced blood pressure (Flagella et al., 1999).

NKCC2 and ***NCC*** are present exclusively in the kidney (Gamba et al., 1994;Payne and Forbush, III, 1994). In humans, inactivating mutations in *SLC12A1* have been shown to underlie the pathophysiology of Bartter's disease (Simon et al., 1996a), and mutations to the *SLC12A3* have been shown to be the cause for Gitelman's syndrome. Bartter's disease is characterized by severe polyuria and electrolyte imbalance (Simon et al., 1996a), whereas Gitelman's disease is defined by hypokalemia, hypomagnesaemia, and metabolic alkalosis (Gitelman et al., 1969;Simon et al., 1996b).

1.2.2 K^+-Cl^- cotransporter

KCC mediates the coupled transport of K^+ and Cl^- across plasma membranes, and was first identified as a swelling-and *N*-ethylmaleimide-activated K^+ efflux pathway in red blood cells (Dunham et al., 1980;Lauf and Theg, 1980;Dunham and Ellory, 1981). K^+-Cl^- cotransport plays a significant role in the ionic and osmotic homeostasis of many cell types and has been implicated in several physiological and pathophysiological processes, including the cell regulatory volume decrease (Lauf et al., 1992), transepithelial salt absorption (Amlal et al., 1994), renal K^+ secretion (Ellison et al., 1985), myocardial K^+ loss during ischemia (Yan et al., 1996), cell growth regulation (Shen et al., 2001), human cervical and ovarian carcinogenesis (Shen et al., 2004), and the control of neuronal Cl^- activity (Rivera et al., 1999). At present, four different isoforms (KCC1-4) have been identified and form the KCC subfamily of CCCs. Within the subfamily, KCC2 and KCC4 form a closely related subgroup, whereas KCC1 is more homologous to KCC3 (Mount et al., 1999).

KCC1 is widely expressed in mammalian tissues and is considered to represent a housekeeping KCC isoform that maintains volume homeostasis in most cells (Gillen et al., 1996). Heterologous expression of KCC1 in *Xenopus laevis* oocytes reveals minimal KCC activity under isotonic conditions, but a strong cell swelling-induced activation of transport under hypotonic conditions (Su et al., 1999;Mercado et al., 2000). However, when KCC1 is expressed in human embryonic kidney (HEK-293) cells, the carrier displays significant isotonic activity which is further activated by cell swelling (Gillen et al., 1996;Holtzman et al., 1998). Compared to other KCCs that display very similar transport kinetics, KCC1 exhibits very low affinity for the transported K^+ and Cl^- ligands (Gamba, 2005).

KCC1 cDNAs have been obtained from several species (Gillen et al., 1996;Holtzman et al., 1998;Pellegrino et al., 1998;Su et al., 1999). Human KCC1 is expressed as multiple hKCC1 isoforms; there are at least six open reading frames encoding for almost identical hKCC1 isoforms, and several splice variants for hKCC1 have also been described (Pellegrino et al., 1998;Adragna et al., 2004;Crable et al., 2005).

The physiological role of KCC1 is still unclear. There are no human diseases directly linked to mutations in *SLC12A4*, and mice with genetic alterations to KCC1 have not yet been described. The majority of KCC activity in red blood cells has been attributed to KCC1, with some contribution of KCC3 and KCC4

(Lauf et al., 2001;Crable et al., 2005). KCC activity in the process of regulatory volume decrease is physiologically relevant to red blood cell maturation when the erythrocyte attains the final cell volume (Lauf et al., 1992). While mature circulating red blood cells express KCC activity at low or unmeasurable levels, the activity is elevated in reticulocytes and young red blood cells, as well as in red blood cells containing abnormal sickle hemoglobin (Lauf et al., 1992). In this regard, increased KCC activity is thought to play a major role in certain human hemoglobinopathies, such as sickle cell disease (Joiner, 1993). A recent report demonstrated aberrant expression of one of the KCC1 splice variants in sickle cells compared to normal reticulocytes (Crable et al., 2005), indicating a possible role for KCC1 in the pathogenesis of sickle cell disease.

KCC2 displays a neuron-specific expression pattern and is abundantly expressed in most neurons throughout the CNS, but absent from the peripheral nervous system (PNS) (Payne et al., 1996;Rivera et al., 1999). The cotransporter plays a crucial role in determining intracellular Cl^- activity and thus the neuronal response to inhibitory neurotransmitters GABA and glycine (Rivera et al., 1999). KCC2 is unique among the KCCs by mediating constitutive KCC activity under isotonic conditions, as assessed in both *X. laevis* oocytes and mammalian cells (Payne, 1997;Strange et al., 2000;Song et al., 2002;Gagnon et al., 2006). This unique feature of KCC2 activity is due to a 15 residue domain in the C-terminus, which is not present in other KCC isoforms (Mercado et al., 2006). Similar to other KCC isoforms, KCC2 is also activated in response to cell swelling (Strange et al., 2000;Song et al., 2002;Mercado et al., 2006).

KCC2 functions postsynaptically to extrude Cl^- from the cell and thus establishes the fast hyperpolarizing inhibitory responses mediated by GABA and glycine (Rivera et al., 1999;Hubner et al., 2001b). The importance of KCC2 in the control of neuronal excitability is underscored in KCC2-deficient mouse models. KCC2 KO mice show significantly increased depolarizing GABA responses and die immediately after birth due to deficits in the respiratory system (Hubner et al., 2001b), and KCC2 knock-down animals retaining 5-8% of normal KCC2 levels exhibit frequent generalized seizures and die shortly after birth (Woo et al., 2002). In addition, impaired expression and/or activity of KCC2 has been implicated in several forms of neuronal injury (Nabekura et al., 2002;Toyoda et al., 2003;Jin et al., 2005), and in the genesis of neuropathic pain and temporal lobe epilepsy (Cohen et al., 2002;Coull et al., 2003).

KCC3 is expressed in multiple tissues, including kidney, heart, brain, muscle, and lung, and has been identified at the molecular level from human and mouse tissues (Mount et al., 1999;Hiki et al., 1999;Race et al., 1999;Pearson et al., 2001;Mercado et al., 2005). KCC3 is activated by cell swelling, and displays isotonic activity when functionally expressed in HEK-293 cells, but not in the *Xenopus* oocyte expression system (Hiki et al., 1999;Race et al., 1999;Mercado et al., 2005).

The human KCC3 gene contains 26 coding exons and harbors two alternative first coding exons (exons 1a and 1b) resulting in two major KCC3 isoforms, KCC3a and KCC3b, respectively (Howard

et al., 2002). The KCC3a sequence predicts a 1150 residue protein with a molecular weight of 128 kDa, whereas KCC3b is 1099 residue protein with a molecular weight of 122 kDa (Mount et al., 1999;Mercado et al., 2005). KCC3a expression is more widespread than that of KCC3b, which is particularly abundant in kidney (Pearson et al., 2001;Mercado et al., 2005). Furthermore, a recent report stated the existence of three additional N-terminal isoforms of mouse and human KCC3 proteins (Mercado et al., 2005).

Loss-of-function mutations in *SLC12A6* are the cause of peripheral neuropathy associated with agenesis of the corpus callosum (Howard et al., 2002). This disorder, also known as Anderman's disease, is an autosomal recessive disease characterized by progressive sensorimotor neuropathy, mental retardation, dysmorphic features, and complete or partial agenesis of the corpus callosum (Dupre et al., 2003). In addition, rare variants of *SLC12A6* have been associated with bipolar disorder (Meyer et al., 2005). Homozygous inactivation of *Slc12a6* in mice replicates the peripheral neuropathy phenotype of Anderman's disease, but not the morphological changes in corpus callosum associated with the disease (Howard et al., 2002;Boettger et al., 2003). In addition to peripheral neuropathy, KCC3 KO mice display arterial hypertension and slowly progressive deafness (Boettger et al., 2003). Furthermore, experiments in cultured cells suggest a role for KCC3 in cell growth regulation (Shen et al., 2001).

KCC4 shows a wide tissue distribution pattern and is highly expressed in kidney, heart, lung, spinal cord, and the PNS (Mount et al., 1999;Velazquez and Silva, 2003;Karadsheh et al., 2004). Functional expression of KCC4 in *Xenopus* oocytes reveals robust activation of KCC transport by cell swelling, with minimal activity under isotonic conditions (Mount et al., 1999;Mercado et al., 2000;Gamba, 2005).

Physiological roles for KCC4 have been elucidated in KCC4 KO mice. These mice were normal at birth, but the cochlear hair cells rapidly degenerated after beginning of hearing at postnatal day 14 (P14), which led to a quick deterioration of hearing ability and to almost complete deafness after the second week of life (Boettger et al., 2002). In addition, KCC4 KO mice develop renal tubular acidosis (Boettger et al., 2002), indicating a role for KCC4 in acid-base metabolism.

1.2.3 Cotransporter-interacting-protein and solute carrier family 12 member 8

SLC12A8 and CIP1 are the two most distant members of the CCC family (Caron et al., 2000;Hewett et al., 2002). Transcripts for these proteins are expressed widely in several tissues, including brain and kidney (Caron et al., 2000;Hewett et al., 2002). The precise functions of CIP1 and SLC12A8 have remained elusive. Functional expression flux studies have not been able to identify the transport substrates for these proteins, and thus these family members remain orphan members of the CCC gene family. CIP1 is, however, able to inhibit the functional expression of NKCC1 by directly interacting with endogenous NKCC1 *in vitro* (Caron et al., 2000), and suggestive evidence for the role of SLC12A8 in psoriaris pathogenesis is accumulating (Hewett et al., 2002;Huffmeier et al., 2005).

1.2.4 Expression of cation-Cl^- cotransporter isoforms within the nervous system

Except for NCC and NKCC2, all other members of the CCC family have been

described within the nervous system. These proteins are expressed broadly and show wide expression patterns with cell-specific variations throughout the adult nervous system (Payne et al., 1996;Plotkin et al., 1997b;Kanaka et al., 2001;Pearson et al., 2001;Yan et al., 2001;Wang et al., 2002;Mikawa et al., 2002;Ueno et al., 2002;Okabe et al., 2002;Okabe et al., 2003;Balakrishnan et al., 2003;Karadsheh et al., 2004;Bartho et al., 2004;Le Rouzic et al., 2006).

NKCC1. In the adult rodent brain, NKCC1 mRNA expression is widely distributed but generally low, with the strongest expression restricted mainly to the granule cells of the cerebellum and choroid plexus (Plotkin et al., 1997a;Kanaka et al., 2001;Mikawa et al., 2002). NKCC1 transcripts are expressed in both neuronal and non-neuronal cells (Kanaka et al., 2001). The neuronal NKCC1 mRNA expression is generally low, but exists in several regions of the nervous system including the olfactory bulb, hippocampus, trigeminal nuclei, amygdala, thalamus, piriform cortex, spinal cord, and somatosensory neurons (Sung et al., 2000;Kanaka et al., 2001;Wang et al., 2002;Okabe et al., 2002;Toyoda et al., 2005;Wang et al., 2005). NKCC1 expression is high in white matter tracts, such as corpus callosum, internal capsule and cerebral peduncle (Kanaka et al., 2001;Hubner et al., 2001a), and has also been detected in astrocytes within cortex, corpus callosum, hippocampus, and cerebellum (Yan et al., 2001). In addition, NKCC1 expression has been detected in cultured spinal cord oligodendrocytes (Hoppe and Kettenmann, 1989;Wang et al., 2003), and in cerebral microvascular endothelial cells (O'Donnell et al., 1995).

In the postnatal rat brain, NKCC1 expression peaks in the first week of postnatal life and then declines from P14 to adult (Plotkin et al., 1997b;Wang et al., 2002). On P7, NKCC1 mRNA expression is strongest in the cortex, hippocampus, and cerebellum (Plotkin et al., 1997b;Mikawa et al., 2002).

KCC1. The expression profile of KCC1 mRNA resembles the NKCC1 mRNA expression pattern in the adult nervous system (Kanaka et al., 2001). The expression of KCC1 transcripts is wide but generally weak, and restricts mainly to the cerebellar granule cells and choroid plexus (Kanaka et al., 2001;Mikawa et al., 2002). KCC1 mRNA is expressed in neuronal and non-neuronal cells, and displays a moderate to low expression within several regions of the nervous system such as the olfactory bulb, hippocampus, superior colliculus, cerebral cortex, piriform cortex, thalamus, amygdala, spinal cord, dorsal root ganglia, posterior hypothalamic nucleus, and trigeminal nuclei (Kanaka et al., 2001;Okabe et al., 2002;Okabe et al., 2003;Toyoda et al., 2005). In addition, KCC1 mRNA is expressed in white matter, including internal capsule and corpus callosum, thus suggesting glial expression (Kanaka et al., 2001;Wang et al., 2002).

KCC2. KCC2 displays a neuron-specific expression pattern (Payne et al., 1996). In the adult CNS, KCC2 mRNA is expressed by most neurons and shows a particularly strong expression in pyramidal neurons of CA1-CA3 regions of the hippocampus and the cortical layer V, granule cells and Purkinje neurons of the cerebellum, granule neurons of the olfactory bulb, spinal cord motoneurons, and piriform cortex (Lu et al., 1999;Kanaka et al., 2001;Le Rouzic et al., 2006). KCC2 transcripts are also expressed strongly in all nuclei of the amygdala, with the strongest expression in the medial nuclei

(Kanaka et al., 2001;Okabe et al., 2003). In the basal ganglia, KCC2 mRNA is highly expressed in the caudate putamen and nucleus accumbens, and moderately in the globus pallidus (Kanaka et al., 2001). In the thalamus, KCC2 is abundant in all thalamic nuclei except the reticular nucleus (Bartho et al., 2004). The thalamic KCC2 expression is most prominent in the primary sensory relay nuclei (first order nuclei), being strongest in the ventral posterolateral and ventral posteromedial nuclei (Bartho et al., 2004). In addition, KCC2 mRNA is highly expressed in the lateral dorsal thalamic nucleus, zona incerta, rhomboid thalamic nucleus, subthalamic nucleus, parafascicular thalamic nucleus, and ventral genigulate body (Kanaka et al., 2001). In the hypothalamic nuclei, the expression levels of KCC2 mRNA vary a lot (Kanaka et al., 2001). A very strong KCC2 mRNA expression is observed in the lateral hypothalamic area, ventromedial hypothalamic nucleus, dorsal premammillary nucleus, medial preoptic area, and supraoptic nucleus (Kanaka et al., 2001;Le Rouzic et al., 2006). KCC2 mRNA is not, however, expressed in the dorsolateral part of the paraventricular nucleus, suprachiasmatic nucleus, and ventromedial part of the supraoptic nucleus (Kanaka et al., 2001). In the midbrain, KCC2 mRNA expression is strong in the interpeduncular nucleus, central gray, red nucleus, and moderate in the substantia nigra pars compacta and pars reticulata (Kanaka et al., 2001). KCC2 is not expressed in the dopaminergic neurons of substantia nigra (Gulacsi et al., 2003). In the pons and medulla, KCC2 mRNA expression is strong in the pretectal area, superior colliculus, superior olivary complex, ventral lateral lemniscus, inferior colliculus, and moderate in most nuclei including the dorsal tegmental nucleus, motor trigeminal nucleus, principal trigeminal nucleus, cerebellar nuclei, ventral cochlear nucleus, medial vestibular nucleus, solitary nucleus, spinal trigeminal nucleus, hypoglossal nucleus, facial nuclei, and inferior olive nucleus (Kanaka et al., 2001;Ueno et al., 2002;Balakrishnan et al., 2003). KCC2 mRNA expression is, however, absent in the mesencephalic trigeminal nucleus (Kanaka et al., 2001;Toyoda et al., 2005). Finally, KCC2 transcripts are moderately expressed in the dorsal and vetral horns of the spinal cord (Kanaka et al., 2001), and not detected in the peripheral nervous system (Rivera et al., 1999;Hubner et al., 2001b;Toyoda et al., 2005).

Electron microscopy analyses in rat hippocampus and thalamus have revealed strong KCC2 expression in the vicinity of excitatory inputs (Gulyas et al., 2001;Bartho et al., 2004). In rat hippocampus, KCC2 expression was shown to accumulate on dendritic spine heads and at the origin of spines of CA1 pyramidal cells, as well as on the thorny excrecences of CA3 pyramidal cells (Gulyas et al., 2001), which are known to be sites of intense excitation. In addition, KCC2 expression was more strongly expressed in interneuron types receiving stronger excitatory input, for example in parvalbumin-containing interneurons (Gulyas et al., 2001). A similar, but less pronounced, KCC2 expression profile was found in relay cells of rat thalamus (Bartho et al., 2004). In these cells, KCC2 was mainly localized on the extrasynaptic membranes of thick and thin dendrites, which are the prime target of excitatory corticothalamic terminals (Bartho et al., 2004). In addition, KCC2 was found on relay cell somata and in close association with excitatory synapses formed by cortical afferents (Bartho et al., 2004).

Compared to the developmental expression pattern of NKCC1 mRNA, KCC2 transcripts show the opposite profile (Plotkin et al., 1997b;Rivera et al., 1999). In rat hippocampus, KCC2 mRNA expression is minimal at birth, low during the first postnatal week, and comparable with adults at P14-15 (Lu et al., 1999;Rivera et al., 1999).

KCC3. The expression of KCC3 mRNA is extensive in most adult rodent brain areas, such as cerebral cortex, hippocampus, brainstem, hypothalamus, cerebellum, white matter, chroid plexus, and piriform cortex (Pearson et al., 2001;Boettger et al., 2003;Le Rouzic et al., 2006). The strongest KCC3 expression is, however, detectable in the highly myelinated tracts of the dorsal columns of spinal cord (Pearson et al., 2001). Interestingly, the development of myelin in rodent CNS correlates with the ontogeny of KCC3 (Pearson et al., 2001), implying a possible role for KCC3 in the physiology of myelination. In the PNS, KCC3 expression is observable in dorsal root ganglia (Pearson et al., 2001;Boettger et al., 2003). In the prenatal rodent brain, KCC3 mRNA shows a low general expression level, but is slightly up-regulated until birth when expression is clear in the olfactory bulb, cortical plate, and spinal cord (Boettger et al., 2003).

