GABA\textsubscript{A} Receptor Mediated Signalling in the Brain:
Inhibition, Shunting and Excitation

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Academic dissertation

To be presented for public criticism, with the permission of the Faculty of Biological and Environmental Sciences, University of Helsinki, in the Lecture Hall 1041 of Biocenter 2, on the 23\textsuperscript{rd} of June 2010 at 12 o’clock noon.

Helsinki, 2010
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ISBN 978-952-92-7507-6 (paperback)
ISSN 1795-7079

Yliopistopaino
Helsinki 2010
1 ORIGINAL PUBLICATIONS

This Thesis is based on the following articles, referred to in the text by their Roman numerals. Unpublished results are also presented in the text.


1 The author contributed to a design of electrophysiological work and performed preliminary recordings on SNC dopaminergic neurons, which were needed to resolve a suitable methodology for data production. The author participated to data analysis and to the writing of manuscript.

2 The author designed and performed the BDNF pre-incubation experiments on acute hippocampal slices, executed the intracellular recordings on CA1 pyramidal cells, analyzed aforementioned data and contributed to the writing of manuscript.

3 The author contributed to a design of experiments and performed most of the electrophysiological work (which include field potential, intracellular, whole-cell, extracellular ion selective, iontophoretic and pressure injection microelectrode techniques), analyzed data and participated to the writing of manuscript.
2 PUBLICATIONS NOT INCLUDED IN THE THESIS


### 3 ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>5-HT₃R</td>
<td>5-hydroxytryptamine (serotonin) receptor type 3</td>
</tr>
<tr>
<td>a</td>
<td>activity</td>
</tr>
<tr>
<td>AAC</td>
<td>axo-axonic cell</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BCECF</td>
<td>2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain derived neurotrophic factor</td>
</tr>
<tr>
<td>BMI</td>
<td>bicuculline methiodide</td>
</tr>
<tr>
<td>CAI–3</td>
<td>cornu ammonis 1–3 areas of hippocampus</td>
</tr>
<tr>
<td>CA</td>
<td>carbonic anhydrase</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CCCs</td>
<td>cation chloride cotransporters</td>
</tr>
<tr>
<td>CCK</td>
<td>cholecystokinin</td>
</tr>
<tr>
<td>CGP55845</td>
<td>(2S)-3-{{[1S]-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl} (phenylmethyl) phosphinic acid</td>
</tr>
<tr>
<td>CIP</td>
<td>cotransporter interacting protein</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>DF</td>
<td>driving force</td>
</tr>
<tr>
<td>DG</td>
<td>dentate gyrus area of hippocampus</td>
</tr>
<tr>
<td>DIDS</td>
<td>4,4´-diisothiocyanato-stilbene-2,2´-disulfonic acid</td>
</tr>
<tr>
<td>DL-AP5</td>
<td>DL-2-Amino-5-phosphonopentanoic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DRG</td>
<td>dorsal root ganglion</td>
</tr>
<tr>
<td>Eion</td>
<td>equilibrium potential of an ion</td>
</tr>
<tr>
<td>Em</td>
<td>membrane voltage given by the GHK equation</td>
</tr>
<tr>
<td>EGABA</td>
<td>reversal potential of GABA_A receptor mediated responses</td>
</tr>
<tr>
<td>E₃rd</td>
<td>E_GABA after 3rd HFS pulse (t~35ms)</td>
</tr>
<tr>
<td>Elast</td>
<td>E_GABA after last HFS pulse (t~405ms)</td>
</tr>
<tr>
<td>EPSP/C</td>
<td>excitatory postsynaptic potential/current</td>
</tr>
<tr>
<td>EZA</td>
<td>ethoxyzolamide</td>
</tr>
<tr>
<td>F</td>
<td>Faraday’s constant</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>FRS–2</td>
<td>FGF receptor substrate 2</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-amino butyric acid</td>
</tr>
<tr>
<td>GABA_A</td>
<td>GABA_A receptor</td>
</tr>
<tr>
<td>GABA_B</td>
<td>GABA_B receptor</td>
</tr>
<tr>
<td>GHK</td>
<td>Goldman-Hodgkin-Katz constant field equation</td>
</tr>
<tr>
<td>GlyR</td>
<td>glycine receptor</td>
</tr>
<tr>
<td>HFS</td>
<td>high frequency stimulation (40 pulses/100Hz)</td>
</tr>
<tr>
<td>HEK–293</td>
<td>human embryonic kidney cell line 293</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid N-(2-hydroxyethyl) piperazine-N´-(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>IGF–1</td>
<td>insulin like growth factor 1</td>
</tr>
<tr>
<td>iGluR</td>
<td>ionotrophic glutamate receptor</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td>$I_m$</td>
<td>membrane current</td>
</tr>
<tr>
<td>$I_m$</td>
<td>membrane current given by the GHK equation</td>
</tr>
<tr>
<td>IPSP/C</td>
<td>inhibitory postsynaptic potential/current</td>
</tr>
<tr>
<td>KCC1–4</td>
<td>$K^+/Cl^-$ cotransporters 1–4</td>
</tr>
<tr>
<td>$K_m$</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>LGIC</td>
<td>ligand gated ion channel</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>$\mu$</td>
<td>electrochemical potential</td>
</tr>
<tr>
<td>nAChR</td>
<td>nicotinic acetylcholine receptor</td>
</tr>
<tr>
<td>NBQX</td>
<td>2,3-dioxo-6-nitro-1,2,3,4-tetrahydronbenzo[f]quinoxaline-7-sulfonamide disodium salt</td>
</tr>
<tr>
<td>NG</td>
<td>neurogliaform class of interneuron</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
</tr>
<tr>
<td>NKCC</td>
<td>Na$^+$ driven $K^+/Cl^-$ cotransporter</td>
</tr>
<tr>
<td>NT–4</td>
<td>neurotrohin–4</td>
</tr>
<tr>
<td>O-LM</td>
<td>oriens-stratum moleculare</td>
</tr>
<tr>
<td>OSR 1</td>
<td>oxidative stress responsive kinase 1</td>
</tr>
<tr>
<td>$p$</td>
<td>ion permeability, relative</td>
</tr>
<tr>
<td>$P$</td>
<td>ion permeability</td>
</tr>
<tr>
<td>$P_n$</td>
<td>postnatal day</td>
</tr>
<tr>
<td>PiTX</td>
<td>picrotoxin</td>
</tr>
<tr>
<td>PLC$\gamma$</td>
<td>phospholipase C$\gamma$</td>
</tr>
<tr>
<td>PP</td>
<td>protein phosphatase</td>
</tr>
<tr>
<td>PV</td>
<td>parvalbumin</td>
</tr>
<tr>
<td>$R$</td>
<td>gas constant</td>
</tr>
<tr>
<td>$R_{in}$</td>
<td>input resistance</td>
</tr>
<tr>
<td>$R_{in}$</td>
<td>input resistance given by the GHK equation</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>$s$</td>
<td>second</td>
</tr>
<tr>
<td>she</td>
<td>src homology 2 domain containing transforming protein</td>
</tr>
<tr>
<td>sp</td>
<td>stratum pyramidale</td>
</tr>
<tr>
<td>SPAK</td>
<td>STE20/SPS1 related proline/alanine-rich kinase</td>
</tr>
<tr>
<td>SLC12</td>
<td>gene family 12 of mammalian solute carriers</td>
</tr>
<tr>
<td>SNc</td>
<td>Substantia Nigra pars compacta</td>
</tr>
<tr>
<td>SNr</td>
<td>Substantia Nigra pars reticulata</td>
</tr>
<tr>
<td>sr</td>
<td>stratum radiatum</td>
</tr>
<tr>
<td>STE20</td>
<td>sterile 20-kinase family</td>
</tr>
<tr>
<td>$T$</td>
<td>absolute temperature</td>
</tr>
<tr>
<td>TeMA</td>
<td>tetramethylammonium ion</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane segment of a protein</td>
</tr>
<tr>
<td>TrkB</td>
<td>tyrosine receptor kinase B</td>
</tr>
<tr>
<td>TTX</td>
<td>tetrodotoxin</td>
</tr>
<tr>
<td>$V_m$</td>
<td>membrane potential</td>
</tr>
<tr>
<td>WNK</td>
<td>with-no-lysine kinase family</td>
</tr>
<tr>
<td>$z$</td>
<td>valence of an ion</td>
</tr>
</tbody>
</table>
4 SUMMARY