KCC4. In the adult rat nervous system, KCC4 is present in both the CNS and PNS, with higher expression in the peripheral nerves and spinal cord than in the whole brain (Karadsheh et al., 2004). Within the brain, the cerebral cortex, hippocampus, and cerebellum display minimal KCC4 expression, whereas midbrain and brainstem demonstrate higher levels (Karadsheh et al., 2004). In the brainstem, however, KCC4 expression is restricted to the cranial nerves and nuclei such as facial (VII) nerve nucleus, abducens (VI) nerve, and vestibular (VIII) nerve (Karadsheh et al., 2004). In these cranial nerves, KCC4 is expressed in both neurons and oligodendrocytes (Karadsheh et al., 2004). In the spinal cord, KCC4 is highly expressed in the white matter tracts of dorsal and ventral columns, but displays very low expression in the central gray matter (Karadsheh et al., 2004). In addition, KCC4 is expressed very strongly in the apical membrane of the choroid plexus and in the suprachiasmatic nucleus of the hypothalamus (Karadsheh et al., 2004;Le Rouzic et al., 2006). KCC4 expression is high at birth but then declines throughout development (Karadsheh et al., 2004).

The two orphan members of the CCC family, CIP1 and SLC12A8, are expressed in the brain as assessed by real-time quantitative PCR and Northern blot analyses (Caron et al., 2000;Hewett et al., 2002). Unfortunately, no detailed analysis concerning the cell-specific expression patterns of these proteins is available.

1.3 Cation Cl^- cotransporters and inhibitory neurotransmission

1.3.1 γ-Aminobutyric acid

The amino acids GABA and glycine serve as the predominant fast-acting inhibitory neurotransmitters in the adult mammalian nervous system. GABA was first discovered in the mammalian brain over a half a century ago and was soon identified as an inhibitory neurotransmitter in both vertebrate and invertebrate nervous systems (Owens and Kriegstein, 2002). GABA is synthesized primarily from glutamate through enzymatic decarboxylation catalyzed by two glutamic

acid decarboxylase (GAD) enzymes, GAD65 and GAD67 (Erlander et al., 1991), which are expressed only in neurons that use GABA as their neurotransmitter. It is estimated that only 10-20% of neurons are GABAergic in cortical circuits (Gulyas et al., 1999). However, these GABAergic interneurons form a dense network of synapses innervating the somata and dendrites of principal cells as well as other inhibitory interneurons, and thus play a major role in the generation of oscillations and other patterns of neuronal activity that are critical in major brain functions, such as memory and the sleep-wake cycle (Ben Ari and Holmes, 2005). GABA exerts its actions by interacting with ionotropic ($GABA_A$) and metabotropic ($GABA_B$) receptors (McKernan and Whiting, 1996;Couve et al., 2000;Korpi and Sinkkonen, 2006).

Glycine receptors are ligand-gated ion channels comprised of five subunits (Lynch, 2004). These receptors are found in many regions of the nervous system, but show particularly abundant expression in spinal cord and brain stem where they are involved in the control of motor rhythm generation, coordination of reflex responses, and processing of sensory signals (Laube et al., 2002).

1.3.2 Maturation of γ-aminobutyric acid type A receptor-mediated transmission

Consistent with the critical role of $GABA_A$ receptors in CNS information processing, $GABA_A$ receptor dysfunction is implicated in many pathological processes such as epilepsy, pain, and anxiety (Cherubini and Conti, 2001). The pentameric $GABA_A$ receptors are members of the ligand-gated ion channel superfamily (Leite and Cascio, 2001), and characteristic of these receptors ligand binding is followed by a conformational change in the receptor complex that opens an integral ion channel. $GABA_A$ receptors are comprised of diverse subunits (α1-α6, β1-β3, γ1-γ3, δ, θ, ε, π, and ρ1-ρ3), and native receptors usually consist of two α and two β subunits with an additional subunit, typically γ, δ, or ε (Whiting et al., 1999). Depending on the ion selectivity of a ligand-gated channel, fast neuronal depolarization or hyperpolarization results. In the case of ionotropic $GABA_A$ receptors, the anion channel is permeant to Cl^- and to a lesser extent to bicarbonate (HCO_3^-) ions (Kaila and Voipio, 1987). Thus the electrochemical reversal potential of $GABA_A$ receptors (E_{GABA-A}) is set by the electrochemical gradients of Cl^- and HCO_3^- ions across the transmembrane.

In most immature neurons, the activation of $GABA_A$ receptors depolarizes the neuronal membrane potential, which can open voltage-gated Ca^{2+} channels and lead to a transient increase in intracellular Ca^{2+} concentration (Owens and Kriegstein, 2002;Ben Ari, 2002). These depolarizing GABA responses have been observed in developing cells from many brain regions including neonatal rat hippocampus and Purkinje cells of the cerebellum (Leinekugel et al., 1995;Leinekugel et al., 1997;Eilers et al., 2001), embryonic and early postnatal rat neocortex and spinal cord (Wu et al., 1992;Gao and Ziskind-Conhaim, 1995;Owens et al., 1996;Maric et al., 2001), and early postnatal mouse hypothalamus (Wang et al., 2001;Gao and Van Den Pol, 2001). The early GABA-mediated communication is believed to influence many Ca^{2+}-dependent developmental processes such as cell proliferation, synaptogenesis and circuit formation (Ben Ari et al., 1994;Owens and Kriegstein, 2002;Fiszman and Schousboe, 2004).

The ionic basis for depolarizing $GABA_A$ receptor-mediated responses depends on high $[Cl^-]_i$ levels and the resultant E_{Cl} that is more positive than the resting membrane potential (Ben Ari, 2002). Thus, activation of the $GABA_A$ receptor results in Cl^- efflux and membrane depolarization. In immature neurons, the high internal Cl^- is largely maintained by the action of the Cl^--accumulating NKCC1 cotransporter, which shows robust expression in immature neurons during early postnatal development (Plotkin et al., 1997b;Sung et al., 2000;Yamada et al., 2004;Dzhala et al., 2005). In contrast, the Cl^--extruding KCC2 displays only minimal expression at this stage in rat cortical neurons and hippocampal pyramidal cells (Rivera et al., 1999;Dzhala et al., 2005). The developmental shift in E_{GABA-A} from depolarized levels towards more hyperpolarized potentials results from an ontogenetic decrease in $[Cl^-]_i$ (Luhmann and Prince, 1991;Owens et al., 1996). This is due to the developmentally up-regulated expression of functional KCC2 that results in increased neuronal Cl^- extrusion (Lu et al., 1999;Rivera et al., 1999;Hubner et al., 2001b).

The role for KCC2 in creating and maintaining the low neuronal $[Cl^-]_i$ was first identified by Rivera and collaborators, who showed that in functionally mature hippocampal pyramidal neurons, antisense oligonucleotide inhibition of KCC2 expression produced a marked positive shift in E_{GABA-A} and a consequent reduction or abolishment of normally hyperpolarizing GABA responses (Rivera et al., 1999). Consistent with this, deletion of the *KCC2* gene results in mice that show significantly increased depolarizing GABA responses in spinal cord motoneurons compared to wild-type control mice (Hubner et al., 2001b). These mice die immediately after birth due to abnormal muscle tonus, defects in motor control, and inability to breath (Hubner et al., 2001b). In addition, over-expression of KCC2 in immature neurons at an early developmental stage, when the protein is normally expressed at low levels, results in a substantial reduction in intracellular Cl^- (Lee et al., 2005;Chudotvorova et al., 2005;Fiumelli et al., 2005), and decrease or abolish GABA-elicited Ca^{2+} responses (Lee et al., 2005). Interestingly, this ectopic expression of KCC2 increases the number of GABA synapses and the frequency and amplitude of miniature postsynaptic potentials, suggesting that KCC2 may be involved in the regulation of the construction of GABAergic networks (Chudotvorova et al., 2005).

While KCC2 is considered as the major regulator of neuronal $[Cl^-]_i$ in the adult CNS, KCC3 seems to have a similar, but less pronounced, role (Boettger et al., 2003). This is apparent in KCC3 knock-out mice that display elevated $[Cl^-]_i$ and weakened, but still hyperpolarizing, GABA-responses as assessed in the cerebellar Purkinje neurons (Boettger et al., 2003).

1.3.3 γ-Aminobutyric acid type A receptor-mediated effects on membrane polarization

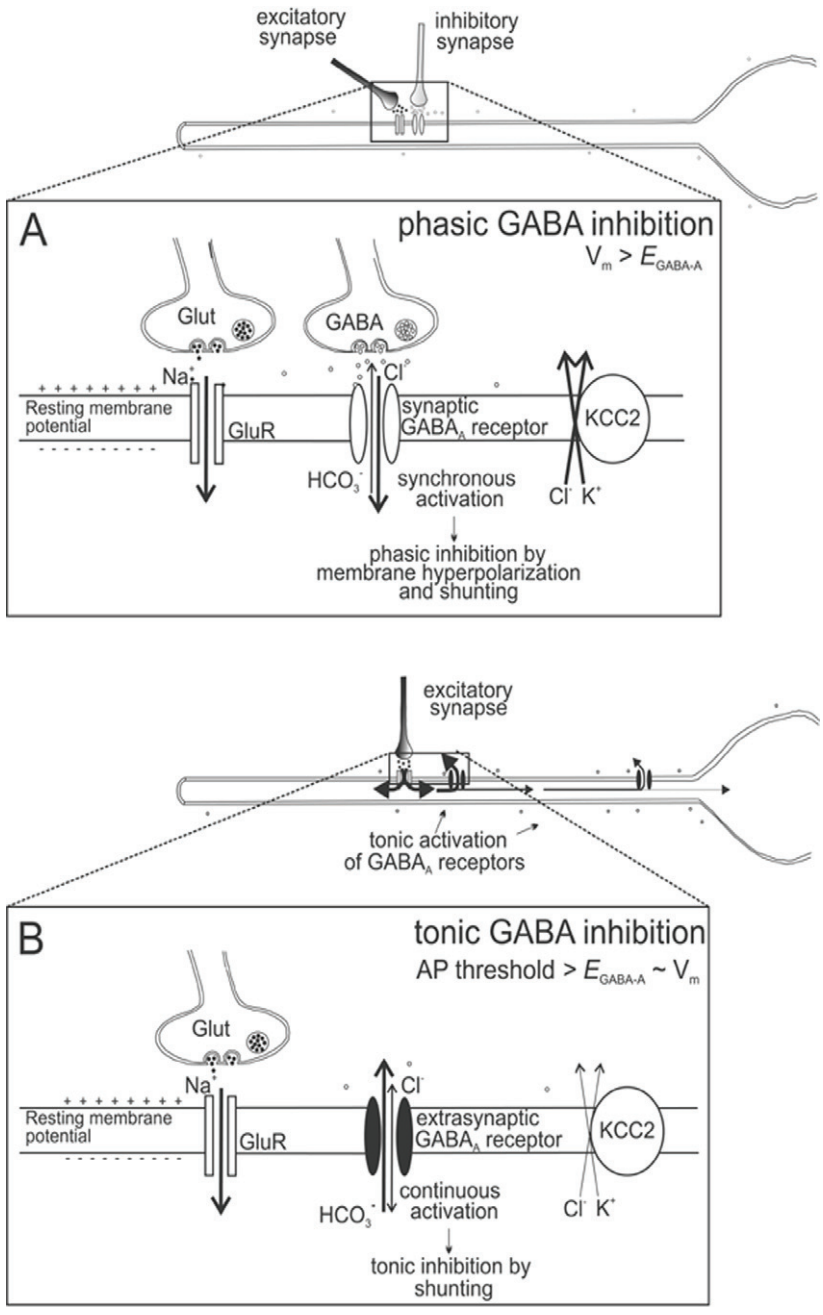
The ionotropic $GABA_A$ receptor-mediated events have two effects on the postsynaptic membrane (Fig. 2). One is a shunting effect due to increased postsynaptic conductance that decreases the amplitude and duration of a voltage response generated by excitatory current (London and Hausser, 2005). The time course for the shunting effect is limited to the opening time of $GABA_A$ receptor (Staley and Mody, 1992). The second effect is due to the synaptic

'battery' to which the $GABA_A$ receptor-mediated conductance is connected, leading to depolarizing (excitatory) or hyperpolarizing (inhibitory) currents depending on the E_{GABA-A} value relative to the actual membrane potential. The time course for these GABAergic events outlasts the conductance change because the time constant of the membrane prolongs the depolarization or hyperpolarization of the membrane (Gulledge and Stuart, 2003;Stein and Nicoll, 2003).

As already stated, in most mature neurons the intracellular Cl^- is low due to Cl^- extruding activity of KCC2 (Rivera et al., 1999), and consequently the E_{Cl} is more negative than the resting membrane potential (~-65 mV). Thus $GABA_A$ receptor activation typically results in net entry of Cl^- ions, and the classically described hyperpolarizing postsynaptic potentials (IPSPs). The $GABA_A$ receptor is, however, permeable to HCO_3^- ions as well (the permeability ratio for HCO_3^- to

Fig. 2. Different roles for KCC2 activity in tonic and phasic GABAergic neurotransmission. **A)** Illustrated is a simplified neuron receiving simultaneous excitatory and inhibitory inputs at a dendritic location. A presynaptic release of glutamate (black circles) activates synaptic glutamate receptors causing an inward postsynaptic current. Simultaneous presynaptic release of GABA activates those postsynaptic $GABA_A$ receptors that are clustered in the membrane immediately beneath the release site. Chloride extrusion by the activity of KCC2 results in reversal potential for $GABA_A$ receptors (E_{GABA-A}) that is more negative than the resting membrane potential (V_m). Thus phasic activation of synaptic $GABA_A$ receptors results in net entry of Cl^- ions, and the classic hyperpolarizing postsynaptic potential. The $GABA_A$ receptor is also permeable to bicarbonate (HCO_3^-) ions (the permeability ratio for HCO_3^- to Cl^- ions being approximately 0.25). The equilibrium potential for HCO_3^- is more positive than V_m and thus the direction of HCO_3^- ion flux results in outwardly directed (depolarizing) current. Therefore, the reversal potential of $GABA_A$ receptor-gated currents is determined by the electrochemical gradients of Cl^- and HCO_3^-. In this case, the internal concentration of Cl^- is so low that E_{GABA-A} remains more negative than the resting membrane potential, despite the depolarizing HCO_3^- current. The $GABA_A$ receptor-mediated effect on the postsynaptic membrane occurs by both the shunting effect and the hyperpolarization of the membrane. The time course for these GABAergic events outlasts the conductance change because the time constant of the membrane prolongs the depolarization or hyperpolarization of the membrane. **B)** Illustrated is a similar neuron receiving an excitatory input at a dendritic location (spine). A release of glutamate activates glutamate receptors causing an inward postsynaptic current that spreads to the soma. A low concentration of ambient GABA (white circles) tonically activates high-affinity $GABA_A$ receptors at extrasynaptic locations, through which depolarizing current leaks out (shunting). In these cells, E_{Cl} may be at equilibrium (or even above the resting membrane potential) due to low KCC2 activity. If E_{Cl} is at equilibrium (as shown in B), GABA is depolarizing due to the contribution of HCO_3^- ions to the ion flux mediated by $GABA_A$ receptors, making E_{GABA-A} more positive than E_{Cl}. E_{GABA-A} may or may not be close the resting membrane potential, but is below the threshold potential for action potential (AP) generation. Thus the increase in membrane conductance (shunting) through the tonic extrasynaptic $GABA_A$ receptors always inhibits the depolarized membrane potential from reaching the action potential threshold, and the shunting is effective even if E_{GABA-A} is above the V_m and GABA responses are depolarizing.

Cl^- being approximately 0.25) (Bormann et al., 1987). In contrast to E_{Cl}, the E_{HCO3} is more positive than the resting membrane potential (around -10 mV), and hence the driving force is significantly stronger for HCO_3^- than for Cl^- at the resting membrane potential. Thus in some mature neurons, even in the presence active Cl^- extruding mechanism, GABAergic neurotransmission can be depolarizing under basal conditions due to the contribution of HCO_3^- ions to the ion flow through $GABA_A$ receptors,



thereby making E_{GABA-A} in some cases more positive than E_{Cl} (Kaila et al., 1993).

The depolarizing GABA responses can still be inhibitory due to the shunting mechanism. However, the effect of shunting depends on its timing and location in relation to excitatory inputs and occurs when the conductance change is temporally or spatially nonseparated from excitatory inputs (Gulledge and Stuart, 2003). The timing of shunting inhibition is important because the depolarizing GABA responses have a longer time course than the underlying conductance change. Therefore, if the depolarizing GABA conductance occurs near (± 2 ms) the onset of excitatory input, the GABA response will first be inhibitory due to conductance change and then excitatory due to depolarized membrane potential which has not yet decayed back to rest. If the shunting GABA conductance is temporally separated from the excitatory stimulation by several milliseconds, the shunting conductance change may be over before the excitatory input and no inhibition of the excitation occurs.

When the shunting conductance and excitatory inputs are temporally coincident, the spatial relation of these inputs determines whether GABA is excitatory or inhibitory. If GABA applications to dendritic locations are paired with excitatory subthreshold somatic or more proximal dendritic depolarizations, the effect of GABA (measured in soma) is purely excitatory. This is because the GABA-induced membrane depolarization, in contrast to the GABA-induced conductance increase, will spread electrotonically to the soma. However, if the dendritically applied GABA is paired with suprathreshold excitatory inputs located at more distal dendritic sites than the GABA application site, the GABA response will inhibit the generation of action potential by shunting. Similarly, somatic GABA responses that are coincident with somatic or dendritic excitatory inputs are inhibitory (Gulledge and Stuart, 2003).

1.3.4 Phasic and tonic activation of γ-aminobutyric acid type A receptors

Ionotropic $GABA_A$ receptors mediate two spatially and temporally distinct modes of inhibition (Fig. 2); 'phasic' fast and transient inhibitory postsynaptic currents at synapses following presynaptic release of vesicular GABA, and continuous 'tonic' conductance at extrasynaptic sites activated by low ambient concentrations of extracellular GABA (Farrant and Nusser, 2005). The classic phasic form of $GABA_A$ receptor activation is characterized by hyperpolarizing IPSPs, which are dependent on the KCC2 activity. Synaptic GABAergic inhibition is generally thought to play an important role in several basic physiological processes of the CNS, including prevention of neuronal overexcitability as well as the regulation and synchronization of the neuronal network activity (Cherubini and Conti, 2001).

In contrast to the transient (phasic) form of GABAergic inhibition, the tonic form of GABA inhibition is mediated by extrasynaptic $GABA_A$ receptors (Farrant and Nusser, 2005). These receptors mediate their inhibitory effects predominantly through the shunting mechanism (Brickley et al., 1996;Mitchell and Silver, 2003). Tonic GABAergic currents are critically involved in the regulation of neuronal network excitability (Semyanov et al., 2003) and information processing (Mitchell and Silver, 2003;Chadderton et al., 2004), and have been identified from

several brain regions, including cerebellar granule cells (Kaneda et al., 1995;Brickley et al., 1996;Wall and Usowicz, 1997), hippocampal interneurons, pyramidal cells, and dentate granule neurons (Semyanov et al., 2003;Stell et al., 2003;Yeung et al., 2003;Wei et al., 2004;Caraiscos et al., 2004), and thalamic neurons (Belelli et al., 2005;Cope et al., 2005).

The subunit composition of $GABA_A$ receptors determines not only the pharmacological properties and function of the receptors, but also distribution within the cellular membrane (Korpi et al., 2002a). For example, receptors that contain the benzodiazepine-sensitive γ2 subunit are preferentially located in the synapses, whereas the δ subunit is present predominantly in extrasynaptic receptors (Somogyi et al., 1996;Nusser et al., 1998). The δ subunit $GABA_A$ receptors have a highly specific regional distribution and are the major contributors to the tonic GABA current in the cerebellar and dentate granule cells as well as in thalamic neurons (Brickley et al., 1996;Pirker et al., 2000;Nusser and Mody, 2002;Stell et al., 2003;Belelli et al., 2005;Cope et al., 2005). In the cerebellar granule cells, the δ subunit is intimately associated with the α6 subunit (Jones et al., 1997;Nusser et al., 1999), whereas in the dentate granule cells it probably associates with the α4 subunit (Sur et al., 1999a). In hippocampal pyramidal neurons, tonic inhibition has been reported to be mediated primarily by extrasynaptic α5 subunit-containing $GABA_A$ receptors (Caraiscos et al., 2004), whereas in CA1 interneurons at least part of the tonic current is mediated by γ subunit-containing $GABA_A$ receptors (Semyanov et al., 2003).

1.3.5 Cation-Cl^- cotransporter isoform 2 and epilepsy

Epilepsy is a chronic neurological disorder that affects approximately 1-2% of the population worldwide. The disease is characterized by recurrent spontaneous seizures which are the clinical manifestation of an underlying transient abnormal neuronal activity (Steinlein, 2004). This hyperactivity is conventionally thought to occur when there is imbalance between neuronal excitation and inhibition, where glutamate and GABA, respectively, play important roles. This view is, however, an oversimplification as the GABA-mediated neurotransmission cannot be described as purely 'inhibitory', as discussed above (1.3.3). In addition, GABAergic interneurons are heterogenous and have many roles that are not restricted to the straightforward concept of 'inhibition of the target' (Freund, 2003;Cossart et al., 2005). Defects in the GABAergic neurotransmission system do, however, contribute to the synchronous hyperexcitable activity of the epileptic brain. This is confirmed by the effectiveness of anticonvulsant drugs which enhance GABAergic transmission, and by *in vitro* studies using cortical slices in which the application of GABA blockers causes the appearance of epileptiform discharges.

In epileptic tissue, modifications to the GABAergic system take place at various levels within the GABAergic networks. These modifications may include changes in $GABA_A$ receptor subunit composition and selective loss of some GABAergic interneuron types (Cossart et al., 2005;Magloczky and Freund, 2005). Recently, depolarizing $GABA_A$ receptor-mediated transmission has been described as one of the potential mechanism

contributing to the pathophysiology of epilepsy (Cohen et al., 2002). In hippocampal-subicular preparations obtained from mesial temporal lobe epilepsy patients, spontaneous synchronous events could be observed in the subiculum (Cohen et al., 2002). These discharges were dependent on both glutamatergic and GABAergic transmission, and the network of neurons discharging during population events comprised subicular interneurons and a subgroup of pyramidal cells. The pyramidal cells displayed depolarizing rather than hyperpolarizing actions of GABA (Cohen et al., 2002), suggesting that in this context, GABA may serve a pro- rather than an anti-epileptic role. The depolarizing GABA responses in subicular pyramidal cells were presumably due to reduction in KCC2 levels which thus lead to accumulation of Cl^- and enhanced neuronal excitability. Indeed, the role of KCC2 in the pathophysiology of epilepsy has been confirmed in KCC2-deficient mice that retain 5-8% of the protein, as these mice exhibit frequent seizure activity and severe brain injury (Woo et al., 2002). In addition, hippocampal kindling-induced seizures *in vivo* and interictal-like activity in slices lead to brain-derived neurotrophic factor (BDNF)-mediated down-regulation of KCC2 mRNA in rodents (Rivera et al., 2002;Rivera et al., 2004). Interestingly, in slice preparations of chronically injured epileptogenic neocortex, Cl^- transport can be largely altered with only minor changes in resting E_{GABA-A} (Jin et al., 2005), suggesting that impaired Cl^- extrusion due to reduced KCC2 may be sufficient to maintain $[Cl^-]_i$ under resting conditions but not during periods of intense GABAergic interneuron activity that might occur at seizure discharge.

2. AIMS OF THIS STUDY

The aims of this thesis were to understand the physiological role of KCC2 *in vivo* by producing transgenic KCC2-deficient mice and analyzing the biological consequences of the gene-deficiency. Another aim was to elucidate the role for different CCC family members in the regulation of CNS development by analyzing the expression patterns of KCC1-4 and NKCC1 in the rodent brain during embryonic development.

The specific aims were to:

1. To reveal the expression patterns of NKCC1, KCC1, KCC2, KCC3, and KCC4 in the rodent CNS during embryonic development.
2 To produce mice with genetically inactivated or down-regulated expression of KCC2.
3 To investigate the significance of reduced KCC2 levels on adult mouse behavior.
4 To study the requirements for KCC2 in tonic versus phasic $GABA_A$ receptor-mediated inhibition in the adult CNS *in vivo*.

3. MATERIALS AND METHODS

The methods used have been described in more detail in the respective publications (I-IV)

3.1 Generation of cation-Cl^- cotransporter isoform 2-deficient mice (I, III, IV)

Generation of KCC2-deficient mice is described in detail in (I). Briefly, the mutant allele-producing gene targeting vectors were developed by utilizing an *in vitro* DNA transposition-based technique. For the KCC2 null allele, a neomycin phosphotransferase (*neo*) resistance gene cassette was inserted into exon 4 of the *Slc12a5* gene to disrupt the reading frame, whereas in the KCC2 hypomorphic allele, a reversely directed *neo* cassette was introduced into intron 3. Both mutant alleles were generated in R1 embryonic stem cells, and chimeric mice derived from these cells were bred to wild-type C57BL/6JOlaHsd and 129S2/SvHsd female mice (Harlan) to obtain F1 heterozygotes ($KCC2^{wt/null}$ and $KCC2^{wt/hy}$). For the first characterization of the mutation, $KCC2^{null/null}$, $KCC2^{hy/hy}$ and wild-type littermates were established from matings of F1 $KCC2^{wt/null}$ and $KCC2^{wt/hy}$ mice, respectively (I, III). Thereafter, heterozygous $KCC2^{wt/hy}$ and $KCC2^{wt/null}$ were backcrossed to wild-type C57BL and 129S2 mice for at least five generations. Intercrossing of the $KCC2^{wt/hy}$ (in C57BL/6) and $KCC2^{wt/null}$ (in 129S2) produced heteroallelic $KCC2^{hy/null}$, heterozygous $KCC2^{wt/null}$ and $KCC2^{wt/hy}$, as well as wild-type littermates in a (C57BL6 x 129S2)F1 hybrid background (Banbury conference on genetic background in mice, 1997) that were used in (III, IV). The animals were kept on a normal day and night cycle (lights on 07.00, off 19.00 h) and were given free access to food and water. The experimental protocols were approved by the committee for Animal Experiments of the University of Helsinki.

3.2 *In situ* hybridization (I)

The protocol for *in situ* hybridization analyses is described in the original article and references therein (II), probes are depicted in table 2. In experiments where embryonic tissues were used (II, III), the animals were mated overnight and inspected the following morning for a vaginal plug to confirm copulation. The day of plug confirmation was regarded as E0.5.

3.3 Reverse transcriptase-polymer chain reaction (I, III)

The protocols for total extraction of mRNA from mouse brain and the PCR amplification for semiquantitative reverse transcriptase-polymer chain reaction (RT-PCR) analysis are described in (II, III). The PCR primers used for relevant purposes are listed in table 3.

3.4 Immunohistochemistry and Western blotting (I, II, III)

The procedures for both immunohistochemistry (IHC) and Western blotting (WB) are described in (I, II, III). The antibodies used in both procedures are presented in table 4. In the immunohistochemical analysis of embryonic rat brain, antibodies against the vimentin and the class III β-tubulin (βIII) TUJ1 were used as specific markers to detect radial glia and postmitotic neurons, respectively. T4 monoclonal antibody was used to detect NKCC1 expression in both

Table 2. List of probes used for *in situ* hybridization

Probe	Accession/source	Nucleotides	Insert bp	Region
Mouse KCC1	AA185691 (EST)	3295-3746	495	3'-UTR
Mouse KCC2	AA982489 (EST)	4605-5566	1039	3'-UTR
Rat KCC2	Payne et al., 1996	5-834	830	3'-UTR
Mouse KCC3	BF020529 (EST)	2832-3348	682	3'-CDR
Mouse KCC4	AI592647 (EST)	2508-3248	745	3'-CDR
Mouse KCC4	RT-PCR	72-275	203	5'-UTR
Mouse NKCC1	AA980272 (EST)	3498-4346	964	3'-CDR+ UTR
Mouse NKCC1	RT-PCR	2742-3006	265	CDR

Table 3. List of primers used for RT-PCR quantification

Probe	Primers	Product bp	Used in
Mouse KCC1	5'-TCGGGACCAGTTTGACATCT-3' 5'-CGATGTCAGCCACCACATAC-3'C	301	III
Mouse KCC3	5'-GCAGTGAGGTCATCACCATTT-3' 5'-TGGAATCCCCTTAGTTCAGC-3'	700	II, III
Mouse KCC4	5'-CATGCCCACGAACTTTACG-3' 5'-TTCTTCCCCTCGAAGTAGCTC-3'	203	III
Mouse cyclophilin	5'-GGTCAACCCCACCGTGTTCTTCGACAT-3' 5'-GGACAAGATGCCAGGACCTGTATGCT	294	II
Mouse GAPDH	5'-GCAAAGTGGAGATTGTTGCCAT 5'-CCTTGACTGTCGCGTTGAATTT	150	III

immunohistochemistry and WB. KCC2-specific antibodies were used to evaluate the KCC2 protein levels and distribution in wild-type and KCC2 mutant mice by WB and immunohistochemistry. Antibodies against parvalbumin, synaptophysin, and calbindin were used to evaluate the gross morphology of the $KCC2^{hy/null}$ mouse brain. The neurofilament H and neuron-specific class of III β-tubulin specific-antibodies were used as loading controls in WB.

3.5 Mouse behavioral studies (III, IV)

The behavioral testing is described in detail in the respective publications (III, IV), except for the previously unpublished prepulse inhibition (PPI). General characteristics of some key experimental setups used in this thesis are discussed below.

3.5.1 Elevated plus-maze

The elevated plus-maze is a nonshock, unconditioned, and natural conflict model of anxiety-related behaviors (Lister, 1990). The test apparatus consists of two open arms and two arms that are enclosed by high walls (Handley and Mithani, 1984;Pellow et al., 1985). The elevated plus-maze rests on the conflict between the tendency of mice to explore a novel environment and the aversive properties of a high and open area, which is manifested in the typical behavior of mouse in the model: greater exploration of the closed arms and less exploration of the open arms (Handley and Mithani, 1984;Pellow et al.,

Table 4. Primary antibodies used in immunohistochemistry and Western blotting

Primary antibody	Host/Antibody	Source/Ref.	Dilution	Method	Used in
KCC2	Rb polyclonal	Williams et al.	1:2000	WB	I
	Rb polyclonal	1999	1:200	IHC	III
Neurofilament H	Rb polyclonal	NA1211, Affiniti	1:2000	WB	I
Class III β-tubulin (βIII)	Mo monoclonal	Clone TUJ1,	1:5000	IHC	II
		BABCO			
NKCC1, clone T4	Mo monoclonal	Hybridoma Bank,	1:1000	IHC	II
		University of Iowa	1:2000	WB	III
*β*III	Rb polyclonal	BABCO	1:2000	IHC	II
			1:1000	WB	III
vimentin	Rb polyclonal	Sigma	1:20	IHC	II
parvalbumin	Rb polyclonal	Swant	1:400	IHC	III
calbindin	Rb polyclonal	Swant	1:400	IHC	III
synaptophysin	Rb polyclonal	Zymed	1:50	IHC	III

1985;Lister, 1987). Advantages of the plus-maze test are that it is an efficient and simple test which does not involve lengthy training as it is based on spontaneous animal behavior. In addition, the total number of entries into all arms provides a built-in control measure for general hyperactivity or sedation (Crawley, 2000). The disadvantage of the model is the non-continous arrangement of the arms. This is apparent when the mice stay in the central start box and may repeatedly return to the start box rather than fully enter an arm (Crawley, 2000).

3.5.2 Open field

The open field test consists of the measurement of behaviors elicited by placing a mouse in a novel, wall-surrounded arena (either circular, square, or rectangular) (Walsh and Cummins, 1976). These behaviors involve spontaneous motor activity and behaviors related to emotionality, and are elicited by a variety of factors such as the removal of the animal from a familiar environment, exposing the mouse to the test environment, and prior experience of the test situation (Walsh and Cummins, 1976). Motor activity is evaluated by calculating the overall locomotion score, whereas the emotional (e.g. anxiety, fear) behavior is generally measured by monitoring the time the animal spends in the central part, the latency to enter the central part, grooming activity, or rearing activity (Archer, 1973;Crawley et al., 1997;Belzung, 1999). Differences in the experimental setup of the test apparatus (e.g. light illumination, background noise, field size) as well as the genetic background of the animals may have an effect on the animal performance (Walsh and Cummins, 1976;Crabbe, 1986;Mathis et al., 1994).

3.5.3 Rotarod

Performance on the rotarod measures the ability of an animal to remain on a rotating rod. As motor performance is a behaviorally complex phenomenon, the rotarod task may require the animal to employ not only motor coordination and sensorimotor control, but also vision, balance, adequate muscle strength, and attention (Jones and Roberts, 1968;Crabbe et al., 2005). In addition, for rotarod tasks where multiple trials are given, motivation and ability to learn and remember may be critical factors (Crawley, 2000;Crabbe et al., 2005). After practice, motor performance becomes a more automatic, unconscious behavior, which is controlled by motor cortex and divisions of the basal ganglia and cerebellum (Hikosaka et al., 2002). In accelerating rotarods, the rotational speed gradually increases, and thus requirements for the mouse to stay on the rod gradually increase. The duration of the test may affect the animal performance, as a 5 min test period may have a fatiguing effect on the animal.

3.5.4 Water maze

The Morris water maze is used to evaluate spatial learning and memory of rats and mice (Morris, 1981). The experimental setup consists of a pool of water (22-23°C) and a hidden escape platform submerged beneath the surface. The task requires spatial navigation as mice are required to learn the location of the platform by using visual cues that surround the maze (Morris, 1981). Platform finding is based on the principle that rodents are highly motivated to escape from the water by the quickest, most direct route. Disadvantage of the model is that some rodent strains enjoy swimming, whereas some strains

sink rather than swim (Crawley, 2000). Transfer tests (probe trials) reveal whether animals are using a spatial search strategy to locate the platform (Morris, 1981).

Performance on the task is highly influenced by the genetic background of the animal (Upchurch and Wehner, 1989;Owen et al., 1997). For example, within some inbred mouse strains one substrain may perform well on the task, whereas some other substrain performs poorly in the task (Montkowski et al., 1997;Balogh et al., 1999). The motivational factors as well as perceptual and motor abilities also influence the water maze performance (Lipp and Wolfer, 1998). In addition, mice that display enhanced anxiety may be more sensitive to the stressful component of the swimming task, or may alternatively display reduced problem-solving abilities or memory (Morley et al., 2001;Herrero et al., 2006). These confounding factors may be controlled by testing the animals in the visible platform version of the Morris water maze task. In this variant, the escape platform is made visible by a colored marker directly above the escape platform, and this serves as a useful control of any impairments seen in the hidden platform version (Morris, 1981). Finally, possible hypothermia caused by the exposition of animals to water may affect the swimming speed of the mice (Panakhova et al., 1984;Rauch et al., 1989). This problem can be alleviated by increasing the intertrial interval time, as demonstrated in mice by increasing the intertrial interval from 30s to 13 min. The cooling effect observed with 30s intervals was removed when the intertrial interval time was increased to 13 min (Iivonen et al., 2003). In the experimental setup used in this thesis, the intertrial interval was approximately 3 min. During this time the animals were able to dry themselves and, consequently, no evidence of slowing down the swimming speed was observed. No additional control of body temperature was used.

3.5.5 Hot plate, tail withdrawal, and von Frey hairs

The hot plate test measures thermal pain reflexes due to footpad contact with a heated surface. The mouse is placed on a horizontal surface heated to 52-55ºC and the latency to lift and lick the paws is the measure of pain sensitivity or analgesia. The reflex measured by the hot plate test requires intact circuitry in the brain as well as in the spinal cord (Malmberg, 1999).

Tail withdrawal (hot-water tail flick) measures a spinal reflex and the dependent variable is the latency for the mouse to withdraw the tail when immersed in a beaker of water maintained usually at 52.5ºC (Gonzales-Rios et al., 1986;Hole and Tjolsen, 1993).