The simple division of chemical synaptic transmission to depolarizing excitation mediated by glutamate and hyperpolarizing inhibition mediated by γ-amino butyric acid (GABA), found in the majority of present day textbooks, is evidently an oversimplification. It has been evident for long, that the nature of chloride dependent GABA transmission is not rigid, but might differ between neuron types (Misgeld et al., 1986; Kaila et al., 1993; Gulledge & Stuart, 2003) and may even change its sign in response to strong stimuli or trauma (Alger & Nicoll, 1979; Andersen et al., 1980; Lambert et al., 1991; Grover et al., 1993; Staley et al., 1995; Kaila et al., 1997; Smirnov et al., 1999; Lamsa & Taira, 2003). Recent findings demonstrate that the GABA_\text{A} receptor (GABA_\text{AR}) mediated responses can be of opposite sign even within a single resting cell, due to the compartmentalized distribution of cation chloride cotransporters (CCCs) (Szabadics et al., 2006). The K^+/Cl^- cotransporter 2 (KCC2), member of the CCC family, promotes K^+ fuelled Cl^- extrusion and sets the reversal potential of GABA evoked anion currents typically slightly below resting membrane potential, whereas the other CCC family member, the Na^+ driven K^+/Cl^- cotransporter 1 (NKCC1) is capable of accumulating Cl^- into neurons and thus promotes depolarizing GABA_\text{AR} responses (Payne et al., 2003; Blaesse et al., 2009). This interesting “ionic plasticity” property of GABAergic signalling emerges from the short-term and long-term alterations in the intraneuronal concentrations of GABA_\text{AR} permeable anions (Cl^- and HCO_3^-) (Voipio & Kaila, 2000; Farrant & Kaila, 2007). The short-term effects arise rapidly (in the time scale of hundreds of milliseconds) and are due to the GABA_\text{AR} activation dependent shifts in anion gradients, whereas the changes in expression, distribution and kinetic regulation of CCCs are underlying the long-term effects, which may take minutes or even hours to develop.

In this Thesis, the differences in the reversal potential of GABA_\text{AR} mediated responses between dopaminergic and GABAergic cell types, located in the substantia nigra, were shown to be attributable to the differences in the chloride extrusion mechanisms. The stronger inhibitory effect of GABA on GABAergic neurons was due to the cell type specific expression of KCC2 whereas the absence of KCC2 from dopaminergic neurons led to a less prominent inhibition brought by GABA_\text{AR} activation.

In addition to the cell type specific expression pattern of KCC2 present in the substantia nigra, the levels of KCC2 protein exhibited activity dependent alterations in hippocampal pyramidal neurons. Intense neuronal activity evoked by kindling, leading to a massive release of brain derived neurotrophic factor (BDNF) \textit{in vivo}, or application of tyrosine receptor kinase B (TrkB) agonists
BDNF or neurotrophin-4 in vitro, were shown to down-regulate KCC2 protein levels which, in turn, led to a reduction in the efficacy of Cl⁻ extrusion within 2–4 hours.

The GABAergic transmission is interestingly involved in an increase of extracellular K⁺ concentration. A substantial increase in interstitial K⁺ tends to depolarize the cell membrane and promote excitation. The effects that rapidly varying ion gradients had on the generation of biphasic GABAₐR mediated responses were addressed, with particular emphasis on the novel idea that the electroneutral K⁺/Cl⁻ extrusion via KCC2 is accelerated in response to a rapid accumulation of intracellular Cl⁻ (Voipio & Kaila, 2000). The KCC2 inhibitor furosemide produced a large reduction in the GABAₐR dependent extracellular K⁺ transients. Thus, paradoxically, the efficient KCC2 activity may promote excitation.

To summarize, the GABAergic responses are controlled by a concerted action of ion permeation and regulation mechanisms. Changes in the activity or expression of CCCs, which cause, or are followed by the alterations in neuronal activity, accentuate the role of ion transporters as key players in the modulation of neuronal signalling (Kaila, 1994; Payne et al., 2003; Blaesse et al., 2009). Chloride regulation is of crucial importance in defining the nature of GABA mediated responses. The existence of diverse GABAₐR dependent responses reflects that the chloride gradient, governed by various anion transporters that are regulated both developmentally and kinetically, can vary considerably both in time and in space.
5 REVIEW OF THE LITERATURE

5.1 GABA

Although high concentrations of GABA were found in mammalian brain extracts already at the beginning of the 50’s (Awapara et al., 1950), its functional role as an inhibitory neurotransmitter was not recognized until some twenty years later (Krnjevic & Schwartz, 1967; Dreifuss et al., 1969). Since then GABA has substantiated its role as the most abundant inhibitory neurotransmitter in the CNS, where up to 30% of all synapses and neurons have been estimated to contain GABA (Bloom & Iversen, 1971; Markram et al., 2004). The physiological effects of GABA are mediated by the activation of its receptors type A and type B (Olsen & Sieghart, 2008; Huang, 2006), which are both ubiquitous. Practically every neuron in the brain is sensitive to GABA (Bowery & Smart, 2006).

5.2 Receptors of GABA

As mentioned above, GABA receptors are categorized to type A ionotrophic receptors (GABA_AR) and type B metabotropic receptors (GABA_BR). The GABA_AR is a channel permeable to anions (Bormann et al., 1987) and the GABA_BR is connected via G-protein cascades to K^+ and Ca^{2+} channels (Hill & Bowery, 1981; Dutar & Nicoll, 1988; Kaupmann et al., 1997). The prominent effect of GABA_BR activation is, on the presynaptic side, reduced transmitter release caused by the inhibition of presynaptic Ca^{2+} channels and, on the postsynaptic side, prolonged hyperpolarization caused by the activation of K^+ channels (Couve et al., 2000; Bettler & Tiao, 2006). Most data presented in this Thesis were produced under a pharmacological blockade of GABA_BRs, so their properties are not discussed further. The literature of ionotrophic GABA_ARs is exhaustive and several excellent reviews on function, pharmacology and regulation of various receptor isoforms have been published quite recently (Sieghart & Sperk, 2002; Farrant & Kaila, 2007; Fritschy et al., 2003; Kittler & Moss, 2003; Mohler, 2006; Olsen & Sieghart, 2008).

5.3 Molecular properties of GABA_AR receptors

GABA_ARs belong to the cysteine-loop superfamily of ligand gated ion channels (LGIC), which all respond to a ligand binding by opening a pore permeable to ions (Simon et al., 2004). The nicotinic acetylcholine (or nicotinicoid) receptor subgroup of LGIC family is exemplified by the nicotinic
acetylcholine receptor (nAChR), other members being the 5-hydroxytryptamine (serotonin) type 3 receptor (5-HT$_3$R), glycine receptor (GlyR) and GABA$_A$R (Barry & Lynch, 2005; Cascio, 2006; Hille, 2001). Members of this family share the characteristic pentameric, barrel-like structure constructed of subunits having four transmembrane (TM) segments M1–M4. A large N-terminal extracellular loop connected to M1 forms the main ligand binding structure of some subunits and a long intracellular loop between M3 and M4 mediates the contacts between receptor complexes and cytoskeletal structures. The M2 segments of each subunit are delineating the central pore and form the ionic selectivity filter of a particular channel type. The nAChRs and 5-HT$_3$Rs are permeable to monovalent cations, mainly to Na$^+$ and K$^+$, while GlyRs and GABA$_A$Rs prefer anions, especially chloride (Moss & Smart, 2001; Barry & Lynch, 2005; Bowery & Smart, 2006).