Von Frey hairs are a set of very fine gauge calibrated metal or nylon fibers used to measure the sensitivity of the mouse for mechanical stimulus (Fruhstorfer et al., 2001). The experimental setup includes an elevated platform, of which the surface is a wide gauge wire mesh. The mouse is placed on the mesh and the von Frey hair is inserted from below to poke the plantar surface of the hindpaw (Pitcher et al., 1999). When a straight elastic fiber of constant diameter is pressed vertically against the skin, the force it exerts on the skin grows until the fiber starts bending. Once bent, the vertical force remains fairly constant even if the fiber is bent further. Applying hairs of different force is done to establish the paw withdrawal threshold (Fruhstorfer et al., 2001). Pain sensitivity in rodents is affected by stress (Lewis et al., 1980;Calcagnetti and Holtzman,

1990). The confounding effects of novelty and handling stress can be avoided by prior handling of the animals and habituation to the test environment (Crawley, 2000).

3.5.6 Prepulse inhibition (unpublished data)

Prepulse inhibition (PPI) is a cross-species measure of sensorimotor gating, which is the ability of a weak sensory event to inhibit "gate" the motor response to an intense sensory stimulus (Swerdlow et al., 2001). Deficits in PPI have been associated with a number of psychiatric diseases, including schizophrenia (Braff et al., 2001).

A group of 3-5-month-old littermate mice (29 wild-type males, 24 $KCC2^{hy/null}$ males, 28 wild-type females, 32 $KCC2^{hy/null}$ females) was subjected to a PPI experiment, which was performed in MED Associates (St. Albans, VT, USA) chambers. The isolation chambers contained a transparent acrylic cylinder (4.5 cm diameter, 10 cm length) for restraining the animal on the platform. The restrainer was placed on a platform containing a movement-sensitive load cell that produces a voltage when the mouse's movements are transmitted through the restrainer bottom. The resulting acoustic startle response or prepulse inhibition data is the displacement of the load cell (as expressed in Volts). A fan and a red light were provided inside the chamber for the comfort of the animal while inside the enclosed chamber. Data acquisition was performed using Med Associates software.

Mice were placed in the startle chamber with a background white noise of 65 dB and left undisturbed for 5 minutes. Testing was performed in 12 blocks of 5 trials and 5 trial types were applied. One trial type was a 40 ms, 105 dB white noise acoustic startle stimulus presented alone. This was used as the measure of baseline startle-sensitivity. In the remaining 4 trial types the startle stimulus was preceded by the acoustic prepulse. The 20 ms prepulse stimuli were white noise bursts of 68, 72, 76 and 80 dB. The delay between onsets of prepulse stimuli and startle stimuli was 100 ms. The 1^{st} and 12^{th} block consisted of startle stimulus-alone trials. In the remaining blocks the startle stimulus and prepulse stimulus-plus-startle stimulus trials were presented in pseudorandomized order such that each trial type was presented once within a block of 5 trials. The intertrial interval ranged between 10 and 20 seconds and experiment lasted 30 min per animal. The startle response was recorded for 65 ms starting with the onset of the startle stimulus. The maximum startle amplitude recorded during the 65-ms sampling window was used as the dependent variable. The startle response was averaged over 10 trials from blocks 2-11 for each trial type. The PPI for each prepulse stimulus was calculated using the following formula: 100-[(startle response on prepulse stimulus-plus-startle stimulus trials / startle response on startle stimulus trials) x 100].

3.6 Electrophysiology (IV)

Electrophysiological recordings from acute hippocampal slices using whole-cell- and perforated-patch configurations were done as described in (IV).

4. RESULTS AND DISCUSSION

4.1 Expression of cation-Cl^- cotransporter isoforms in the developing CNS (I)

In order to elucidate the possible roles for CCC isoforms in the control of CNS development, we conducted an analysis of the distribution patterns of KCC1-4 and NKCC1 in rodent CNS during embryonic development using immunohistocemistry and *in situ* hybridization. Results from this analysis (I) and other equivalent reports are summarized in table 5. The developmental stages (E12.5-P1) presented in the table 5 correspond to the embryonic mouse development. The rat nervous system development is about two days behind from the mouse during the second week of gestation, and about three to four days behind at birth (Alvarez-Bolado and Swanson, 1996). In table 5, results from the developing rat nervous system have been extrapolated to match the mouse developmental stage (e.g. E14.5 in rat corresponds to the mouse developmental stage at E12.5 in mouse). The developmental time period in mouse (E12.5-P0) corresponds roughly to the human developmental time window spanning the late 1st semester and the early 2nd semester (Clancy et al., 2001).

4.1.1 Na^+-K^+-Cl^- cotransporter isoform 1 is highly expressed in the proliferative zones of subcortical regions

NKCC1 transcripts were detectable in the developing mouse CNS already at E12.5, when a moderate expression was observed in the developing choroid plexus invagination and in the proliferative ventricular zones (VZs) where neurogenesis takes place, including the the lateral ganglionic eminence of the telencephalon. By E14.5 in mouse, NKCC1 mRNA expression had increased significantly in the telencephalon and was robust in the VZs of lateral and medial ganglionic eminences, and moderate in the VZ of isocortex. At this stage, NKCC1 mRNA expression was not detectable in the cortical plate (CP) layer or in the subventricular zone. A moderate expression of NKCC1 transcripts was detected throughout the ventricular layers of the developing embryo (table 5). As indicated by the general co-localization of NKCC1 protein with radial glia marker vimentin, the majority of NKCC1 expressing cells in the VZ layers were radial glia and neuronal precursor cells. Towards later stages of embryonic development (E17-P0), NKCC1 mRNA expression disappeared from the VZ layers and shifted to the CP layer where the transcripts were expressed in postmitotic migrating or differentiating neurons. Throughout embryonic development, NKCC1 expression was strong in the choroid plexus.

Our results of embryonic rodent NKCC1 expression were consistent with other reports (Hubner et al., 2001a;Wang et al., 2002), which demonstrated strong NKCC1 expression in the embryonic choroid plexus and premature cells of neuroepithelia.

4.1.2 Cation-Cl^- cotransporter isoform 2 expression follows neuronal maturation

The expression of KCC2 mRNA was first apparent in the ventral part of the cervical spinal cord at E12 in rat embryos. Thereafter, KCC2 expression gradually spread in caudal-rostral progression following the pattern of neuronal maturation, as indicated by the

coexistence of KCC2 mRNA with TUJ1 (class III β-tubulin) labeled brain regions. Interestingly, in newly differentiated regions most TUJ1 expressing cells were KCC2-negative, suggesting that KCC2 is not expressed in immature, newly born neurons.

In E14.5 rat embryos, KCC2 transcripts were abundant in rhombencephalon, diencephalon, and basal ganglia. At this developmental stage, these brain regions are known to contain postmitotic neurons (Altman and Bayer, 1995). In the diencephalon, KCC2 mRNA was high in ventral thalamus, ventral lateral geniculate complex, lateral hypothalamic area, and in the subthalamic nucleus. Especially high levels of KCC2 expression were detected in the olfactory bulb and the lower parts of the brainstem; the tegmental region of medulla and the caudal part of the spinal nucleus of the trigeminal nerve, consistent with the more advanced maturation of neurons in these regions (Altman and Bayer, 1995). In P0 rat brain, KCC2 expression was spread over the complete spinal cord and was present in all parts of the brain except for cortex and hippocampus.

In conclusion, consistent with other reports (Clayton et al., 1998;Rivera et al., 1999;Hubner et al., 2001b;Wang et al., 2002;Stein et al., 2004), the up-regulation of KCC2 mRNA expression in the rodent brain during embryonic development follows the neuronal maturation in all CNS regions.

4.1.3. Cation-Cl^- cotransporter isoforms 1 and 3 exhibit low expression in the embryonic brain

The expression of KCC1 mRNA in the embryonic mouse brain was generally low and displayed a slight up-regulation over the course of development, consistent with a previous report (Clayton et al., 1998). However, inconsistent with the *in situ* hybridization report from (Wang et al., 2002), we did not observe a strong KCC1 mRNA expression in the prenatal cortical plate, cerebral cortex hippocampus, or caudate putamen. At E14.5 in mouse brain, we detected strong KCC1 mRNA expression only in the choroid plexus.

KCC3 mRNA expression was generally weak in the early embryonic rodent brain. At E14.5 in mouse, KCC3 mRNA was detectable in diencephalon, mesencephalon, and rhombencephalon, and in the newly differentiated regions of the preplate layer of telencephalon where postmitotic neurons organize into layers. KCC3 mRNA expression in the brain was moderately up-regulated at later stages of development and displayed an increase in the cortex and strong signal in the olfactory bulb in the P0 embryonic brain. The observed developmental up-regulation of KCC3 mRNA expression is consistent with another report (Boettger et al., 2003), demonstrating minimal expression of KCC3 in the E14.5 mouse embryo and strong expression in mouse P0 brain.

4.1.4 Cation-Cl^- cotransporter isoform 4 expression is high in the proliferative zones

Throughout embryonic mouse development, KCC4 mRNA expression was abundant in the subcortical proliferative zones where neurogenesis occurs. In the E14.5 mouse embryo, KCC4 transcripts were strongly expressed in the ventricular layers of telencephalon and rhombencephalon, and moderately in the VZs of diencephalon and mesencephalon. In contrast, no KCC4 was detectable in the intermediate zones and the preplate of the

Table 5: The expression of CCC isoforms in the embryonic and adult rodent nervous system

	E12.5-13.5					E14.5-16.5					E17-P1					ADULT				
Anatomical structure	KCC1	KCC2	KCC3	KCC4	NKCC1	KCC1	KCC2	KCC3	KCC4	NKCC1	KCC1	KCC2	KCC3	KCC4	NKCC1	KCC1	KCC2	KCC3	KCC4	NKCC1
Telencephalon	NA	(+)	0	(+)	+	0	(+)	(+)	++	++	(+)	+	+	+	+	+	++	++	(+)	(+)
Cortical plate		0	0	0	0	0	0	(+)	0	(+)	+	(+)	+	0	++					
Ventricular zone		0	0	(+)	+	0	0	0	++	++	0	0	0	+	(+)					
Olfactory system		+	0	NA	+	NA	++	NA	NA	+	(+)	++	++	0	+	+	+++	NA	0	++
Basal ganglia		(+)	0	0	0	0	++	(+)	0	+	(+)	+	0	0	(+)	(+)	++	0	0	(+)
Caudate putamen						0	+	NA	NA	+	(+)	+	0	0	(+)	(+)	++	0	0	(+)
Piriform cortex						0	+	(+)	0	+	(+)	+	NA	0	(+)	(+)	+++	++	0	(+)
Hippocampus						0	(+)	(+)	NA	+	(+)	+	++	(+)	(+)	+	+++	++	(+)	+
Amygdala						0	0	(+)	NA	0	(+)	++	NA	0	0	(+)	++	0	0	(+)
Cerebral cortex											+	(+)	++	(+)	+	+	++	++	(+)	(+)
Diencephalon	NA	++	0	(+)	(+)	0	++	(+)	+	+	(+)	++	+	0	(+)	(+)	+++	++	(+)	(+)
Ventricular zone		0	0	(+)	+	0	0	0	+	+	0	0	0	0	0					
Thalamus		++	0	0	0	0	++	(+)	(+)	+	(+)	++	+	0	(+)	(+)	+++	NA	0	(+)
Ventral thalamus		++	0	0	0	0	++	(+)	(+)	+	(+)	++	+	0	(+)	(+)	++	NA	0	(+)
Dorsal thalamus		(+)	0	0	0	0	(+)	(+)	(+)	+	(+)	++	+	0	(+)	(+)	++	NA	0	(+)
Hypothalamus		++	0	0	0	0	++	(+)	(+)	+	(+)	++	+	NA	(+)	+	+++	++	++	(+)
Mesencephalon	NA	++	0	(+)	(+)	0	++	(+)	+	+	(+)	++	+	(+)	0	+	+++	++	+	+
Ventricular zone		0	0	(+)	+	0	0	0	+	+	NA	0	0	NA	0					
Tectum/Tegmentum		++	0	0	0	0	++	(+)	(+)	(+)	(+)	++	+	(+)	0	+	+++	++	+	+

Table 5 continuing

	E12.5-13.5					E14.5-16.5					E17-P1					ADULT				
Anatomical structure	KCC1	KCC2	KCC3	KCC4	NKCC1	KCC1	KCC2	KCC3	KCC4	NKCC1	KCC1	KCC2	KCC3	KCC4	NKCC1	KCC1	KCC2	KCC3	KCC4	NKCC1
Rhombencephalon	NA	++	0	(+)	(+)	0	++	(+)	++	+	+	++	+	(+)	0	+	+++	++	++	++
Ventricular zone		0	0	(+)	+	0	0	0	++	(+)	NA	0	0	NA	0					
Pons/medulla		++	0	0	0	0	++	(+)	+	(+)	+	++	+	+	0	+	+++	++	++	++
Cerebellum											0	++	+	(+)	0	++	+++	++	(+)	+
Purkinje cells																++	+++	++	NA	0
Granule cells																++	+++	0	NA	++
Spinal cord	+	++	++	0	+	0	++	++	(+)	(+)	(+)	+++	++	++	(+)	(+)	++	+++	++	(+)
Choroid plexus						++	0	0	++	++	++	0	(+)	+	+++	++	0	++	++	+++
Peripheral nervous system	+	0	+	NA	+	0	0	+	++	+	NA	0	++	NA	+	+	0	0	+++	+
References	F	I, F, N	I, F	I	I, E, F, S	I, B, G	I, B, G, L, N	I, L	I	I, B, E, G, S	I, B, F, G, H, M	I, B, F, G, H, K, L, N	I, D, F, L, M	I, M	I, A, B, E, F, G, H, K, S	B, C, G, H, I, J, Q	B, C, G, H, I, J, P, Q, R	D, R	O, R	A, B, C, E, G, H, I, J, Q

The table is based on the published mRNA *in situ* hybridization, RT-PCR, and protein immunohistochemical and WB data of I and (Plotkin[A] et al., 1997b;Clayton[B] et al., 1998;Kanaka[C] et al., 2001;Pearson[D] et al., 2001;Hubner[E] et al., 2001a;Hubner[F] et al., 2001b;Wang[G] et al., 2002;Mikawa[H] et al., 2002;Okabe[I] et al., 2002;Okabe[J] et al., 2003;Balakrishnan[K] et al., 2003;Boettger[L] et al., 2003;Becker[M] et al., 2003;Stein[N] et al., 2004;Karadsheh[O] et al., 2004;Bartho[P] et al., 2004;Toyoda[Q] et al., 2005;Le Rouzic[R] et al., 2006;Vanden Heuvel[S] et al., 2006), and should not be used to compare the absolute levels between the CCC isoforms. The brain regional profile for each subunit is given using the same scale even if the absolute concentrations of CCC mRNAs or peptides are different. The data scale is denoted as +++ very high expression, ++ high, + moderate, (+) low/very low, 0 not detected, NA information not available, and no grading (empty field) the brain region not apparent during a specific developmental stage.

isocortex. In addition, moderate expression was observed in the spinal nucleus of the trigeminal nerve of the rhombencephalon and in the dorsal roof plate of the brain stem in the E14.5 mouse embryo. In contrast to other KCCs, KCC4 mRNA was expressed in the peripheral ganglia, such as the vestibular (VIII) ganglion. Towards P0, KCC4 mRNA expression decreased in the neocortex but was clear in the choroid plexus and other epithelial linings as well as in the peripheral ganglia.

4.1.5 Possible roles for Na^+-K^+-Cl^- cotransporter isoform 1 and cation-Cl^- cotransporter isoforms in the regulation of embryonic development

Neuronal precursors and maturing neurons in the embryonic VZ and CP layers express functional $GABA_A$ receptors before the formation of functional synaptic contacts (LoTurco et al., 1995;Owens et al., 1996;Owens and Kriegstein, 2002). These receptors mediate depolarizing GABA currents which are required for trophic GABA effects (LoTurco et al., 1995;Owens et al., 1996;Owens and Kriegstein, 2002). The depolarizing GABA responses of VZ and CP evidently result from the Cl^- intruding activity of NKCC1, which is highly expressed in the immature neurons of these layers (Sung et al., 2000;Yamada et al., 2004;Dzhala et al., 2005). This has been shown also in adult newborn neurons, in which NKCC1 maintains the depolarized $E_{GABA\text{-}A}$ essential for correct synapse formation and dendritic development (Ge et al., 2006). Knock-down of NKCC1 mRNA in newborn neurons results in marked defects in denritic arborization as the total dendritic length and branch number as well as the dendritic complexity of these neurons is significantly reduced (Ge et al., 2006). In addition, NKCC1 may have a role in the regulation of the cell cycle of neuronal precursors of the VZ layer. These cells undergo several cell cycles to generate postmitotic neurons, and an increase in cell size is an important variable in determining the timing of cell division (Gao and Raff, 1997). Thus it is plausible that by mediating the regulatory cell volume increase, NKCC1 might be involved in the control of the neuronal cell cycle. Indeed, experiments in cultured non-neuronal cells have indicated a role for the NKCC-mediated cell volume regulation mechanism in cell proliferation (Panet and Atlan, 1991;Bussolati et al., 1996). However, possible defects in neurogenesis remain to be shown in NKCC1 KO mice.

The expression of NKCC1, KCC1, and KCC4 was strong in the embryonic choroid plexus. The epithelial cells of the choroid plexus secrete cerebrospinal fluid, by a process which involves the transport of Na^+, Cl^-, and HCO_3^- from the blood to the ventricles of the brain (Brown et al., 2004). The transport of ions is unidirectional due to the polarity of the epithelium, i.e. the ion transport proteins in the blood-facing (basolateral) membrane are different to those in the ventricular (apical) membrane (Brown et al., 2004). In adult animals, NKCC1 is confined to the apical membrane of choroid plexus epithelial cells (Plotkin et al., 1997a), but the role of the protein in the apical membrane is unclear. In addition to NKCC1, Na^+-K^+-ATPase is located at the apical membrane and serves as the dominant pathway for secretion of Na^+ to CSF (Brown et al., 2004). NKCC1 may, however, also contribute to the secretion of Na^+, and at the same time enrich the CSF with K^+ to feed the Na^+-K^+-ATPase (Bairamian et al., 1991;Javaheri and Wagner, 1993;Keep et al., 1994). In the

adult nervous system, KCC4 protein is located at the apical membrane and KCC3 is strongly expressed in the basolateral membrane of choroid plexus (Pearson et al., 2001;Karadsheh et al., 2004), whereas the exact localization of KCC1 protein is not known. In the embryonic brain, however, KCC3 is absent from the choroid plexus. Thus it may be plausible that in the embryonic brain, KCC1 substitutes for KCC3 as the basolateral KCC isoform, and KCC4 serves as the apical isoform. In this case, KCC1 may have a significant role in the absorption of K^+ from the CSF to the blood. The hypothesis is supported by the fact that no other transporters or channels capable of mediating K^+ efflux have been identified in the basolateral membrane (Brown et al., 2004). KCC4 might be involved in Cl^- secretion and at the same time contribute to the recycling of K^+ similar to NKCC1.

The expression profiles of KCCs revealed a non-overlapping regional expression in the embryonic rodent brain; KCC2 was present in mature regions and KCC3 in newly differentiated regions, whereas KCC4 was abundant in undifferentiated proliferative zones. These KCC isoforms display different characteristics in their kinetics and activation (Mercado et al., 2000;Gamba, 2005), making them suitable for different tasks. For example, among the KCC isoforms, KCC4 displays the most robust cell swelling-induced activity (Mercado et al., 2000), and KCC2 is unique in having constitutive Cl^- extrusion activity. The neonatal expression of KCC2 in the spinal cord and brainstem is crucial for normal spinal cord reflexes and respiratory motoneuron function (Hubner et al., 2001b). KCC3 is mutated in a severe peripheral neuropathy associated with agenesis of the corpus callosum (Howard et al., 2002). As this structure forms during embryogenesis, the callosal agenesis indicates that KCC3 is at least indirectly involved in early axonal migration. The robust expression of KCC4 in the proliferative zones of embryonic brain implies a role for the cotransporter in the cell volume regulation associated with the proliferative cell cycling during embryogenesis.

4.2 Generation of cation-Cl^- cotransporter isoform 2-deficient mice (II)

4.2.1 Construction of gene targeting vectors

The mouse is the leading vertebrate experimental model because its genome can be efficiently and precisely engineered by various means. Targeted mutagenesis into ES cells is a generally used technique for the production of mutant mice to study a gene function *in vivo* (Nagy and Rossant, 1996). In order to characterize the *in vivo* roles for KCC2, we generated transgenic mice with KCC2 null and hypomorphic alleles. For this purpose, we developed a novel *in vitro* DNA transposition-based system for the generation of mutant allele-producing targeting vectors.

Transposons are mobile genetic elements that can relocate between genomic sites by a special form of DNA recombination called DNA transposition (Mizuuchi, 1997;Hayes, 2003). DNA transposition-based methods are utilized widely in molecular biology techniques, such as DNA sequencing, gene mapping, and insertional mutagenesis (Hayes, 2003). The main advantage of using transposons in genetic engineering is their ability to integrate efficiently and relatively randomly into target DNA. One of the

best-characterized DNA transposition systems is an *in vitro* system derived from Bacteriophage Mu transposition (Mizuuchi, 1983;Haapa et al., 1999b), which only requires three purified macromolecular components: transposon DNA, transposase protein MuA and target DNA.

In order to construct appropriate gene targeting vectors for KCC2 null, hypomorphic, and conditional alleles, we first customized a 1418 bp mini-Mu transposon DNA called Neoflox-Mu. At its ends this transposon contained, as inverted repeats, 50 basepairs of R-end DNA (Savilahti et al., 1995). Between these recognition sites of the Mu transposon were: 1) a marker neomycin phosphotransferase (*neo*) resistance gene under the control of eukaryotic thymidine kinase promoter (p_{tk}) and 2) the marker gene flanking *loxP* sites. The *loxP* sites enable the excision of the DNA segment between pairs of these sites in cells expressing functional Cre recombinase (Sauer, 1998;Nagy, 2000). As target DNA, we used a 6688 bp genomic clone of the mouse *Slc12a5* gene including exons 2-6 (Haapa et al., 1999a).

The transposon Neoflox-Mu was introduced into the genomic *Slc12a5* clone by *in vitro* DNA transposition (Haapa et al., 1999a). The suitable gene targeting vectors were then screened for by PCR analysis. For the null mutant allele, the PCR reaction was designed to identify sense orientated transposon integrations into exon 4. One of the integrants contained the transposon 14 bp downstream from the 5'-end of exon 4, and was chosen as the vector for targeting the knock-out allele (Fig 3A).

The insertion of *neo* cassette into introns, as well as in 5'- or 3'-flanking, or untranslated regions of the gene, can interfere with the expression of the targeted allele (Lewandoski, 2001). In some cases, it has been determined that intronic *neo* insertion creates hypomorphic alleles either through cryptic splicing signals in *neo* that lead to a frameshift, or by down-regulating gene expression through an unknown mechanism (Jacks et al., 1994;Meyers et al., 1998;Nagy et al., 1998). Thus, for the generation of the hypomorphic allele, we chose a construct that harbored an antisense orientated transposon in intron 3 (Fig. 3B). Intronically positioned *neo* may, however, lead to unexpected inhibitory effects on the expression of neighboring genes (Olson et al., 1996). This type of phenomenon seems to arise when the targeted gene is a part of a multigene group such as $GABA_A$ receptor β2-α6-α1-γ2 subunit gene cluster (Uusi-Oukari et al., 2000), *Hox* gene clusters (Rijli et al., 1994), or β-globin gene cluster (Fiering et al., 1993). The clustered organization of multigene groups implies that transcriptional elements might be shared by two or more genes, or that elements controlling one gene may be located within DNA sequences of another. The mechanisms for the *neo*-induced neighboring effect is not yet identified, but may include direct inhibition of neighboring genes, or indirect inhibition of local transcriptional elements shared by many genes (Uusi-Oukari et al., 2000).

The *Slc12a5* gene locus is located on mouse chromosome 2H2-H3 (Sallinen et al., 2001). Genes in the immediate neighbourhood of this loci (spanning ~70 kb up- or downstream) include *Mmp9* (encoding matrix metallopeptidase 9 (MMP9)), *Ncoa5* (encoding nuclear receptor coactivator (Ncoa)), *LOC629777* (encoding hypothetical protein LOC629777)), *Zpf335* (encoding zinc finger protein (Zfp335)), and *Cd40*

(encoding antigen CD40) (NCBI). This group of genes does not form a cluster of related genes, and it is not obvious that these genes would be sharing transcriptional elements. However, we cannot exclude the possibility that *neo* in *Slc12a5* locus might interfere with the expression of above mentioned genes, or other genes at more distant locations. Thus the interpretation of the phenotypes from the KCC2-deficient mice might be confounded by the disrupted expression of the genes located near the intended target. From the neighboring genes, MMP-9 and CD40 are known to be expressed in the CNS. MMP-9 belongs to a family of proteolytic matrix metalloproteinases, which expression in the CNS is customarily thought to be associated with injury or pathology (Yong et al., 2001). Some MMP isoforms, such as MMP-9, may have a role in normal ontogeny, and this isoform has been shown to be necessary for oligodendrocyte process extension and myelination (Uhm et al., 1998;Oh et al., 1999;Larsen et al., 2006), and is possibly required for hippocampal late-phase long-term potentiation and memory (Nagy et al., 2006). CD40 is a member of the tumor necrosis factor receptor family participating in the regulation of pro-inflammatory and immune responses in the brain (Chen et al., 2006). These two gene products display distinguishly different functions compared to KCC2, and the phenotypes observed in KCC2-deficient mice (III, IV) might not be readily explained by the *neo*-induced neighboring gene effect. The function of the other genes neighboring the *Slc12a5* locus is unknown.

The conditional allele-containing gene targeting construct was generated from the hypomorphic allele-containing clone by introducing an additional *loxP* site into a unique *Hpa*I site residing in intron 1 of the *Slc12a5* gene. We did not, however, proceed to generate the KCC2 conditional allele mouse line.

The selected constructs for null and hypomorphic alleles were targeted into the endogenous *Slc12a5* locus by homologous

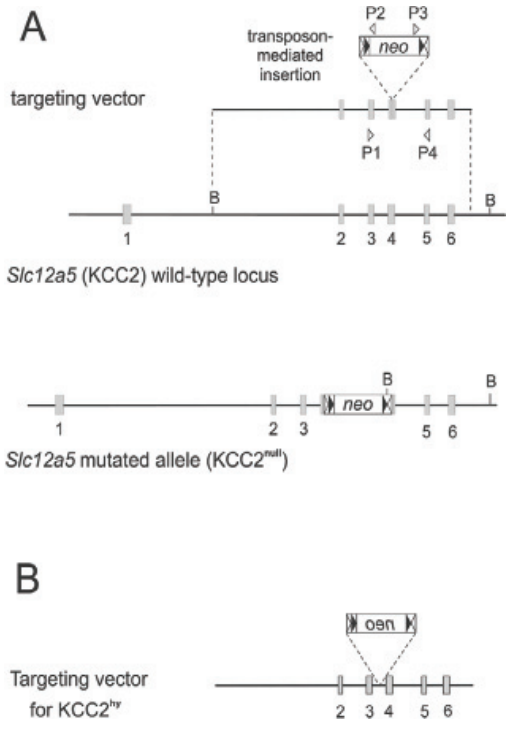


Fig. 3. Targeting of *Slc12a5*. **A)** The targeting construct for the generation of the KCC2^{null} allele. The construct contained a *neo* selection cassette inserted into exon 4 of *Slc12a5*. Depicted also are the wild-type locus *Slc12a5*, and the resultant null mutant allele. Neoflox-Mu transposons are shown as rectangles containing *LoxP* sites (black arrowheads) and transposon ends (white arrowheads). The intron-exon sequence and the relevant restriction sites (B, *Bam*HI) and the primers for genotyping (P1-P4) are shown. **B)** The targeting construct for KCC2 hypomorphic allele that contains an inverted neocassette in intron 3 of a genomic *Slc12a5* clone.

recombination. For the null mutant allele, disruption of exon 4 with the *neo* cassette caused a protein truncation and complete loss of the protein, whereas the hypomorphic mutant allele with an inverted *neo* in intron 3 resulted in the anticipated down-regulation of KCC2 expression. Mice homozygous for the hypomorphic allele ($KCC2^{hy/hy}$) showed a 60-70% reduction of normal KCC2 levels in the brain.

4.2.2 General characteristics of cation-Cl^- cotransporter isoform 2-deficient mice (III)

Consistent with the work of Hubner and collaborators (Hubner et al., 2001b), our KCC2 knock-out mice died shortly after birth apparently due to defects in the respiratory system, whereas mice homozygous for the KCC2 hypomorphic allele (retaining 30-40% of normal KCC2 levels) were viable and showed no obvious phenotypes (I). To produce KCC2 hypomorphic mice with more informative phenotypes, we intercrossed heterozygous mice for the null and hypomorphic alleles, which resulted in heteroallelic $KCC2^{hy/null}$ mice retaining 15-20% of normal KCC2 levels in the brain and spinal cord, as assessed by Western blotting. Immunohistochemical analysis with a KCC2-specific antibody showed that the reduction of KCC2 expression in $KCC2^{hy/null}$ mice was universal as the overall KCC2 distribution pattern was similar in mutant and wild-type mice. In addition, the mutant mice displayed no compensatory changes in other KCC family members or NKCC1 as detected by RT-PCR or Western blotting.

The $KCC2^{hy/null}$ mice displayed normal reproduction and life span, but had a reduced body weight (-20% in both sexes). The weight difference emerged two weeks after birth and persisted throughout the adult life of the animals. The mechanism behind the reduced weight remains unknown. Apart from the mild weight reduction, the mutant mice displayed no other obvious phenotypes, as the body position, spontaneous activity, respiration rate, palpebral closure, fur condition, gait, pelvic elevation, trunk curl, limb grasping, visual placing, and grip strength of the mutant mice was similar to wild-type control mice. Standard histological analysis of the brain morphology with haematoxylin and eosin staining revealed no gross changes between the genotypes. Moreover, a descriptive light microscope analysis suggested that the density and morphology of cell bodies and the density of synapses in the hippocampus and cortex were normal in the $KCC2^{hy/null}$ mice. This was addressed by immunohistochemical analysis with antibodies against parvalbumin, calbindin, and synaptophysin.

4.3. Behavioral and *in vivo* pharmacological characterization of cation-Cl^- cotransporter isoform 2 mutant mice (III,IV)

The role for KCC2 in hyperpolarizing GABA inhibition has been well characterized (Rivera et al., 1999;Hubner et al., 2001b;Woo et al., 2002;Zhu et al., 2005), but little is known concerning the role of KCC2 at the systems level. In addition, no experimental evidence exists regarding the role of KCC2 in the tonic form of GABA inhibition. To address these questions, we decided to study the significance of reduced KCC2 levels on adult mouse behavior, and test the relative importance of KCC2 in the tonic versus phasic GABAergic inhibition in the adult CNS, and the related behavior *in vivo*.

Consistent with the role of KCC2 in establishing hyperpolarizing GABA responses (Rivera et al., 1999;Hubner et al., 2001b;Woo et al., 2002;Zhu et al., 2005), the $KCC2^{hy/null}$ mice displayed significantly depolarized E_{GABA-A} values in hippocampal CA1 pyramidal cells compared to wild-type control mice (E_{GABA-A}, WT: -69 ± 4 mV, n=6, hy/null: -48±5 mV, n=5) (R Riekki et al., unpublished results). Consequently, the normally hyperpolarizing GABA responses in hippocampal pyramidal cells were abolished in KCC2-deficient mice.

4.3.1 Potential limitations to the genetically modified mouse models in the investigation of the γ-aminobutyric acid type A receptor neurotransmission system

Experimental data from genetically modified KO or KI (knock-in) mice associated with the $GABA_A$ neurotransmission has proven this system to be critically involved in the control of a variety of physiological behaviors, including motor coordination, spontaneous locomotion, learning and memory, nociception, anxiety, panic, and epileptogenesis (table 6). A limitation to the GABA receptor subunit KO studies is the possible compensation by the remaining $GABA_A$ family members or by other molecules in unrelated systems (Brickley et al., 2001;Rudolph and Mohler, 2004). In addition, targeted inactivation of the $GABA_A$ receptor subunits may result in a substantial loss of total $GABA_A$ receptors in brain (Sur et al., 2001;Kralic et al., 2002a). As discussed below, these phenomena may mask the true function of the targeted gene and thus make interpretation of the results difficult.

$GABA_A$ receptor α1 subunit is the most abundant α subunit variant in the brain and is highly expressed throughout most brain regions (Pirker et al., 2000). Mice that lack the α1 subunit displayed more than 50% loss of total $GABA_A$ receptors, but were viable, fertile, and showed no spontaneous seizures (Sur et al., 2001;Kralic et al., 2002a). These mice exhibited compensatory increases in the expression of α2 and α3 subunits and down-regulation of β2/3 and γ2 subunits in the forebrain (Sur et al., 2001;Kralic et al., 2002a).

$GABA_A$ receptor α6 subunit is exclusively expressed in cerebellar and cochlear nucleus granule cells (Laurie et al., 1992a;Varecka et al., 1994). Mice that lack the α6 subunit showed a compensatory up-regulation of TASK-1 potassium channels and a ~25% loss of δ subunits in cerebellar granule cells (Jones et al., 1997;Brickley et al., 2001). The continuously active TASK-1 channels fully compensated the tonic GABA currents abolished by the deletion of α6 subunit-containing $GABA_A$ receptors, and thus maintained the normal cerebellar granule cell excitability (Brickley et al., 2001).

$GABA_A$ receptor β2 subunit is the most abundant of the β subunits and is expressed in virtually all brain regions (Pirker et al., 2000). Consistent with its widespread distribution, deletion of the β2 subunit results in more than 50% reduction of the total number of $GABA_A$ receptors (Sur et al., 2001). The β2 KO mice displayed, however, surprisingly normal phenotype being viable, fertile, and devoid of spontaneous seizures. The β2 subunit KO mice showed reduced expression levels (~50%) of all six α subunits (Sur et al., 2001).

$GABA_A$ receptor β3 subunit is strongly expressed in the cerebral cortex, hippocampus, hypothalamus, cranial nerve ganglia, and spinal cord in adults, and is

even more widespread and abundant in the prenatal brain (Wisden et al., 1992;Laurie et al., 1992b). Deletion of the β3 subunit produced several behavioral deficits and epilepsy, and resulted in reduction of the total number of brain $GABA_A$ receptors containing α2 or α3 subunits (Homanics et al., 1997;Ramadan et al., 2003).

The $GABA_A$ receptor γ2 subunit is expressed throughout the brain, is incorporated in most postsynaptic $GABA_A$ receptor subtypes, and mediates postsynaptic clustering of $GABA_A$ receptors and gephyrin (Essrich et al., 1998;Pirker et al., 2000;Schweizer et al., 2003). This $GABA_A$ receptor subunit has an obligatory role in benzodiazepine responsiveness (Pritchett et al., 1989). Consistent with this, γ2 subunit KO mice displayed 94% reduction of the benzodiazepine binding site, but showed an unchanged number of $GABA_A$ receptors (Gunther et al., 1995). This may be due to receptor subtypes composed only of α and β subunits, indicating that γ2 subunit is dispensable for the assembly of functional $GABA_A$ receptors (Gunther et al., 1995).

$GABA_A$ receptor δ subunit is primarily expressed in the extrasynaptic membranes and shows abundant expression in the cerebellar granule cells (Nusser et al., 1998), and in forebrain regions such as thalamic relay nuclei, caudate-putamen, molecular layer of dentate gyrus and outer layer of cerebral cortex (Peng et al., 2002). Mice that lacked δ subunit showed an extensive up-regulation of γ2 subunit in the cerebellum and forebrain, whereas the expression of α4 was reduced in the forebrain (Tretter et al., 2001;Peng et al., 2002;Korpi et al., 2002b). In δ subunit KO mice, γ2 seemed to replace δ subunit by coassembling with α6 subunit in the cerebellum and α4 subunit in the forebrain (Tretter et al., 2001;Peng et al., 2002;Korpi et al., 2002b), implying a possible subunit competition during the assembly of $GABA_A$ receptors. The δ subunit KO mice were viable but exhibited attenuated sensitivity to neuroactive steroids (Mihalek et al., 1999).