5.4 Diverse subunits of GABA$_A$ receptors

Since the original cloning of bovine cDNAs coding for $\alpha$1 and $\beta$1 subunits which are capable of forming functional GABA$_A$Rs (Schofield et al., 1987), the family of identified subunit genes has expanded rapidly (Levitan et al., 1988; Simon et al., 2004) and contains 19 members to date (Olsen & Sieghart, 2008). GABA$_A$R genes are divided into eight families, $\alpha$1-$\alpha$6, $\gamma$1-$\gamma$3, $\pi$, $\epsilon$, $\delta$, $\beta$1-$\beta$3, $\theta$, $\rho$1-$\rho$3 and the existence of splice variants of some $\alpha$, $\beta$, $\gamma$, $\epsilon$ or $\rho$ mRNAs increases the list even further. Although the possible subunit combinations are numerous, the list of actually existing subunit combinations seems to be considerably shorter. Nearly all intrinsic properties of GABA$_A$Rs can be constructed in heterologous systems by co-expression of $\alpha$, $\beta$ and $\gamma$ subunits (Pritchett et al., 1989). The prototype assembly contains $\alpha$1$\beta$2$\gamma$2 subunits in a presumed 2$\alpha$:2$\beta$:1$\gamma$ stoichiometry and covers roughly 40% to 60% of known receptors. The $\alpha$2$\beta$2$\gamma$2 combination forms ~20% of receptors, other combinations being less common (Whiting et al., 1999; Sieghart & Sperk, 2002).

The cellular distribution pattern of GABA$_A$Rs is determined by their subunit composition. The $\gamma$2 (or $\gamma$3) subunit is needed to guide the receptors to postsynaptic densities, where they are involved in a classical form of phasic synaptic inhibition. Of the synaptic receptors containing $\gamma$2 subunits, those associated with $\alpha$1 are found on the somatodendritic synapses of hippocampal pyramidal cells. The $\alpha$2 receptor isoforms are concentrated on the axon initial segment and are ubiquitous also on somatic and dendritic regions (Brunig et al., 2002a; Fritschy & Brunig, 2003). The formation of postsynaptic GABA$_A$R clusters is mainly dependent on intracellular molecular chaperones, gephyrin in particular (Essrich et al., 1998) and may proceed independently of GABAergic innervation (Brunig et al., 2002b), but the stabilization of synaptic contacts seems to require proper connections between the post- and presynaptic counterparts (Fritschy et al., 2003).
The presence of δ or α5 (or α6 in cerebellar granule cells) directs the receptors onto extrasynaptic sites, where they mediate tonic inhibition (Nusser et al., 1998). Apparently the γ2 and δ subunits compete for assembly with α and β subunits and the expression levels of γ2 and δ may regulate the balance between pools of synaptic and extrasynaptic receptors (Luscher & Keller, 2004).

### 5.5 Classical GABA<sub>A</sub> receptor mediated inhibition

The GABA<sub>A</sub>Rs are mainly permeable to chloride and to a lesser extent to other small anions (Bormann et al., 1987). Of the non-chloride anions, bicarbonate (HCO<sub>3</sub>⁻) is the only one being physiologically relevant (Kaila & Voipio, 1987; Kaila, 1994). Postulating that the intracellular pH is kept 0.2–0.3 units more acidic than extracellular pH, the current carried by HCO<sub>3</sub>⁻ has a reversal around −12mV to −18mV (E<sub>HCO3</sub>), which is a value considerably above the typical resting membrane potential of neurons. The GABA<sub>A</sub>R mediated Cl⁻ flux is associated with a concomitant outflow of HCO<sub>3</sub>⁻, which has a significant depolarizing effect on the membrane potential (V<sub>m</sub>) (Kaila & Voipio, 1987; Kaila & Voipio, 1990). The classical, phasic form of hyperpolarizing inhibition is attributable to a net influx of Cl⁻, which necessitates the existence of Cl⁻ extrusion mechanisms in the membrane of postsynaptic cell (Lux, 1971; Eccles et al., 1977; Misgeld et al., 1986). This chloride extrusion device of rodent hippocampal neurons was found to be a specific member of the CCC family, named KCC2, the expression of which exhibits postnatal up-regulation that parallels the gradual development of hyperpolarizing GABA<sub>A</sub>R responses (Payne, 1997; Rivera et al., 1999).

Although quite common, the postsynaptic hyperpolarization is not the only phenomenon produced by GABA. For instance, in hippocampal pyramidal cells and interneurons the strong activation of GABA<sub>A</sub>Rs evoke biphasic, initially hyperpolarizing responses which are followed by a prolonged depolarizing phase (Alger & Nicoll, 1979; Andersen et al., 1980; Lambert et al., 1991; Grover et al., 1993; Staley et al., 1995; Kaila et al., 1997; Smirnov et al., 1999; Lamsa & Taira, 2003) and in the neocortical principal cells GABA may exhibit depolarizing effects only (Kaila et al., 1993; Gulledge & Stuart, 2003). Also the granule cells of hippocampus (Misgeld et al., 1986), the principal cells and interneurons of perirhinal area and lateral amygdala (Martina et al., 2001) appear to have a chloride regulation machinery promoting purely depolarizing GABA<sub>A</sub>R responses. Thus depending on the cell type, developmental stage and prevailing conditions, the anion regulation mechanisms may differ and GABA<sub>A</sub>R dependent ionic mechanism may hyperpolarize or depolarize postsynaptic cell membrane.
5.6 GABA_A receptor mediated inhibition in the substantia nigra

The rodent substantia nigra (SN), a component of the basal ganglia, contains two anatomically and physiologically distinct regions, *pars compacta* and *pars reticulata*. Substantia nigra *pars reticulata* (SNr) is formed by GABAergic neurons, which form the major output route of the basal ganglia and substantia nigra *pars compacta* (SNC) is formed by dopaminergic neurons projecting to striatum (Smith *et al.*, 1998; Bolam *et al.*, 2000).

The dopaminergic neurons of SNC receive numerous GABA_AR mediated inputs from other basal ganglia regions, mainly from the striatum and globus pallidus, and from collaterals of GABAergic projection neurons of the nearby SNr (Grofova, 1975; Smith & Bolam, 1989; Grace & Bunney, 1985; Tepper *et al.*, 1995). The projection neurons of the SNr are also innervated by GABA_AR mediated inhibitory inputs (Precht & Yoshida, 1971; Rick & Lacey, 1994), which to a large extend originate from cells located within the same nucleus (Grace & Bunney, 1979; Grofova *et al.*, 1982; Hausser & Yung, 1994; Paladini *et al.*, 1999). Both dopaminergic and GABAergic cells of the SN can be divided to smaller subnuclei based on the expression of neurochemical markers, which may propose functional compartmentalization within dopaminergic and GABAergic cell populations (Gonzalez-Hernandez & Rodriguez, 2000). However, at least the two groups of GABAergic cells expressing either parvalbumin or calretin appear to be physiologically indistinguishable (Lee & Tepper, 2007), suggesting that the local GABAergic connections of SN are not functionally as versatile as in hippocampus (see below).