Such adaptive compensatory changes are not, however, apparent in $GABA_A$ receptor α3 and α5 subunit, GABA transporter-1 (GAT1) and GAD65 KO mice (Kash et al., 1999;Collinson et al., 2002;Jensen et al., 2003;Yee et al., 2005). $GABA_A$ receptor α3 and α5 subunits are of minor abundance in the brain. $GABA_A$ receptors that contain α3 subunit are the main subtypes expressed in monoaminergic and basal cholinergic cells (Gao et al., 1993). In addition, this receptor subtype is expressed in the olfactory bulb, cerebral cortex, reticular thalamic nucleus, superior colliculus, amygdala, and cranial nerve nuclei. The primarily extrasynaptic α5 subunit-containing $GABA_A$ receptors are expressed to a significant extent only in the hippocampus, but display also low expression in the olfactory bulb, cerebral cortex, and the hypothalamus (Pirker et al., 2000;Brunig et al., 2002). The α3 and α5 KO mice showed no alterations in the expression levels of other $GABA_A$ receptor subunit family members (Collinson et al., 2002;Yee et al., 2005). In addition, the GAD65 and GAT1 KO mice showed largely unaffected $GABA_A$ receptor expression (Kash et al., 1999;Jensen et al., 2003). Out of four identified GAT subtypes (GAT1-4), GAT1 is the major subtype and displays a particularly strong expression in hippocampus, neocortex, cerebellum, and retina (Guastella et al., 1990). GAD65 is expressed in all GABAergic neurons (Erlander et al., 1991).

As mentioned earlier, the analysis of possible adaptive compensatory changes in the $KCC2^{hy/null}$ mice was restricted to the remaining KCC family members and NKCC1. Thus we do not know whether the

knock-down mutation of KCC2 induces compensatory changes in the organization and in the functional properties of $GABA_A$ receptors, or in other proteins associated with the GABAergic neurotransmission system. In addition, as discussed before, we cannot exclude the possibility that *neo* cassette might have induced inhibitory effects on the expression of the *Slc12a5* neighboring genes, or genes at more distant locations. For these reasons, we cannot be sure whether the phenotypes we observed in the $KCC2^{hy/null}$ mice are due to the reduced KCC2 expression in the adult animal, or whether the phenotypes results from adaptive compensations in the brain during development or the potential gene regulatory elements expressed in *neo* cassette.

The above mentioned problems have been tried to overcome by a variety of $GABA_A$ receptor KI mice that introduce more subtle point mutations to the mouse genome (Rudolph and Mohler, 2004). The idea is that the point mutation would specifically prevent the modulation of the respective receptor by a defined drug, but would leave the physiological function of the targeted molecule intact (Rudolph and Mohler, 2004). Some of the $GABA_A$ receptor subunit KI mice are presented in table 6 and are discussed more closely later.

Table 6 presents some of the behavioral phenotypes of genetically modified mice associated with the $GABA_A$ neurotransmission system. The behaviors collected in the table are those that we observed to be altered in the $KCC2^{hy/null}$ mice. For the above mentioned reasons, the interpretation of the phenotypes for most of these mouse models may be difficult. In addition, differences in the testing conditions and in the background of the animals may cause confounding results that are not necessarily reflecting the true function of the targeted protein. The behavioral phenotypes of KCC2-deficient mice and the possible relations of these phenotypes to the GABAergic neurotransmission system are discussed in the remaining chapters 4.3.2-4.3.11.

4.3.2 Cation-Cl^- cotransporter isoform 2 mutant mice display normal motor coordination and spontaneous locomotor activity (III)

The major brain regions for control of movement in vertebrates include spinal cord, brain stem, hypothalamus, basal ganglia, cerebral cortex, and cerebellum (Grillner, 2003). $GABA_A$ receptors are the main inhibitory receptors for fast inhibitory neurotransmission in all of these regions, except for the spinal cord where $GABA_A$ and glycine receptors are the main receptors for fast inhibitory tone (Wisden et al., 1992;Laurie et al., 1992a;Laube et al., 2002). Mice with targeted deletions of $GABA_A$ receptor subunit α1 and GAT1 display readily observable tremor and consequently impaired performance in the rotarod (Chiu et al., 2005;Kralic et al., 2005) (table 6). The tremor observed in the $GABA_A$ receptor subunit α1 KO mice resembles a human essential tremor disorder with an unknown etiology (Chen and Swope, 2003;Kralic et al., 2005). Similarly, a gain-of-function $GABA_A$ receptor α1 (S270H) mutated mice, which display increased sensitivity to GABA and prolonged mIPSCs in hippocampal neurons, show intention tremor and impaired rotarod performance. In addition, mice devoid of β3 subunit have difficulties in swimming, walking on grids, and fall of platforms and rotarods (Homanics et al., 1997). Both $GABA_A$ receptor subunits α1 and β3, as well as GAT1, are expressed

Table 6. Selected behavioral phenotypes of genetically modified mice associated with the $GABA_A$ neurotransmission system.

Phenotype	KCC2 KO and knock-down mice	$GABA_AR$ (α1,3,5,6, β1,2, γ2 & δ) KO and knock-down mice	$GABA_AR$ KI mice	GAD65/67 KO	GAT1 KO	References
Seizures	Mice with 5-8% of KCC2 show frequent, spontaneous seizures. $KCC2^{hy/null}$ mice with 15-50% of normal KCC2 show increased seizure susceptibility.	β3 KO mice exhibit spontaneous seizures. α1 and δ KO mice display increased seizure susceptibility.	α1 (H101R) mice show partially reduced anticonvulsant effect of diazepam	GAD 65 KO mice show spontaneous seizures and reduced seizure threshold.	No obvious susceptibility for seizures.	III, (Asada et al., 1996;Homanics et al., 1997;Kash et al., 1997;DeLorey et al., 1998;Rudolph et al., 1999;Stork et al., 2000;Woo et al., 2002;Spigelman et al., 2002;Kralic et al., 2002a;Chiu et al., 2005)
Anxiety-related behavior	$KCC2^{hy/null}$ mice show anxiety-related behavior in several tests.	Heterozygous animals for γ2 KO and knock-down, and homozygous mice for γ2L KO (long variant of γ2) display increased anxiety.	The anxiolytic action of diazepam absent in α2 (H101R) mice.	GAD 65 KO mice show anxious phenotype.	Mild anxiety or nervousness	III, (Asada et al., 1996;Kash et al., 1999;Crestani et al., 1999;Homanics et al., 1999;Stork et al., 2000;Low et al., 2000;Chiu et al., 2005;Chandra et al., 2005)
Motor coordination	Mice with 5-8% of KCC2 show abnormal posture and gait. $KCC2^{hy/null}$ mice show normal rotarod performance.	The α1 KO mice show essential tremor impaired motor coordination. The β3 KO mice show impaired motor abilities and difficulties in swimming. The α6 KO mice show increased sensitivity to diazepam-induced sedation/motor impairment.	Sedative/motor impairing effects of diazepam abolished in α1 (H101R) mice. Gain-of function α1 (S270H) KI mice display tremor, and impaired rotarod performance.	GAD65 mice display normal rotarod performance.	Tremor, gait abnormality and impaired rotarod performance.	III, (Asada et al., 1996;Homanics et al., 1997;DeLorey et al., 1998;Rudolph et al., 1999;Korpi et al., 1999;McKernan et al., 2000;Crestani et al., 2000;Woo et al., 2002;Kralic et al., 2002a;Chiu et al., 2005;Kralic et al., 2005;Homanics et al., 2005;Chandra et al., 2005)
Somato-sensory perception	$KCC2^{hy/null}$ mice show reduced tactile and thermal sensitivity.	The β3 KO animals display increased sensitivity to tactile and thermal stimulus.	The α5 (H105R) KI mice show normal somatosensory perception.	NA	NA	III, (Ugarte et al., 2000;Crestani et al., 2002)

Table 6 continuing

Phenotype	KCC2 KO and knock-down mice	$GABA_AR$ (α1,3,5,6, β1,2, γ2 & δ) KO and knock-down mice	$GABA_AR$ KI mice	GAD65/67 KO	GAT1 KO	References
Locomotor activity	$KCC2^{hy/null}$ mice display normal spontaneous locomotor activity, and reduced sensitivity to diazepam induced sedation/motor impairment.	The β3 KO mice are hyperactive with continuous running in tight circles. The α3 and β2 KO mice show elevated spontaneous locomotor activity. The α1 KO mice show increased sensitivity to diazepam-induced sedation/motor impairment.	Gain-of function mutated α1 (S270H) KI male mice and α5 (H105R) KI female mice show increased spontaneous locomotor activity.	GAD65 mice show normal locomotor activity.	Normal	III, (Asada et al., 1996;Homanics et al., 1997;Rudolph et al., 1999;McKernan et al., 2000;Crestani et al., 2000;Sur et al., 2001;Kralic et al., 2002b;Reynolds et al., 2003;Hauser et al., 2005;Chiu et al., 2005;Yee et al., 2005;Homanics et al., 2005)
Spatial learning	$KCC2^{hy/null}$ animals display impaired performance in water maze.	The α5 KO mice show improved performance in water maze.	NA	GAD65 mice show normal water maze performance	NA	III, (Asada et al., 1996;Collinson et al., 2002)
PPI	$KCC2^{hy/null}$ females show attenuated PPI.	The α3 KO mice display attenuated PPI	The (H105R) KI mice display attenuated PPI.	GAD 65 KO mice display attenuated PPI.	GAT-1 KO animals display attenuated PPI.	III, (Heldt et al., 2004;Hauser et al., 2005;Yee et al., 2005)
Body weight	Mice with 5-8% of KCC2 show marked (~60-70%) reduction in body weight. $KCC2^{hy/null}$ mice show ~20% reduction of body weight.	The α1 KO animals display ~30% reduction in body weight.	Gain-of-function α1 (S270H) KI mice show reduced body weight (~25%).	NA	NA	III, (Sur et al., 2001;Homanics et al., 2005)

NA, information not available

highly throughout the brain including the motor pathways in brainstem, cerebellum, cerebral cortex, and basal ganglia (Wisden et al., 1992;Laurie et al., 1992a;Yasumi et al., 1997;Pirker et al., 2000). As deletion of GAT1 results in elevation of GABA levels (Jensen et al., 2003), these results suggest that tremor and the subsequent impairment in motor coordination can arise from either too little or too much GABAergic neurotransmission throughout the brain.

KCC2-deficient mice did not display tremor. We tested the sensorimotor control and coordination abilities of the mice by normal and accelerating rotarods, in which the performance depends primarily on neuronal circuits in the cerebellum, spinal cord, and brain stem (Grillner, 2003;Schieber and Baker, 2003). In both rotarod setups, the $KCC2^{hy/null}$ displayed normal performance. The results imply that although KCC2 is highly expressed in CNS neurons required for motor coordination, including the granule cells and Purkinje neurons of the cerebellum (Payne et al., 1996), inhibitory synapses throughout the spinal cord (Hubner et al., 2001b;Coull et al., 2003), and brain stem nuclei that control posture and reflex behaviors (e.g vestibular nucleus) (Kanaka et al., 2001), the proper functioning of these systems at the behavioral level was not critically altered by 80% reduction of KCC2 levels.

KCC2-deficient mice were tested for spontaneous locomotor activity in novel open field environment. Dopaminergic neurotransmission has been shown to be critical in the regulation of motor activity, emotion, motivation, and cognition (Holmes et al., 2004), and alterations to the dopaminergic system lead to changes in the rodent spontaneous locomotor activity (Spielewoy et al., 2000;Holmes et al., 2004). Dopaminergic pathways are known to be under the control of GABAergic inhibition, and mice with targeted mutations to $GABA_A$ receptor subunits α3, α5, β2, and β3 show hyperlocomotor activity (Homanics et al., 1997;DeLorey et al., 1998;Sur et al., 2001;Crestani et al., 2002;Blednov et al., 2003;Hauser et al., 2005;Yee et al., 2005). The mechanism for the enhanced locomotion in these mice is unclear, but the phenomenon is not associated with reduced anxiety levels (Crestani et al., 2002;Yee et al., 2005). In addition, β3 KO mice display circling behavior, which is known to be indicative of deficits in the dopaminergic neurons of the basal ganglia (Kaakkola and Teravainen, 1990). These observations imply that impaired GABAergic inhibitory control of the dopaminergic neurons might be one of the factors underlying the enhancement of locomotor activity.

We tested the spontaneous locomotor activity in KCC2 mutant mice in the open field and Y-maze. In both of these tests, the $KCC2^{hy/null}$ mice displayed apparently normal spontaneous locomotor activity. KCC2 is not expressed in the nigrostriatal dopaminergic neurons (Gulacsi et al., 2003), and thus it might be possible that KCC2 is not directly involved in the GABAergic inhibitory regulation of brain dopaminergic systems. Whether KCC2 is expressed in the three other anatomically distinct (mesolimbic, mesocortical, and tuberoinfundibular) dopaminergic pathways is not known. Dopaminergic neurons from the nigrostriatal and mesolimbic pathways express at least $GABA_A$ α2, α3, α4, β1, β3, and γ2 subunits (Okada et al., 2004).

4.3.3 The sedating/motor-impairing effect of diazepam, but not of gaboxadol, is reduced in cation-Cl^- cotransporter isoform 2-deficient mice (IV)

Because tonic GABA inhibition is mainly mediated via a shunting inhibition, which is independent of the inwardly directed Cl^- gradient (Staley and Mody, 1992), we hypothesized that the tonic GABA inhibition would remain effective in KCC2 mutant mice. To investigate the requirements for KCC2 in tonic versus phasic GABA neurotransmission in the adult CNS, we selectively enhanced the activity of synaptic or extrasynaptic $GABA_A$ receptors on $KCC2^{hy/null}$ mice by allosteric $GABA_A$ receptor modulators.

A classical benzodiazepine, diazepam, interacts with and enhances the activation of $GABA_A$ receptors that contain α1, α2, α3, or α5 subunits, but is unable to bind to $GABA_A$ receptors that contain mainly extrasynaptic α4 and α6 subunits (Rudolph and Mohler, 2004). The diazepam binding property of the α1, α2, α3, or α5 subunits is enabled by a conserved histidine residue (H101, rat α1 subunit numbering), whereas α4 and α6 subunits that contain a conserved arginine residue are unable to bind diazepam (Wieland et al., 1992;Benson et al., 1998;Rudolph and Mohler, 2004). The primarily extrasynaptically located δ subunit is found mostly to be associated with α4 and α6 subunits (Wisden et al., 1992;Jones et al., 1997), and there are several reports stating that the δ subunit containing $GABA_A$ receptors are distinguished by their insensitivity to benzodiazepines (Saxena and Macdonald, 1996;Brown et al., 2002). However, although native α4 and α6 subunits do not recognize the classical benzodiazepines (e.g. diazepam), there are certain benzodiazepine site ligands (e.g. flumazenil and Ro15-4513) that are able to bind to these diazepam-insensitive receptor subtypes (Wieland et al., 1992;Derry et al., 2004;Hanchar et al., 2006;Lindquist and Birnir, 2006), and may thus affect tonic GABAergic conductances mediated by the δ subunit-containing $GABA_A$ receptors. In addition, rats homozygous for a naturally occurring point-mutation (R100Q) in the α6 subunit display diazepam and flunitrazepam sensitive α6/δ subunit-containing $GABA_A$ receptors (Santhakumar et al., 2006).

In the granule cells of the cerebellum and dentate gyrus, neocortex, and thalamic relay nuclei tonic GABAergic inhibition is, however, mediated by diazepam-insensitive $GABA_A$ receptors that contain α6 or α4 subunits together with the δ subunit (Brickley et al., 1996;Nusser and Mody, 2002;Stell et al., 2003;Belelli et al., 2005;Drasbek and Jensen, 2005;Cope et al., 2005;Mtchedlishvili and Kapur, 2006). In contrast, in the hippocampal CA1 area, tonic inhibition is mediated by the diazepam-sensitive γ2 subunit-containing $GABA_A$ receptors in interneurons and, to a lesser extent, in pyramidal cells (Semyanov et al., 2003). This may, however, only occur due to experimental conditions that increase ambient GABA concentrations, and at low physiological ambient GABA concentrations the δ subunit-containing $GABA_A$ receptors predominate in mediating the tonic currents in the CA1 area (Scimemi et al., 2005).

Mice with a point mutation in the $GABA_A$ receptor subunit α1 are insensitive to low-dose diazepam-induced sedation and motor impairment (Rudolph et al., 1999;McKernan et al., 2000;Crestani et al., 2000), implying that the majority of diazepam-induced sedation is mediated through the activation of the α1 subunit-containing $GABA_A$ receptors, which are

highly expressed in postsynaptic densities (Somogyi et al., 1996;Farrant and Nusser, 2005). The brain regions that mediate the sedative effects of diazepam are not, however, known.

In order to specifically examine the role of KCC2 in the phasic (synaptic) form of GABAergic inhibition, we tested the locomotor-impairing/sedative effects of diazepam in the $KCC2^{hy/null}$ mice in the open field. This test was chosen because the basal drug-free response of $KCC2^{hy/null}$ mice to novel open field environment did not differ from their wild-type littermates (III). Diazepam (2 mg/kg) significantly depressed the spontaneous motor activity (distance travelled) in wild-type mice, but resulted in only a slight decrease of the spontaneous motor activity in the $KCC2^{hy/null}$ mice. This resistance to diazepam-induced sedation was consistent with the reported role of KCC2 in synaptic GABAergic inhibition (Rivera et al., 1999;Hubner et al., 2001b;Woo et al., 2002;Zhu et al., 2005). In contrast to diazepam, gaboxadol (10 mg/kg) impaired the locomotor activity in the open field test similarly between the genotypes. As the diazepam-insensitive, primarily extrasynaptic δ subunit-containing $GABA_A$ receptors are the main target for gaboxadol (Brown et al., 2002;Krogsgaard-Larsen et al., 2004;Maguire et al., 2005;Belelli et al., 2005;Cope et al., 2005;Chandra et al., 2006), the result suggested that tonic GABA inhibition might be relatively intact in the KCC2-deficient mice.

The difference in the behavioral response of $KCC2^{hy/null}$ mice to drugs that preferentially enhanced either phasic or tonic GABA inhibition was clear. However, because of the lack of *in vitro* pharmacological analysis of the KCC2-deficient mice, we do not know the exact cellular effects of diazepam and gaboxadol, and various other drugs used throughout this thesis. These experiments would provide strong and solid supporting evidence to the *in viv*o pharmacological data presented in this thesis, and will hopefully be a subject of future studies.

4.3.4 Cation-Cl^- cotransporter isoform 2 mutant mice display normal motor impairment responses to alcohol but not to diazepam (IV)

Alcohol is known to directly modulate the activity of a variety of receptors and ion channels, including ionotropic glutamate receptors, potassium channels, adenosine receptors, $GABA_A$ receptors, $5\text{-}HT_3$ receptors, and neuronal nicotinic acetylcholine receptors (Wallner et al., 2006).

Alcohol intoxication alters coordination and motor skills. Although the precise mechanism of action of alcohol in motor impairment is unknown, alcohol may, in part, act by interfering with normal cerebellar functioning (Carta et al., 2004;Hanchar et al., 2005;Valenzuela et al., 2005). Glutamatergic cerebellar granule cells receive sensory information from mossy fibers and relay it to Purkinje neurons via the parallel fibers (Houk and Mugnaini, 2003). Mossy fiber input is filtered at granule cells by inhibitory neurotransmission provided by Golgi interneurons, which profoundly modulates cerebellar information storage capacity (Semyanov et al., 2004). Golgi interneurons provide tonic and phasic GABAergic input to granule cells, which express a limited set of $GABA_A$ receptor α1, α6, β2/3, γ2, and δ subunits, of which the δ subunits are found exclusively in the extrasynaptic membranes (Nusser et al., 1998). The α6 subunit is reported to be expressed exclusively in the cerebellar

granule cells and cochlear nucleus (Laurie et al., 1992a;Varecka et al., 1994). In cerebellar granule cells, the δ subunits subunits partner principally with α6 subunits (Jechlinger et al., 1998;Poltl et al., 2003), and these $GABA_A$ receptor subtypes are necessary for tonic inhibition (Brickley et al., 2001).

There are controversial results with respect to the significance of ethanol on tonic GABA currents generated by extrasynaptic $GABA_A$ receptors. When recombinantly expressed in *Xenopus* oocytes, the α4β2/3δ and α6β2/3δ combinations of $GABA_A$ receptors have been reported to show high sensitivity to low ethanol concentrations (Sundstrom-Poromaa et al., 2002;Wallner et al., 2003;Hanchar et al., 2005), suggesting that low ethanol selectively potentiates GABA currents mediated by δ subunit-containing $GABA_A$ receptors. This claim was not, however,confirmed by a recent consortium study from four different independent laboratories using identical experimental conditions (Borghese et al., 2006), or by a recent report using mammalian cell lines that expressed $GABA_A$ receptor subtypes α4β2δ, α6β2δ, and α6β3δ (Yamashita et al., 2006). In addition, in cultured cerebellar granule cells, the effect of ethanol on α6β2δ $GABA_A$ receptors ranged from a slight potentiation to a slight inhibition of GABA currents, suggesting that changes in the intracellular milieu might play a role in the ethanols effects on δ-containing $GABA_A$ receptors (Yamashita et al., 2006).

Conflicting results have also been obtained from studies using rats with a point-mutation (R100Q) in the $GABA_A$ receptor α6 subunit (Hanchar et al., 2005;Valenzuela et al., 2005). This polymorphism occurs naturally in outbred strains of Sprague-Dawley rats and was reported to render rats homozygous for the mutation more sensitive to low (1.25 g/kg) ethanol-induced motor-impairment (Hanchar et al., 2005). The same report also demonstrated low ethanol enhancement of tonic GABA currents in cerebellar granule cells in slice preparations, and that this enhancement was potentiated in homozygous α6(R100Q) rats (Hanchar et al., 2005). This tonic current enhancing effect of α6(R100Q) polymorphism was not confirmed by another report using inbred alcohol-nontolerant and alcohol-tolerant rats, which were homozygous for the α6(R100Q) and α6(R100R) genotypes, respectively (Valenzuela et al., 2005). This report, however, confirmed the effect of low ethanol in enhancing tonic GABA currents in cerebellar granule cells in slice preparations (Valenzuela et al., 2005). Finally, a recent report demonstrated that the low ethanol-induced increase in tonic GABA currents in cerebellar granule cells might be due to increased presynaptic GABA release, rather than a postsynaptic effect of ethanol (Carta et al., 2004).

Despite the controversial reports regarding the postsynaptic $GABA_A$ targets for ethanol, the mechanisms by which ethanol enhances tonic GABA currents, or whether ethanol has any role in modulating tonic GABAergic currents (see discussion above), the fact remains that three independent groups have shown that low ethanol induces an enhancement of tonic GABA currents *in vitro* in cerebellar granule cells in slice preparations (Carta et al., 2004;Hanchar et al., 2005;Valenzuela et al., 2005).

We tested the motor-impairing effect of low (1.25 g/kg, i:p.) dose of ethanol on $KCC2^{hy/null}$ mice on an accelerating rotarod. The mice were tested before (pretrial) and after the ethanol injection. Importantly, motor abilities were similar between the

genotypes in the drug-free pretrial test. Ethanol injection significantly impaired motor coordination in both genotypes, and the alcohol-induced motor impairment revealed no differences between the genotypes. As KCC2 is highly expressed in the cerebellar granule cells (Payne et al., 1996;Rivera et al., 1999;Kanaka et al., 2001;Mikawa et al., 2002), this result is consistent with a recent report showing that blockade of almost all (~80%) of KCl cotransport in cerebellar granule cells in slices is not affecting tonic GABA currents (Hamann et al., 2002). As the cerebellar information processing is profoundly modulated by these shunting GABA currents in the cerebellar granule cells (Mitchell and Silver, 2003), the results presented here imply the integrity of tonic GABAergic inhibition in the cerebellum of KCC2-deficient mice. We still, however, lack the key experiment of directly measuring the effect of low ethanol on cerebellar granule cell tonic GABA currents in the KCC2-deficient mice.

To further compare the effect of phasic *vs* tonic enhancement of GABAergic inhibition in the $KCC2^{hy/null}$ mice in the same behavioral task, we repeated the accelerating rotarod test by replacing the ethanol by diazepam. Again, the pretrial test showed no differences in the motor abilities between the genotypes. Diazepam (3 mg/kg, i.p.) significantly impaired the motor abilities in both genotypes, but the diazepam-induced motor impairment/ sedation was considerably stronger in the wild-type mice. This was consistent with the open field results, and suggests that the phasic GABAergic inhibition mediated by the α1 subunit-containing $GABA_A$ receptors is impaired in the $KCC2^{hy/null}$ mice. The difference between the genotypes was smaller using higher dose of diazepam (10 mg/kg, i.p.), that cause more severe impairment of rotarod performance. This is consistent with previous results that the sedative and myorelaxant actions of higher dose of diazepam are mediated also via other $GABA_A$ receptors than synaptic α1 subunit-containing $GABA_A$ receptors (Rudolph et al., 1999;McKernan et al., 2000;Crestani et al., 2000).

4.3.5 Neurosteroid-induced hypnosis and anticonvulsant potency of propofol are normal in cation-Cl^- cotransporter isoform 2 mutant mice (III, IV)

Extrasynaptic $GABA_A$ receptors that contain the δ subunit are highly sensitive to neuroactive steroids *in vitro* (Wohlfarth et al., 2002;Stell et al., 2003). The *in vivo* effect of neurosteroids on these receptors has been demonstrated in δ subunit KO mice, which show reduced duration of loss of righting reflex (LORR) in response to neurosteroids pregnanolone and alphaxalone (Mihalek et al., 1999). In addition, *in vitro* studies demonstrate that pregnanolone exhibits a clear separation of efficacy between γ2 and δ subunit-containing $GABA_A$ receptors, showing a particularly high efficacy for α4β3γ $GABA_A$ receptor subtype (Pillai et al., 2004). The neuroanatomical targets for the hypnotic action of neurosteroids are not known, but may include thalamocortical relay neurons and granule cells of the dentate gyrus (Stell et al., 2003;Belelli et al., 2005). As thalamocortical neurons are a known target for general anaesthetics (Rudolph and Antkowiak, 2004), and the thalamocortical neurons of the ventrobasalis complex display δ subunit containing $GABA_A$ receptor-mediated tonic currents (Belelli et al., 2005), it may be that the hypnotic action of neurosteroids is mediated at least partly by this area. In

addition to this region, neurosteroids show selective enhancement of tonic δ subunit-mediated GABA currents in dentate gyrus granule cells (Stell et al., 2003). In both of these cell types, the relay neurons of the thalamus and granule cells of the dentate gyrus, KCC2 shows a high expression (Gulyas et al., 2001;Bartho et al., 2004).

In animal studies, LORR is used as a marker for the hypnotic state, which is commonly defined as a drug-induced impairment of the cognitive functions required for responding to environmental stimuli (Rudolph and Antkowiak, 2004). Thus, in order to test the neurosteroid effect in KCC2 mutant mice, we conducted a pregnanolone induced (8 mg/kg, i.v) LORR in these mice. The results revealed no difference in the duration of LORR between the $KCC2^{hy/null}$ mice and wild-type littermates.

Propofol is a widely used anaesthetic, and has been shown to possess anticonvulsant properties against seizures induced by electroshock and chemoconvulsants (Lowson et al., 1990;Ahmad and Pleuvry, 1995). Propofol acts on $GABA_A$ receptors that contain any of the β subunits β1, β2, and β3 (Smith et al., 2004), but also interacts with targets distinct form the $GABA_A$ receptor, including sodium channels (Ratnakumari and Hemmings, Jr., 1997), potassium channels (Magnelli et al., 1992), cation channels (Ih) (Higuchi et al., 2003), calcium channels (Inoue et al., 1999), and glutamate channels (Orser et al., 1995). In hippocampal CA1 interneurons and pyramidal cells, electrophysiological studies have shown that propofol enhances $GABA_A$ receptor-mediated responses predominantly through the shunting effect (Bai et al., 1999;Bieda and MacIver, 2004). Tonic inhibition in these cells has been reported to be mediated by α5 and γ subunit-containing $GABA_A$ receptors (Bai et al., 2001;Semyanov et al., 2003;Caraiscos et al., 2004), but this receptor subtype may only be recruited when ambient GABA concentration increases, and at low ambient GABA concentrations, activation of δ subunit-containing $GABA_A$ receptors predominates (Scimemi et al., 2005). Propofol substantially enhances both γ and δ subunit-containing $GABA_A$ receptors *in vitro*, but is more potent on the receptors that contain the γ subunit (Feng and Macdonald, 2004). This implies that in hippocampal pyramidal cells, propofol enhances both phasic mIPSCs and tonic GABA currents, but may be more potent in enhancing tonic GABA currents when ambient GABA is elevated and the tonic GABA current is mediated by the activation of α5 and γ containing $GABA_A$ receptors.

We tested the anticonvulsant potency of this drug in the $KCC2^{hy/null}$ mice in an acute pentylenetetrazole (PTZ)-seizure model. Interestingly, the protective effect of propofol against seizures revealed no difference between the $KCC2^{hy/null}$ and control littermates. As propofol has a potent role in enhancing tonic GABA currents, we interpret this result to be consistent with the gaboxadol-induced sedation, the alcohol-induced motor impairment test and neurosteroid-induced LORR. Taken together, the results from these four experiments suggest that a profound (80-85%) reduction in KCC2 levels does not affect the efficiency of tonic GABAergic signalling in $KCC2^{hyä/null}$ mice as assessed at the behavioral level. Further experiments are, however, needed to specifically measure the effect of these drugs at the cellular level.

4.3.6 Electrophysiological measurement of tonic GABA currents in cation-Cl^- cotransporter isoform 2-deficient mice (IV)

Electrophysiological recordings were conducted to measure the tonic GABA currents at the cellular level in the KCC2-deficient mice. When tonic GABAergic current was measured in hippocampal CA3 interneurons by conventional whole-cell-clamp technique, no change in holding current was observed between the genotypes. This implies that tonic $GABA_A$ conductance, receptor expression, and extracellular GABA levels were unaltered in the KCC2-deficient mice. However, without a direct measuring of the efficacy of the $GABA_A$ receptors that mediate the tonic currents or without a quantitative immuhistochemical analysis, we cannot completely exclude the possibility of adaptive plasticity (for example, $KCC2^{hy/null}$ mice might display less $GABA_A$ receptors with higher efficacy and vice versa). When tonic current was measured using the gramicidin-perforated patch-clamp configuration that maintains internal Cl^- homeostasis, tonic current density (change in holding current per cell capacitance) was higher in the KCC2-deficient neurons when the cells were held at -80 mV. This indicates a more positive E_{GABA} of the tonic current in these animals that is consistent with the positive shift in E_{GABA} in synaptically induced $GABA_A$ receptor-mediated responses. This does not, however, necessarily compromise the efficiency of tonic (shunting) inhibition in KCC2-deficient mice as discussed below.

Shunting inhibition is a nonlinear phenomenon that behaves in an on-off like manner (Koch et al., 1983) and is efficient even if GABA is depolarizing (Staley and Mody, 1992). The key element in the shunting inhibition is the increase in membrane conductance, which limits depolarization above E_{GABA} and therefore inhibits the membrane potential from reaching an action potential threshold, provided that the E_{GABA} is below this value (Owens and Kriegstein, 2002). Thus the more positive E_{GABA} of the tonic current observed in $KCC2^{hy7null}$ mice may not manifest at the behavioral level, because E_{GABA} still remains hyperpolarized relative to action potential threshold, and the tonic current can still effectively diminish excitatory inputs. This does not, however, exclude the possibility that under certain conditions (such as during prolonged hyperexcitability) that lead to accumulation of intracellular chloride levels, the altered tonic current (due to reduced chloride extrusion capacity) in $KCC2^{hy/null}$ mice could manifest at the behavioral level, such as increased seizure susceptibility

4.3.7 Cation-Cl^- cotransporter isoform 2-deficiency results in anxiety-like behavior (III)

A diversity of neurotransmitters (e.g. GABA, glutamate, serotonin, and dopamine) and neuromodulators (e.g. cannabinoids, hormones, and neurotrophins) are involved in the induction or inhibition of anxious states, and numerous of interconnected limbic and cortical structures have been implicated in the emotional mechanisms which underlie and modulate anxious states (Millan, 2003). These structures, including regions such as the hippocampus, lateral septum, and amygdala, express high levels of KCC2 and are also known to contain GABAergic pathways that may exert an inhibitory influence upon the release of many neurotransmitters known to

mediate anxiogenic actions (Kanaka et al., 2001;Okabe et al., 2003;Brambilla et al., 2003). For example, GABA exerts an inhibitory tone on corticolimbic noradrenergic, serotonergic, and dopaminercig projections, which are implicated in the pathophysiology of anxiety disorders (Tanaka et al., 2000;Millan, 2003). In addition, a link between GABAergic system and neurosteroid modulation of anxiety states has been reported (Brambilla et al., 2003;Maguire et al., 2005).

Many classical anxiolytic drugs, for example, benzodiazepines, operate by increasing the activity of $GABA_A$ receptors. The $GABA_A$ receptor subtypes responsible for anxiolysis are not clearly determined, but the prime candidates are the receptors that contain either α2 or α3 subunits (Low et al., 2000;Dias et al., 2005). In addition, mice heterozygous for deletion of the predominantly synaptic γ2 subunit show enhanced anxiety in several tests of innate and learned fear (Crestani et al., 1999). These mice show decreased synaptic clustering of $GABA_A$ receptors in the hippocampus and cerebral cortex, consistent with studies in patients suffering from panic attacks (Schlegel et al., 1994;Kaschka et al., 1995;Malizia et al., 1998). In addition, mice with targeted inactivation of GAD65 and GAT-1 display enhanced anxiety-related behavior (Kash et al., 1999;Chiu et al., 2005).

Tonic GABA inhibition has been suggested to play a role in the modulation of anxious states in female mice (Maguire et al., 2005). During the mouse estrous cycle, hormonal changes may alter the expression of δ subunit-containing $GABA_A$ receptors and thus the magnitude of the tonic GABA conductance (Maguire et al., 2005). In late diestrus (high-progesterone phase), the expression of δ subunit-containing $GABA_A$ receptors is increased. This leads to increased tonic inhibition and consequent reduction of neuronal excitability, which correspondingly results in decreased anxiety (Maguire et al., 2005). During the estrus phase (when serum progesterone levels are relatively low), tonic inhibition is reduced by 50%, which correspondingly increases anxiety (Maguire et al., 2005).

The $KCC2^{hy/null}$ mice showed enhanced anxiety-like behavior in several behavioral tests. In the elevated plus-maze, the mutant mice showed an anxiety-like phenotype by avoiding the open arm entries almost completely, displaying less exploratory activity and head dippings, and by defecating more often than their wild-type littermates. Surprisingly, elevated plus-maze revealed that the wild-type littermates displayed highly anxious behavior as well, but these mice were still significantly less anxious compared to the KCC2-deficient mice. In addition, as the results presented in III are from two separate cohorts of mice containing wild-type and $KCC2^{hy/null}$ littermate mice, and no difference between the cohorts were observed (i.e. the $KCC2^{hy/null}$ mice displayed more anxious behavior in both cohorts), we conclude that the $KCC2^{hy/null}$ mice displayed an enhanced anxiety-like behavior in the elevated plus-maze.

The mice were subsequently tested in the open field. In this test, the mice continued to show some signs of increased anxiety (abnormal rearing activity and increased defecation), but were similar to wild-type controls with respect to the time spent in the central part of the area, and the latency to enter the central part. In the light-dark exploration test (another unconditioned test of anxiety), the $KCC2^{hy/null}$ mice continued to show decreased rearing activity and increased

defecation, consistent with the results from the elevated plus-maze. In this model, however, no differences between the genotypes were detected regarding the conventional measures of anxiety: latency to leave the lit compartment and number of transitions between the light and dark compartments. This may be due to previous testing of the mice in the elevated plus-maze and open field, as exploratory activity and emotionality have been shown to be sensitive to previous handling and testing (Voikar et al., 2004). However, the $KCC2^{hy/null}$ mice did continue to show abnormal rearing activity and increased defecation in the Y-maze test, consistent with the increased anxiety-like behavioral phenotype. Although the increased defecation in KCC2-deficient mice might be interpreted as abnormalities in the gastrointestinal system (i.e. fast bowel, poor digestion), deficits in this system would be reflected in the mouse body weight, or in food/water intake. Indeed, the KCC2-deficient display reduced body weight compared to their wild-type littermates, but this difference arises during the first two postnatal weeks, after which the difference remains the same. In addition, we have measured the food and water intake in $KCC2^{hy/null}$ mice by using a comprehensive lab animal monitoring system (CLAMS) (Columbus Instruments, Columbus, OH), and found no difference in water or food intake between the genotypes (unpublished data not shown). Thus we conclude the increased defecation as a consequence of increased anxiety in the mice.