The dopaminergic and GABAergic cells of the SN respond to GABA_AR activation by a membrane hyperpolarization (Lacey *et al.*, 1989; Hajos & Greenfield, 1994), which necessitates the existence of Cl^- extrusion. The SNr GABAergic cells target mainly somatic and proximal dendritic regions of SNC neurons and are thus ideally located to exert a powerful inhibitory action on dopaminergic cells (Mailly *et al.*, 2003), but the effectiveness of GABA_AR dependent inhibition has been described to be less profound in the dopaminergic neurons than in the GABAergic neurons (Grace & Bunney, 1979; Waszczak *et al.*, 1980; Celada *et al.*, 1999). Among other factors, the differences in chloride extrusion mechanisms may explain the variability in the efficacy of inhibition between SN cell types (Gulacsi *et al.*, 2003). The GABAergic cells of *pars reticulata* exhibit a classical, KCC2 mediated Cl^- extrusion whereas the dopaminergic neurons of *pars compacta* rely on a HCO_3^- dependent Cl^- extrusion, most likely maintained by the Na^+ driven Cl^-/HCO_3^- exchanger (Gulacsi *et al.*, 2003; Farrant & Kaila, 2007).
Hippocampal feed-forward and feedback circuits connected via GABA<sub>A</sub> receptors

The pyramidal neurons of rodent hippocampus *cornu ammonis* I (CA1) region are thoroughly covered with GABAergic synapses (Megias et al., 2001). These inputs arise from both feed-forward and feedback circuits (Alger & Nicoll, 1982a; Andersen et al., 1964) which are constructed by various subclasses of interneurons (for review, see Freund & Buzsaki, 1996; McBain & Fisahn, 2001; Ascoli et al., 2008). In their insightful review in the mid 90’s, Freund & Buzsaki (1996) proposed that the inhibitory circuitry of rat hippocampus could be grossly categorized to two groups. Based on the anatomical and electrophysiological properties of identified GABA-containing interneurons, they suggested that the synapses on perisomatic regions, rising predominantly from feed-forward connections, are ideally located and equipped for the precise control of the output of principal neurons, whereas the interneuron types targeting distal dendrites, receiving input mainly from feedback connections, would control the plasticity and efficacy of excitatory inputs. In line with this functional dichotomy, the somatically targeting interneurons evoke faster and larger GABA<sub>A</sub>R mediated responses than those targeting more distal regions (Maccaferri et al., 2000) and somatic and dendritic GABAergic responses have unique properties both in physiological and pharmacological domains (Alger & Nicoll, 1982b; Pearce, 1993; Buhl et al., 1994a; Miles et al., 1996). However, the inhibitory circuitry appears to be even more versatile (Klausberger & Somogyi, 2008). More than 20 types of interneurons form several local feed-forward and feedback loops, which are incorporated to main excitatory projections targeting the somatic, proximal and distal dendritic regions of pyramidal cells. The majority of known interneuron types are targeting dendritic regions in a domain specific manner (Klausberger & Somogyi, 2008). I shall go through some of those interneuron types, from which a wealth of physiological data exists (McBain & Fisahn, 2001; Somogyi & Klausberger, 2005; Mann & Paulsen, 2007; Buzsaki et al., 2007; Klausberger & Somogyi, 2008).

Main interneuron types targeting perisomatic region

It is often suggested that the major function of perisomatic inhibition is to prime and synchronize neuronal ensembles to fire at specific frequencies (Freund, 2003). Interneurons targeting perisomatic region are axo-axonic cells (AACs) and basket cells positive for either parvalbumin (PV+) or cholecystokinin (CCK+), each cell type forming a network with unique properties (Freund, 2003; Freund & Katona, 2007).

The AACs are the most selectively targeting cell type with distinct morphology that is reflected by their other name “chandelier cells”. In a CA1 region the AAC has a resting input resistance (R<sub>in</sub>) of
75MΩ and a resting membrane time constant of ~8ms, exhibits a short duration (~0.4ms) non-overshooting action potentials which are followed by a steep and fast after-hyperpolarization (Buhl et al., 1994b). The excitatory inputs to AACs generate fast rising excitatory postsynaptic potentials (EPSPs, rise time from 10% to 90% level ~3ms), which can be evoked by stimulation of variable afferents (e.g. Schaffer collaterals, perforant path and alveus). Both the rise time and amplitude of EPSPs show source dependent variation (Buhl et al., 1994b). As the AACs exclusively project to the axon initial segment of principal cells, their activity is presumed to exert powerful gating of principal neuron output, or to transiently decouple the somatic subthreshold computations from output (Howard et al., 2005). At the network level AACs might contribute to the selection of dynamic pyramidal neuron ensembles (Klausberger & Somogyi, 2008).

However, recent experiments have demonstrated that the axon initial segment of neocortical pyramids and hippocampal dentate granule cells posses a high content of Cl− and depolarizing GABAAR responses (Szabadics et al., 2006;Khirug et al., 2008), which might even excite cortical microcircuits (Szabadics et al., 2006). If so, then the postsynaptic affect that AACs produce might depend on the intensity of presynaptic activity. A modest short term activity generates primary excitatory effects, whereas a strong and long lasting stimulation of AACs would promote inhibition, due to the prominent inactivation of voltage activated Na+ channels (Trigo et al., 2008). The AAC network might thus have a homeostatic role, promoting excitation during modest activity and enhancing inhibition when activity increases (Trigo et al., 2008).

A typical parvalbumin positive (PV+) basket cell has short action potentials (~1ms), can fire up to 200Hz without accommodation and has a resting membrane time constant of 10ms and Rm around 60MΩ (Glickfeld & Scanziani, 2006). Frequent excitatory input to PV+ basket cell evokes considerably large, indefatigable EPSPs. The PV+ basket cells receive inputs from nearby hippocampal regions and are the primary mediators of feed-forward inhibition engaged by Schaffer collateral stimulation. The sequential activation of both glutamatergic and GABAergic inputs, combined with the passive and active membrane properties, efficiently shortens the time window for signal integration. Thus each PV+ basket cell is capable of discriminating inputs separated by 3ms only (Glickfeld & Scanziani, 2006).

Compared to a PV+ basket cell, the cholecystokinin positive (CCK+) basket cell has a longer resting membrane time constant (~25ms), higher membrane input resistance (150MΩ) and it generates relatively wide accommodating action potentials. The glutamatergic drive received is not as intense and EPSPs tend to diminish in response to a repetitive stimulation. This means that the CCK+ basket cells can (and must) integrate several excitatory inputs, both from feed-forward and
feedback circuits, to produce an output. As it is the subsequent feedback excitation from CA1 pyramids that usually brings the basket cells over action potential threshold, the CCK+ network is largely mediating feedback inhibition (Glickfeld & Scanziani, 2006). In addition to the projections arising from hippocampal regions, the CCK+ basket cells receive considerable modulatory (cholinergic, serotonergic) inputs from subcortical regions and are responsive to endocannabinoids (Gulyas et al., 1999; Hajos & Freund, 2002). The activation of cannabinoid receptors (CB1) on presynaptic boutons may lead to an inhibition of N-type Ca\(^{2+}\) channels and to a concomitant reduction in GABA release (Wilson et al., 2001b). Taking into account the differing properties of basket cells containing either CCK or PV, it has been hypothesised that the tightly coupled PV+ basket cell network would work as precise, non-plastic clockwork, which provides the rhythm for prevailing network activity, whereas the CCK+ basket cells would behave as modulators and tune up the ongoing activity patterns in hippocampal microcircuits, according to the subcortical inputs conveying information on the motivational and physiological status of the animal (Freund, 2003).