Benzodiazepines mediate their anxiolytic effects predominantly through synaptic $GABA_A$ receptors, and it has been hypothesized, for instance, that panic disorder might be due to abnormal activity of endogenous ligands of the benzodiazepine receptor (Nutt et al., 1990). Thus the anxiety-related phenotype in KCC2 mutant mice might be induced due to compromized effects of endogenous benzodiazepine ligands on synaptic GABAergic receptors. Although estrous cycle may have a significant role in the modulation of anxious states, we did not specifically address this question in the $KCC2^{hy/null}$ mice. We did not, however, observe any gender-specific differences in the anxiety-related phenotype in the KCC2 mutant mice.

4.3.8 Morris water maze learning is impaired in cation-Cl^- cotransporter isoform 2 mutant mice (III)

Several brain regions have been implicated in Morris water maze learning, including hippocampus, striatum, basal forebrain, and cerebellum (D'Hooge and De Deyn, 2001). KCC2 is abundant in all of these regions and shows a particularly high expression in the hippocampal formation (Kanaka et al., 2001). Spatial learning in the water maze appears to depend upon the coordinated action of these different brain regions and the neurotransmitter systems that constitute a functionally integrated neural network. Mice with targeted deletion of $GABA_A$ receptor subunit α5, which is mainly localized to hippocampus (Sur et al., 1998;Sur et al., 1999b), display enhanced water maze performance (Collinson et al., 2002). In addition, activation of the GABA system by benzodiazepines have been shown to interfere directly with the spatial learning abilities (McNaughton and Morris, 1987;McNamara and Skeleton, 1993).

In order to test whether KCC2-deficiency has an effect on the mouse spatial learning capabilities, we tested the performance of $KCC2^{hy/null}$ mice in a water

maze. The mutant mice demonstrated a longer cumulative distance to the target than controls during the learning period, but were similarly able to shorten the distance to the platform. In the first transfer test (also referred to as the probe trial), the $KCC2^{hy/null}$ mice displayed a decreased ability to locate the platform. During the second transfer test (performed after reversal learning) the mutant mice showed no preference for the new or old target location. The $KCC2^{hy/null}$ mice did not, however, differ from controls regarding performance in the visible platform test, thigmotaxis or distance swum during the test, which indicated normal motivation, as well as visual and swimming abilities. Also the swimming speed was not different compared to the wild-type controls. Our results thus suggest a possible role for KCC2 in spatial hippocampus-dependent learning.

4.3.9 Threshold for nociception is increased in cation-Cl^- cotransporter isoform 2-deficient mice (III)

Neuropathic pain that occurs after peripheral nerve injury depends on the hyperexcitability of lamina I neurons in the dorsal horn of the spinal cord (Woolf and Salter, 2000;Ji and Strichartz, 2004;Lewin et al., 2004). The underlying mechanism appears to involve a down-regulation in KCC2 expression and the consequent increase in $[Cl^-]_i$ that causes GABA and glycine released from interneurons to depolarize the lamina I neurons instead of hyperpolarizing them (Coull et al., 2003). The effect of benzodiazepines was not tested in this experimental setup. The KCC2 down-regulation is presumably mediated via BDNF (Rivera et al., 2002), as peripheral nerve injury activates microglia to release BDNF, which then acts on neuronal TrkB receptors causing the depolarizing shift in the neuronal anion gradient (Coull et al., 2005).

To investigate whether the $KCC2^{hy/null}$ mice exhibited abnormalities in their somatosensory processing, we tested the sensitivity of $KCC2^{hy/null}$ mice to tactile and thermal stimuli. Thermal sensitivity was tested by warm water tail withdrawal and hot plate tests. In both tests, the traditionally used temperatures (52°C for hot plate and 49°C for tail withdrawal) revealed no difference between the genotypes. With lower temperatures (49°C for hot plate and 45°C for tail withdrawal), however, the $KCC2^{hy/null}$ displayed decreased responsiveness. The sensitivity for mechanical stimulus was also decreased in the $KCC2^{hy/null}$ mice as assessed by von Frey hairs.

Our results were in obvious contrast with the report of Coull and colleagues (2003) showing that intrathecal KCC2 antisense knock-down increased mechanical and thermal sensitivity in the same tests. The discrepancy may be explained by the universal reduction of KCC2 in $KCC2^{hy/null}$ mice versus the local spinal KCC2 reduction observed by Coull and coworkers (2003). Electrical stimulation in widespread sites in the brain, including the sensory cortex, thalamus, hypothalamus, midbrain, pons, and medulla have been shown to produce inhibitory effects on spinal nociceptive processing (Gebhart, 2004). This descending modulation of spinal nociceptive processing can also be facilitatory as well as biphasic, is often tonically active, and the strength of this supraspinal modulation compared to direct spinal action depends on the activated brain region and on the intensity of the stimulus (Gebhart, 2004). Especially the rostral ventromedial medulla and the

periaqueductal gray have been identified as important sites to descend inhibition of spinal nociceptive processing (Hunt and Mantyh, 2001;Gebhart, 2004). Local microinjections of $GABA_A$ receptor antagonists to these two sites have been shown to result in antinociception in the spinal dorsal horn (Moreau and Fields, 1986;Drower and Hammond, 1988;Heinricher and Kaplan, 1991), and the behavioral responses in tests of nociception, including tail withdrawal, are subject to modulation by these supraspinal sites. Thus a plausible mechanism for the reduced sensitivity in $KCC2^{hy/null}$ mice would be reduced GABAergic inhibition in the central pain modulatory neurons.

4.3.10 Cation-Cl^- cotransporter isoform 2 mutant mice show increased susceptibility to seizures (III)

Epilepsies are characterized by recurrent seizures, which can cause motor, sensory, cognitive, psychic or autonomic disturbances (Steinlein, 2004). The GABA neurotransmission system has been shown to be involved in epilepsy, and the modifications within the GABAergic system leading to epileptic states can occur at all levels of integration, from $GABA_A$ receptors (DeLorey et al., 1998) to interneuron networks (Cossart et al., 2005). Experiments in genetically modified mice imply that $GABA_A$ receptor subunits β3, α1, and δ are associated with alterations in seizure susceptibility (Homanics et al., 1997;DeLorey et al., 1998;Mihalek et al., 2001;Kralic et al., 2002a) (table 6). Targeted deletions of β3 and α1 subunits result in spontaneous seizures or reduced seizure threshold (Homanics et al., 1997;DeLorey et al., 1998;Kralic et al., 2002a). $GABA_A$ receptor δ subunit KO mice show increased seizure susceptibility (Mihalek et al., 2001), and periodic alterations in the δ subunit levels during mouse estrous cycle have been implicated to affect the mouse seizure susceptibility level (Maguire et al., 2005). Finally, mutations in the $GABA_A$ receptor α1, γ2, and δ subunits have been identified in patients with different inherited epileptic disorders (Wallace et al., 2001;Baulac et al., 2001;Harkin et al., 2002;Kananura et al., 2002;Dibbens et al., 2004;Maljevic et al., 2006).

In human subiculum tissues from patients with temporal lobe epilepsy, GABAergic synapses have been shown to be still operative, but to display unique excitatory effects due to a significant positive shift of the reversal potential of GABA-mediated currents (Cohen et al., 2002). This is probably due to alterations in the expression levels of subicular NKCC1 and KCC2, as in epileptic tissues from temporal lobe epilepsy patients, NKCC1 was upregulated and KCC2 was down-regulated (Cohen et al., 2002;Palma et al., 2006). In addition, application of $GABA_A$ receptor antagonists in slice preparations suppressed the ongoing activity, instead of generating epileptic activity as observed in control conditions (Cohen et al., 2002). The results implied that depolarizing GABA currents in the subiculum contribute to the mechanism of cortical epileptic discharges.

In order to investigate whether KCC2 deficiency results in altered seizure susceptibility, we tested the $KCC2^{hy/null}$ mice in a $GABA_A$ receptor antagonist PTZ-induced seizure model, which is thought to mimic generalized seizures in humans (Andre et al., 1998). The $KCC2^{hy/null}$ mice were more susceptible to PTZ-induced seizures than wild-type controls, consistent with the findings in mice heterozygous for a KCC2 mutation (Woo et al., 2002). This

result was not, however, consistent with the results from the epileptic subicular tissues, in which $GABA_A$ receptor antagonists suppressed the interictal activity. This implies that, at least in some neurons of the KCC2-deficient mice, synaptic GABA responses are still inhibitory and PTZ induces seizures by blocking both the synaptic and tonic GABAergic inhibition. An alternative explanation is that the KCC2-deficient mice display depolarizing synaptic GABA responses that are blocked by PTZ, and thus PTZ might have a partial anticonvulsant effect in KCC2-deficient mice. This effect would, however, be overrun by the PTZ blockade of extrasynaptic $GABA_A$ receptors and by the consequent abolishment of all inhibitory GABAergic neurotransmission in the brain. In addition, possible compensations in the GABAergic system may have an affect on the seizure susceptibility level observed in $KCC2^{hy/null}$ mice.

We conclude that the decreased seizure susceptibility in KCC2-deficient mice implies a critical role for KCC2 in the control of neuronal excitability, and polymorphisms in KCC2 are obvious candidates associated with human epilepsies.

4.3.11 Female cation-Cl^- cotransporter isoform 2-deficient mice show disruption in prepulse inhibition (unpublished results)

Disturbances in cortical and hippocampal GABAergic interneuron networks have been linked to a broad array of disorders in cognitive function, like those seen in schizophrenia (Benes and Berretta, 2001;Lewis et al., 2005). GABAergic interneurons are remarkably diverse and complex in nature and engage in complex interactions not only with the excitatory neurons, but also with one another (Woo and Lu, 2006). Thus defects in even one component of these interneuron networks may potentially have profound effects of local circuits as well as larger scale macrocircuits. Within the corticolimbic system, GABAergic networks regulate the generation of oscillatory rhythms, discriminative information processing, and gating of sensory information. All of these functions may be abnormal in patients with cognitive function disorders (Benes and Berretta, 2001).

In humans, disruption of PPI of startle response is manifested in neuropsychiatric disorders characterized by sensorimotor gating deficit. Studies in rats have shown that PPI is mostly regulated by complex neuronal interactions between limbic cortex (including e.g. temporal cortex, medial prefrontal cortex, hippocampus, and amygdala), the ventral striatum, the ventral pallidum, and pontine tegmentum (Swerdlow et al., 2001). The neurotransmitter systems implicated in the proper functioning of this circuitry are known to include dopaminergic and other monoaminergic systems, as well as GABAergic and glutamatergic systems (Carlsson et al., 2001). GABAergic inhibitory control of the dopaminergic and other monoaminergic systems appears to be important for the normal expression of PPI (Swerdlow et al., 2001). Direct evidence for the role of GABAergic neurotransmission in the expression of PPI has been obtained from genetically modified mice (table 6). Inactivation of several components of the GABA neurotransmitter system, including GAT-1, GAD65, and $GABA_A$ receptor subunits α3 and α5, result in mice with deficits in PPI (Heldt et al., 2004;Hauser et al., 2005;Chiu et al., 2005;Yee et al., 2005). In addition, studies in rats have shown that

microinjections of the $GABA_A$ receptor antagonist picrotoxin into amygdala, prefrontal cortex, and hippocampus disrupt PPI (Japha and Koch, 1999;Fendt et al., 2000), whereas intraperitoneal treatment of mice with diazepam improves PPI (Ouagazzal et al., 2001).

To assess the sensorimotor gating of $KCC2^{hy/null}$ mice, we used the paradigm of PPI of the acoustic startle response. The mice showed no difference between the genotypes and the gender in the startle response to the pulse stimulus alone (data not shown, $F_{1.109} = 0.57$, $p > 0.05$). In the PPI analysis, the magnitude of PPI (percent inhibition) increased with increasing intensity of the prepulse stimulus for both wild-type and $KCC2^{hy/null}$ mice (Fig. 3A, B. effect of prepulse intensities, $F_{3.327} = 307$, $p < 0.001$). The wild-type female mice displayed enhanced PPI compared to the wild-type male mice, but this gender difference between the PPI levels was not apparent in KCC2-deficient mice. A marked attenuation of PPI compared wild-type littermates was seen in the female KCC2-deficient mice, but not in male mice. These findings were supported by a 2-way repeated measures ANOVA which yielded significant effects of prepulse intensities ($F_{3.327} = 307$, $p < 0.001$), and of sex ($F_{1.109} = 11.2$, $p < 0.01$), and a significant interaction between sex and genotype ($F_{1.109} = 4.44$, $p < 0.05$). *Post hoc* analysis detected a significant interaction between sex and genotype in female ($p < 0.01$), but not in male mice ($p > 0.05$). This implies that females in our (C57/BL6 x 129S2)F1 hybrid background show enhanced levels of PPI compared to males, and that this enhancement of PPI is abolished by KCC2 deficiency.

Previous studies have identified sex differences in acoustic startle and PPI

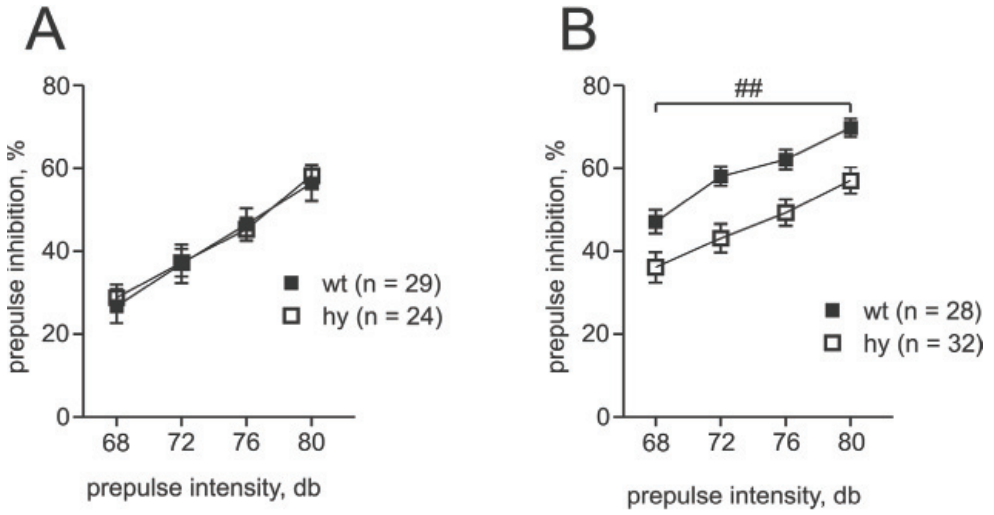


Fig. 4. PPI in $KCC2^{hy/null}$ male and female mice. PPI was indexed by percent inhibition, defined as the percent reduction in reactivity in prepulse-plus-pulse trials relative to pulse alone trials. **A)** Increasing prepulse intensity led to similarly increased magnitude of PPI in both $KCC2^{hy/null}$ male and wild-type male mice. **B)** The normal enhancement of PPI was abolished in $KCC2^{hy/null}$ female mice, a marked attenuation of PPI was seen at every prepulse intensity tested. Data represent mean ± SEM. ##$p < 0.05$ between the genotypes by two way ANOVA followed by Newman-Keuls post-hoc test.

in humans and rodents (Swerdlow et al., 1993;Lehmann et al., 1999). Specifically, PPI fluctuates across the estrous or menstrual cycle, implicating a role for estrogen and progesterone in the regulation of sensorimotor gating (Swerdlow et al., 1993;Swerdlow et al., 1997;Koch, 1998). In addition, studies using inbred mouse strains have shown a significant contribution of genetic influences to the sex-dependent differences in PPI responses (Willott et al., 2003). The sex-specific nature of the PPI attenuation observed in KCC2 deficiency suggests that KCC2 activity or expression might be regulated by sex hormones or that KCC2 expression displays sex-specific differences in certain limbic areas that regulate the normal expression of PPI, such as hippocampus, basolateral amygdala, striatum, and substantia nigra reticulata (Swerdlow et al., 2001). Indeed, sex-specific differences in KCC2 expression has been described in rat substantia nigra (Galanopoulou et al., 2003). In addition, sex hormones have been shown to regulate the expression of KCC2 mRNA in juvenile rat substantia nigra (Galanopoulou and Moshe, 2003). In this brain region, testosterone and dihydrosterone were shown to up-regulate KCC2 mRNA in both sexes, whereas estradiol down-regulated the transcript expression in males but not in females (Galanopoulou et al., 2003). Unfortunately, the possible influence of the estrous cycle upon KCC2 mRNA expression has not been described.

5. CONCLUDING REMARKS

- KCC isoforms and NKCC1 undergo dramatic spatiotemporal changes in their expression during rodent embryonic CNS development. KCC2 mRNA expression displays developmental up-regulation and follows the rostro-caudal progression pattern of neuronal maturation. NKCC1 and KCC4 transcripts are highly expressed in the proliferative zones of subcortical regions at the peak of neurogenesis. These expression profiles suggest a role for CCC isoforms in the maturation of synaptic responses as well as in the regulation of neuronal proliferation during embryogenesis.

- KCC2 is a critical player in creating and maintaining low neuronal $[Cl^-]_i$, which is required for hyperpolarizing GABA and glycine neurotransmitter-mediated responses. Complete disruption of the KCC2-encoding *Slc12a5* gene in mice leads to death immediately after birth, whereas KCC2 knock-down animals that retain 15-20% of the normal KCC2 levels are viable and display several behavioral phenotypes. These mice exhibit anxiety-like behavior and seizure susceptibility as well as impaired water-maze performance and alterations in nociceptive processing. This thesis provides the first report concerning the role of KCC2 in adult mouse behavior and suggests that KCC2 may have implications for human neurological and psychiatric disorders, such as epilepsy, anxiety, and pain.

- The role of KCC2 in GABAergic neurotransmission is emphasized in the fast phasic inhibition mediated by postsynaptic $GABA_A$ receptors. KCC2 deficiency leads to compromised hyperpolarizing GABA responses which are directly correlated with mice behavior, whereas reduced tonic GABA currents are not necessarily manifested in the animal behavior.

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