### 5.9 Main interneuron types of dendritic regions

#### 5.9.1 Proximal dendritic region

Two interneuron types targeting the proximal region of basal and apical dendrites of hippocampal principal cells are the trilaminar and bistratified cells (Buhl et al., 1994a; Sik et al., 1995). These cells are mainly driven by the Schaffer collaterals arising from CA3 region, or by the feedback projections of CA1 principal cells, and are thus part of both feed-forward and feedback circuits. The trilaminar cells, as its name implies, send their axons to stratum oriens, stratum pyramidale and stratum radiatum regions and have their somas near the stratum pyramidale. The somas of bistratified cells are usually found near the oriens-alveus border, from where their axons target the oriens and radiatum layers (Somogyi & Klausberger, 2005). Both cell types share similar intrinsic membrane properties (\(R_{in} 130–170 \text{M}\Omega\), resting time constant 16–19ms) and exhibit fast and regular spiking rate. The amplitude of EPSP/Cs is slightly larger and the rise time bit faster in trilaminar cells than in bistratified cells (Gloveli et al., 2005). The inhibitory postsynaptic responses in CA1 pyramids generated by the bistratified cells have smaller amplitude and slower kinetics than the IPSCs generated by basket cells and AACs targeting close or directly somatic regions (Maccaferri et al., 2000).
5.9.2 Distal dendritic region

Some of the synaptic GABA responses arising in distal apical sites were shown to have an exceptionally slow kinetics (Pearce, 1993). These are now thought to represent inputs from a neurogliaform (NG) class of interneurons, which form both electrically and synaptically connected networks (Price et al., 2005). The NG cells have their soma in the stratum lacunosum-moleculare, target exclusively the distal dendritic regions of pyramids and form inhibitory feed-forward loops to the perforant path input, but respond to Schaffer collateral inputs also. An input resistance is around 200MΩ and the NG cells respond to a membrane depolarization with delayed action potentials, which exhibit accommodating or bursting behaviour. The excitatory drive to NG cells via the perforant path shows time-dependent behaviour, it is first facilitated and then depressed, whereas the repetitive inhibitory responses between NG cell pairs fade rapidly due to the GABABR mediated presynaptic depression (Price et al., 2005).

The other cell type targeting apical distal regions, aligned with perforant path inputs, is the oriens-stratum moleculare (O-LM) interneuron. These cells have a high input resistance (>300MΩ), long resting membrane time constant (>30ms) and the EPSP/Cs received are slow and have a low amplitude, when compared to the glutamatergic responses of fast-spiking interneurons targeting to perisomatic regions (Gloveli et al., 2005). Their axons originate from somata located at the stratum oriens and are assumed to mediate feedback inhibition only (Freund & Buzsaki, 1996). The unitary IPSCs in CA1 pyramids generated by the O-LM cells are small in amplitude and very slow in kinetics (Maccaferri et al., 2000). In addition the O-LM cells have intrinsic membrane currents which promote spontaneous spiking at the so-called theta frequency range of 4−10Hz (Maccaferri & McBain, 1996; Chapman & Lacaille, 1999).

5.10 Tonic inhibition

In the rodent brain, the tonic inhibition was first described and has been mostly studied in cerebellar granule cells (Kaneda et al., 1995), but has also been detected in various cell types including hippocampal CA1 principal neurons, dentate gyrus (DG) granule cells and interneurons (Stell & Mody, 2002; Caraiscos et al., 2004; Glykys et al., 2007). Extrasynaptic and perisynaptic receptors containing δ or α5 (or α6 in cerebellar granule cells) subunits (Nusser et al., 1998) exhibit a very high affinity to GABA (in the μM range) and virtually no desensitization, thus being tonically activated by low levels of ambient GABA (Farrant & Kaila, 2007; Glykys & Mody, 2007). The GABA concentration of extracellular space is determined by the activity of vesicular transmitter
release machinery and by the GABA uptake mechanisms, both which are dynamic processes (Richerson & Wu, 2003; Semyanov et al., 2003). Due to the alterations in release or uptake mechanism the ambient levels of GABA may fluctuate, which in turn affects the activation level and the composition of receptor populations that are contributing to the tonic inhibition (Scimemi et al., 2005). Under nanomolar GABA levels, apparently the δ-subunit containing receptors are primarily responsible for the tonic inhibition, whereas the α5 containing receptors appear to be recruited in response to elevated levels of GABA, established with either uptake blockers or exogenous application (Scimemi et al., 2005; Farrant & Kaila, 2007).

The sustained increase in membrane conductance brought by tonic inhibition dampens the voltage changes generated in response to a given synaptic input and restricts the space and time of signal integration. Thus tonic inhibition affects the input-output relation of a particular neuron, increasing the amount of excitatory drive needed to produce a spike. In other words, it reduces the excitation-spike coupling of neurons (Mitchell & Silver, 2003). Recently the tonic inhibition mediated by δ subunit containing receptors of hippocampal DG granule cells was shown to be strongly augmented by physiological levels of stress-related neuroactive steroids, whereas the tonically active α5 receptors of CA1 pyramids were unaffected (Stell et al., 2003), suggesting that the tonic inhibition has cell specific features. The tonic conductance of DG networks can be modulated independently of CA1 circuitry, according to the current physiological state of the animal. The high sensitivity of α4β2δ receptors to alcohol (Sundstrom-Poromaa et al., 2002), and the importance of α5 containing receptors in learning and memory formation highlight the importance of tonic inhibition in mammalian cognition (Caraiscos et al., 2004; Glykys & Mody, 2007).

5.11 Rhythmic activity patterns within neuronal networks

The crucial involvement of GABAergic transmission in the generation and maintenance of oscillatory activity patterns is well established (Mann & Paulsen, 2007; Singer, 1996; Whittington & Traub, 2003; Bartos et al., 2007). Oscillations restricted to certain frequency bands (theta (θ), 4–10Hz; gamma (γ), 40–100Hz; sharp wave ripples 100–200Hz) are readily detected in hippocampus both in vivo and in vitro and the mechanisms and supposed functions of oscillations have been studied intensively (Buzsaki, 2005; Lisman & Buzsaki, 2008; Fries et al., 2007). Interneurons targeting the perisomatic region of pyramidal neurons are thought to participate in the formation of local, synchronous cell groups firing in the γ-frequency, whereas cells projecting to the distal dendritic regions seem to have a preference to be entrained to the θ-rhythm (Klausberger et al., 2003; Gloveli et al., 2005).
5.12 Cation Chloride Cotransporters

Active transport mechanisms present at various cell membranes can be divided into two groups based on their source of energy. The primary active transport is fuelled by enzyme-catalysed reactions utilizing adenosine triphosphate (ATP) as an energy source. For example during the transport cycle of P-type ion motive ATPases (Na⁺/K⁺ATPase, Ca²⁺ATPase), a phosphate group of ATP is first incorporated to and then removed from carrier protein and the associated changes in the energy state of transporter fuel the cargo delivery. The secondary active transport uses energy stored in ion gradients to translocate molecules or other ions across cellular membranes (for an interesting early review on carrier mediated transport models, see Crane, 1977).

The CCCs are secondary active transporters, which utilize the chemical gradients of K⁺ and/or Na⁺ generated by the primary active Na⁺/K⁺ATPase. CCCs transport chloride across the cell membrane together with at least one potassium and/or sodium ion. The existence of electroneutral Na⁺/Cl⁻ cotransport (NCC) mechanism was first demonstrated at the mid 70’s in the urinary bladder of winter flounder (Renfro, 1975; Renfro, 1977). A few years later, an electrically silent, furosemide sensitive but ouabain insensitive, cotransport of Na⁺/K⁺/Cl⁻ was described in Ehrlich cells. The transport process had a presumed stoichiometry of 1:1:2 and was suggested to be able to increase cell volume, (Geck et al., 1980). This mechanism was subsequently identified in several epithelial and non-epithelial cells. The presence of a nearly identical, but kidney specific electroneutral Na⁺/K⁺/2Cl⁻ transport mechanism was found in the mammalian thick ascending loop, where it is responsible for the Cl⁻ reabsorption (Greger & Schlatter, 1981). The more abundant isoform of Na⁺/K⁺/2Cl⁻ transporters became later known as NKCC1 and the renal specific version as NKCC2.

The outwardly directed K⁺/Cl⁻ cotransport (KCC) mechanism was first described in red blood cells as a swelling- and N-ethylmaleimide- (NEM) activated K⁺ efflux pathway, having a 1:1 stoichiometry and low affinity constants for both ions (Dunham et al., 1980; Lauf & Theg, 1980). Four KCC isoforms have been identified to date (Gamba, 2005). The widely expressed and swelling activated KCC1 is suggested to be a part of mechanisms involved in the regulatory volume decrease (Gillen et al., 1996). The neuron specific KCC2 reduces the intracellular Cl⁻ concentration and has its role in the development and functional modulation of hyperpolarizing neurotransmission mediated by GABA and glycine (Payne, 1997; Rivera et al., 1999). The KCC3 transcripts are abundant (Hiki et al., 1999; Mount et al., 1999; Race et al., 1999) and within the CNS the KCC3 serves a role similar to that of KCC2 and lowers the intracellular Cl⁻ level and may also contribute to the neuronal volume regulation (Boettger et al., 2003). In addition the KCC3 transport activity has been suggested to participate in the epithelial cell growth regulation (Shen et al., 2001). KCC4
isoform is also ubiquitous and strongly activated by cell swelling (Mount et al., 1999; Mercado et al., 2000).

Several knock out mouse strains that lack the expression of NKCC1, KCC1, KCC3 and KCC4 have been developed (Delpire et al., 1999; Flagella et al., 1999; Pace et al., 2000; Boettger et al., 2002; Boettger et al., 2003; Rust et al., 2007). The mice devoid of KCC2 die soon after birth, due to the severe motor deficits causing respiratory failure (Hubner et al., 2001b), but the hypomorphic mice expressing <20% of normal levels of KCC2 are viable (Tornberg et al., 2005). The knockdown of KCC2 have been successful e.g. in hippocampal organotypic slices in vitro and in spinal laminar I neurons in vivo (Rivera et al., 1999; Coull et al., 2003).

5.13 Basic properties of NKCC and KCC

The ion transport processes of NKCC and KCC (Russell, 2000; Lauf & Adragna, 2000) share four fundamental features: 1) All ions translocated must be present on the same side of the membrane, termed cis-side requirement. 2) All ions are translocated simultaneously in an electroneutral manner. 3) The direction of transport depends only on the sum of the chemical potential gradients of the transported ions. 4) The loop-diuretics furosemide and bumetanide bind to the protein and block the transport of all ions.

The research of CCCs has been complicated due to the co-existence of several transport processes in various cell types and due to the lack of selective pharmacological tools. A common strategy to assess the contribution of KCC2 on intraneuronal Cl\(^{-}\) level is to use furosemide (0.1–2 mM) to inhibit the K\(^{+}\)/Cl\(^{-}\) cotransport (Misgeld et al., 1986; Hochman et al., 1995; Jarolimek et al., 1996; Fukuda et al., 1998). Within the abovementioned concentration range furosemide will block the NKCC transporter as well. However, when added on top of low levels of bumetanide (1–10 µM), the furosemide can be used to isolate K\(^{+}\) fuelled Cl\(^{-}\) cotransporters from Na\(^{+}\) driven K\(^{+}\)/Cl\(^{-}\) cotransporters (Payne et al., 2003). Bumetanide reversibly inhibits NKCC in various preparations in a concentration dependent manner, with a half-inhibitory constant of \(\sim 1 \times 10^{-7}\) M (Russell, 2000) and has been successfully used to identify NKCC1 activity in neurons (Yamada et al., 2004). It should be noted that all currently known KCC isoforms are inhibited by bumetanide in concentrations above 10 µM and a near complete block of K\(^{+}\)/Cl\(^{-}\) cotransport is achieved in the millimolar range (Payne, 1997). Recently several new drug molecules were characterized that inhibit the KCC isoforms without affecting the NKCC transport (Delpire et al., 2009).
5.14 NKCC

The classical squid giant axon has been a versatile preparation in ion transport studies on the NKCC1 isoform. The internal dialyzation method, developed at the late 60’s solved problems related to isotopic exchange in flux measurements (Brinley, Jr. & Mullins, 1967). Due to the large size of the axon (500µm × 6–7cm), it was possible to insert a small dialysis capillary inside it. This allowed the experimenters to control both the intra- and the extracellular fluid composition while measuring unidirectional ion fluxes. Series of experiments revealed that the presence of all three ion species on the cis-side is obligatory for the ion transport (Russell, 1979; Russell, 1983). On the contrary to the inwardly directed transport process, the return of binding sites to the outward facing conformation does not require intracellular binding, translocation and the release of ions to extracellular space.

5.14.1 Electroneutrality

An electroneutral transporter does not generate membrane currents. However, a transporter protein itself may be sensitive to the membrane potential even if it mediates electroneutral transport. Thus the electroneutrality can be a difficult question to assess, but two neuronal preparations have been useful in this, the squid giant axon and frog dorsal root ganglion (DRG) cells. The membrane potential of internally dialysed giant axon was pharmacologically manipulated using the Na+ channel opener veratridine and the Na+ channel blocker tetrodotoxin (TTX). The effects of drugs on the $V_m$ were recorded using intracellular microelectrodes and bumetanide sensitive Cl\(^-\) influx was measured with \(^{36}\text{Cl}\). The application of veratridine depolarized the membrane by nearly 30mV, an effect that was reversed upon the subsequent application of tetrodotoxin. The bumetanide sensitive component of Cl\(^-\) influx was shown to be unaffected by the changes in $V_m$, supporting the view that NKCC mediated ion influx is electroneutral (Russell, 1984).

Alvarez-Leefmans et al. (1988) used ion sensitive microelectrodes to study Cl\(^-\) regulation in frog dorsal root ganglion (DRG) cells. Two-barrelled microelectrode recordings, suitable for simultaneous $V_m$ and intracellular Cl\(^-\) activity measurements, revealed intracellular chloride levels considerably higher than expected from passive distribution. The ion substitution experiments of Cl\(^-\), K\(^+\) and Na\(^+\) demonstrated that the maintenance of [Cl\(^-\)] was compromised if any of these ions was omitted from the frog ringer solution. In addition, bumetanide considerably slowed down the recovery of intracellular Cl\(^-\) activity after Cl\(^-\) depletion, suggesting that NKCC is the mechanism producing high Cl\(^-\) levels in DRG neurons. The membrane potential of neurons was modulated by
varying the extracellular potassium concentration without a clear effect on the intracellular Cl\(^-\) activity, strongly arguing for the electroneutrality of NKCC mediated transport process responsible for Cl\(^-\) accumulation (Alvarez-Leefmans et al., 1988). Interestingly, during the Cl\(^-\), K\(^+\), or Na\(^+\) free periods, the calculated E\(_{\text{Cl}}\) of DRG neurons dropped below recorded V\(_m\) revealing the existence of active chloride extrusion in these cells. Taking together, the results of Russell and Alvarez-Leefmans and their co-workers provided direct and strong evidence on behalf of the electroneutral nature of NKCC mediated ion transport. More recent studies have not challenged this conclusion (for review, see Russell, 2000).

### 5.14.2 Stoichiometry

The electroneutrality of NKCC imply that the net charge of transport load must be equal to zero. The simplest ion combination fulfilling that demand would thus be 1 Na\(^+\):1 K\(^+\) and 2 Cl\(^-\), the very same result to which Geck et al. (1980) ended up in one of the earliest studies of NKCC stoichiometry. They used K\(^+\) depleted and Na\(^+\) enriched Ehrlich ascites tumour cells and measured the net furosemide sensitive Na\(^+\), K\(^+\) and Cl\(^-\) uptake as a function of each ion’s extracellular concentration. Their results demonstrated that the net uptake of Cl\(^-\) was twice as large as the Na\(^+\) uptake, and that Na\(^+\) and K\(^+\) uptakes were approximately equal as a function of extracellular K\(^+\) concentration. The same flux ratios were obtained by varying [Na\(^+\)]\(_o\) and [Cl\(^-\)]\(_o\). The conclusion was that the NKCC is most likely transporting ions with 1 Na\(^+\):1 K\(^+\):2 Cl\(^-\) stoichiometry. Since then, the NKCC stoichiometry has been shown to be very close to that in the majority of cell types examined (Russell, 2000).

Early experiments done with squid giant axons hinted that the stoichiometry of its NKCC transport might differ from the common 1Na\(^+\):1 K\(^+\):2 Cl\(^-\). To study this issue more thoroughly, the coupled K\(^+\)/Cl\(^-\) and K\(^+\)/Na\(^+\) influxes were measured using intracellular solutions devoid of Na\(^+\) and Cl\(^-\), thus obtaining Na\(^+\) and Cl\(^-\) fluxes relative to K\(^+\) fluxes (Russell, 1983). The effects of 0.3mM furosemide and [Cl\(^-\)]\(_i\) shifts from 150mM to 0mM and back were tested. Both manipulations produced similar results, demonstrating that the NKCC mediated Cl\(^-\) flux is three times and Na\(^+\) flux is two times larger than the K\(^+\) flux. These findings provided strong support for the conclusion that the NKCC of squid has a deviant, though still electroneutral, stoichiometry of 2Na\(^+\):1K\(^+\):3Cl\(^-\) (Russell, 1983).
5.14.3 Kinetic model of NKCC transport

Based on their results on norepinephrine activated NKCC on duck red blood cells, Lytle et al. (1998) constructed a model of NKCC transport. According to their model (stoichiometry $1\text{Na}^+:1\text{K}^+:2\text{Cl}^-$) an ordered binding and de-binding of ions can characterize the transport process, each step being associated with minor changes in the protein conformation. For a typical inward delivery, ions bind on the outside in an order of $\text{Na}^+$, $\text{Cl}^-$, $\text{K}^+$, $\text{Cl}^-$ and the intracellular release obeys first in, first out behaviour, known as glide symmetry. Only the fully loaded carrier can transfer ions across the membrane. The return of completely empty transporter for reloading conformation is rate-limiting, explaining the $\text{K}^+/$$\text{K}^+$ exchange in high $\text{K}^+$ cells and $\text{Na}^+/$$\text{Na}^+$ exchange in high $\text{Na}^+$ cells as partial reactions of the full cotransport cycle (Lytle et al., 1998). In high $\text{Na}^+$ cells the vacant $\text{Na}^+$ site is rapidly occupied by an intracellular $\text{Na}^+$ ion before the first $\text{Cl}^-$ ion is released and in high $\text{K}^+$ cells the exchange of extracellular to intracellular counterpart in the $\text{K}^+$ binding site takes place before the release of the last $\text{Cl}^-$. The fully loaded carrier then returns back to the external conformation and the result is an exchange of $\text{Na}^+$ or $\text{K}^+$ without a net transport.

5.15 KCC

Most of the early research assessing the properties of KCC was done using erythrocytes of various species (Lauf et al., 1992). Ouabain insensitivity, tight 1 to 1 coupling between transported $\text{K}^+$ and $\text{Cl}^-$ and the inevitable electroneutrality were shown to be the basic features of red blood cell KCC. Since then, the rapidly increasing interest towards KCCs of other cell types has shown that, regardless of the tissue of origin, all known isoforms share these properties (Lauf & Adragna, 2000; Adragna et al., 2004).

5.15.1 Electroneutrality

Brugnara et al. (1989) examined the properties of swelling activated $\text{K}^+$ and $\text{Cl}^-$ transport in human red blood cells. The transport mechanism under study was revealed after the inhibition of $\text{Cl}^-/$$\text{HCO}_3^-$ exchange and rapid $\text{CO}_2$ hydration. The membrane potential was held constant using anions more permeable than $\text{Cl}^-$ but not transported by the $\text{K}^+/$$\text{Cl}^-$ cotransport system. Under these conditions, an outwardly directed $\text{Cl}^-$ gradient promoted $\text{K}^+$ efflux against inwardly directed $\text{K}^+$ gradient suggesting tight electroneutral coupling between $\text{K}^+$ and $\text{Cl}^-$ (Brugnara et al., 1989). In the subsequent work done with human erythrocytes, at that time new and very selective $\text{Na}^+$ ionophore
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hemisodium was used to assess the effects of $V_m$ on $K^+/Cl^-$ cotransport. The membrane potential was measured by the change in fluorescence of the carbocyanine dye diS-C$_3$-5. By applying sequential concentrations of hemisodium $V_m$ was varied within the range of $-8mV$ to $-90mV$, without any effect on the swelling activated fluxes of $K^+$ and $Cl^-$, supporting the electroneutral nature of transport (Kaji, 1993).

5.15.2 Stoichiometry

The work of Lauf & Adragna (1996), done with pH and volume clamped low $K^+$ sheep erythrocytes, gave further support for the 1:1 stoichiometry of $K^+/Cl^-$ transport. They measured flux reversal potentials in various $[Rb^+]_o$ and plotted the results as a function of $Cl^-$ gradient prevailing across the cell membrane. Thermodynamic considerations revealed that when the concentration ratios of anions and cations transported through KCC were plotted against each other, the slope was very close to unity, underpinning the 1:1 coupling of $K^+$ and $Cl^-$. In addition, their analysis of flux reversal potentials and ion gradients verified the prediction that the chemical gradients of transported ions define the sole driving force of KCC (Lauf & Adragna, 1996; Lauf & Adragna, 2000). A direct estimate of KCC stoichiometry came from the work of Jennings & Adame (2001), who measured the NEM stimulated fluxes of $^{36}Cl^-$ and $^{86}Rb^+$ in rabbit red blood cells clamped to around 0mV with the protonophore 2,4-dinitrophenol. After correction of $^{68}Rb^+$ fluxes to match $K^+$ fluxes, the ratio of $K^+$ and $Cl^-$ fluxes turned out to be on the average 1.12 confirming the electroneutral stoichiometry of KCC (Jennings & Adame, 2001).

5.15.3 Kinetic model of KCC transport

Using hypo-osmotically swollen low $K^+$ sheep red blood cells, Delpire & Lauf (1991) measured the influxes and effluxes of $K^+$ (or $Rb^+$) ions under both zero $K^+$ and $Rb^+$ trans conditions. Varying concentrations of intra- and extracellular $K^+$ (or $Rb^+$) and $Cl^-$ were used to assess the kinetic properties of $K^+/Cl^-$ cotransport. Their results demonstrated that the rate-limiting step seemed to be the “translocation” of the fully loaded carrier. Ion interactions with KCC were random on the intracellular side, but ordered on the extracellular face, $Cl^-$ binding prior to $K^+$ (Delpire & Lauf, 1991). The currently known Michaelis constant ($K_m$) values for extracellular ions differ considerably between KCC isoforms (Lauf & Adragna, 2000). The affinity of KCC1 is lowest, having $K_m$ values $>25mM$ for $K^+$ and $>50mM$ for $Cl^-$. The KCC2 is otherwise very much alike
KCC1, but it has a very high affinity for extracellular $K^+$, ($K_m$ 5–7mM). The binding constants of KCC3 mediated transport for $K^+$ and $Cl^-$ are around 10mM and 32mM, for KCC4 the $K_m$ values of $K^+$ and $Cl^-$ are 18mM and 16mM.

5.16 Thermodynamics of secondary active transporters

The energetic prerequisites of secondary active transport can be evaluated by considering the overall change in the free energy. To this end, the electrochemical potentials of transported ions (defined for ion $i$ as $\hat{\mu}_i = \mu_o + RT \ln a_i + z_i F V$, where $\mu_o$ is constant, $a$ is activity, and $R$, $T$, $z$ and $F$ have their usual meanings) are calculated on both the cis and on the trans side of the membrane. Then the trans-cis difference in the electrochemical potential of each ion ($\Delta \hat{\mu}_i$) is calculated and summed up to get the total change during transport ($\Sigma \Delta \hat{\mu}$). At the equilibrium $\Sigma \Delta \hat{\mu} = 0$, and values below zero suggest conditions favourable for a net transport in the assumed direction. In the following, this approach is applied on the electroneutral cotransport process of NKCC1 and KCC2.

As discussed above strong evidence is supporting the electroneutral nature and 1Na+:1K+:2Cl$^-$ stoichiometry of NKCC1 in mammalian CNS (but see (Brumback & Staley, 2008). Under ionic conditions typical for a neuron with a low $[Cl^-]_i$ (see Table 1.) the NKCC1 mediates net inward transport. Assuming that the Na$^+/K^+$ATPase can maintain the gradients of Na$^+$ and K$^+$ relatively stable, the thermodynamic equilibrium of NKCC1 would not be reached until $[Cl^-]_i$ reach a level as high as 56mM. However the intracellular Cl$^-$ level in neurons is very seldom, if ever, that high. Apparently NKCC1 is shut down before the equilibrium is reached (Breitwieser et al., 1996).

The same thermodynamic examination can be used to define the equilibrium condition of KCC2 mediated transport of $K^+$ and $Cl^-$. Applying the conditions described in Table 1. KCC2 mediates an efflux of $K^+$ and $Cl^-$ and appears to operate close to equilibrium in the dendrites of mature pyramidal neurons (Khirug et al., 2005). The high affinity of KCC2 ($K_m$=5.2mM) makes it very sensitive to changes in $[K^+]_o$ and a slight increase in $[K^+]_o$ from 3mM to ~5mM would turn the transport direction from outward to inward, if chloride gradient is assumed to stay constant (Payne, 1997). The sensitivity of KCC2 to $[K^+]_o$ (Payne, 1997) led to the suggestion that KCC2 would work in reverse mode during epileptic seizures (see also Bihi et al., 2005;Fröhlich et al., 2008), which are associated with a prominent increase in $[K^+]_o$ (Heinemann & Lux, 1977;Heinemann et al., 1986;Fröhlich et al., 2008).
Table 1. Extracellular ([ion]_o) and intracellular ([ion]_i) ion concentrations and Nernst equilibrium potentials (E_{ion}) for a typical mammalian neuron. The experimental work presented in the Thesis was done under standard CO₂/HCO₃⁻ buffered conditions and under nominally CO₂ free, HEPES buffered conditions, where the GABA AR permeable weak acid anion HCO₃⁻ was replaced with formate, when necessary. Note that in CO₂/HCO₃⁻ solution the E_{Cl} is very close to E_K and represents the most negative E_{GABA} values encountered during the experiments. Such a low [Cl⁻]_i level approaches the limit that KCC2 mediated Cl⁻ extrusion can achieve (E_{K}=E_{Cl}).

<table>
<thead>
<tr>
<th></th>
<th>[ion]_o</th>
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<tr>
<td>CO₂/HCO₃</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na⁺</td>
<td>150 mM</td>
<td>10 mM</td>
<td>+72 mV</td>
</tr>
<tr>
<td>K⁺</td>
<td>110 mM</td>
<td>3 mM</td>
<td>−95.3 mV</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>131 mM</td>
<td>3.6 mM</td>
<td>−95.1 mV</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>25 mM</td>
<td>15.8 mM</td>
<td>−12 mV</td>
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<tr>
<td>Ca²⁺</td>
<td>2 mM</td>
<td>100 nM</td>
<td>+130 mV</td>
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<tr>
<td>pH</td>
<td>7.3</td>
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<tr>
<td>HEPES/formate</td>
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<tr>
<td>Na⁺</td>
<td>150 mM</td>
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<td>K⁺</td>
<td>110 mM</td>
<td>3 mM</td>
<td>−95.3 mV</td>
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<tr>
<td>Cl⁻</td>
<td>111 mM</td>
<td>10 mM</td>
<td>−63.7 mV</td>
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<tr>
<td>formate</td>
<td>20 mM</td>
<td>10 mM</td>
<td>−18 mV</td>
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<tr>
<td>Ca²⁺</td>
<td>2 mM</td>
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<td>pH</td>
<td>7.3</td>
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5.17 Molecular Characterization of CCCs

The mammalian solute carrier 12 family (SLC12) (Hebert et al., 2004; Gamba, 2005) can be divided into three branches, of which the first includes Na⁺ coupled Cl⁻ carriers and contains three members. Genes named SLC12A1, SLC12A2 and SLC12A3 are coding the Na⁺/K⁺/2Cl⁻ transport isoforms 2 and 1 (NKCC2 and NKCC1) and Na⁺/Cl⁻ (NCC) transporter, respectively. The second branch contains four genes named SLC12A4−7, which produce K⁺ coupled Cl⁻ transporters (KCC1 to KCC4). The third group contains two genes SLC12A8 and SLC12A9, so called orphan members of the family, which presumably do not promote ion transport. In coinjection and coimmunoprecipitation studies, the SLC12A9 product was shown to inhibit and bind to NKCC1, and was thus named Cotransporter Interacting Protein (CIP) (Caron et al., 2000). The subgroup SLC12A1−3 constitutes of proteins from 1002 to 1212 amino acids (Gamba, 2005), and SLC12A4−7 members contain 1011 to 1150 residues, with slight variation between species. SLC12 members may exhibit as low as 25% identity between their amino acid sequences, but the hydropathy analysis following the algorithm by Kyte and Doolittle suggests a structure common for all CCCs (Gamba, 2005). However, the secondary structure is confirmed only for NKCC1 (Gerelsaikhan & Turner, 2000). Apparently the folded CCC polypeptide has intracellular amino and carboxy terminals, which flank the central hydrophobic domain of 12 TM α-helixes. The location of a relatively large, extracellular hydrophilic loop differs. In Na⁺ coupled carriers the loop contains two to three putative N-glycosylation sites and is located between α-helixes 7 and 8, whereas in K⁺ coupled carriers the loop is larger, has four N-glycosylation sites and is located between TM segments 5 and 6 (Gamba, 2005). Splice variants are found for several CCCs (Mercado et al., 2004; Gamba, 2005; Uvarov et al., 2007) and all CCCs can assembly to hetero- and homo-oligomers, which may construct the functional transporters on the membrane (de Jong et al., 2003; Moore-Hoon & Turner, 2000; Blaesse et al., 2006; Simard et al., 2007; Uvarov et al., 2009; Watanabe et al., 2009).

5.18 Expression of CCCs in the mammalian CNS

The expression of several CCCs has been reported to take place in the mammalian central neurons (Delpire et al., 1994; Gillen et al., 1996; Payne et al., 1996; Mount et al., 1999) and more detailed expression pattern studies within neuronal structures have revealed that apparently NCC and NKCC2 are the only isoforms not present in the central nervous system (Plotkin et al., 1998; Delpire et al., 1994; Gillen et al., 1996; Payne et al., 1996; Mount et al., 1999).
5.18.1 NKCC1

Two functional isoforms NKCC1a and NKCC1b have been identified, both which are ubiquitous. The NKCC1b form seems to be more abundant in adult murine brain and lacks the protein kinase A consensus site present in NKCC1a (Randall et al., 1997). In the murine CNS NKCC1 mRNA and protein expression levels display abundant, but relatively faint distribution covering both neuronal and non-neuronal cell types (Plotkin et al., 1997a; Plotkin et al., 1997b; Kanaka et al., 2001; Hubner et al., 2001a). In a study by Plotkin et al. (1997a) NKCC1 was reported to be mainly restricted to the cerebellum, although mRNA levels from low to moderate were detected in neurons of nearly all brain areas examined. High levels were found in lateral reticular nucleus and inferior olive of the medulla as well as in the magnocellular preoptic nucleus of the diencephalon, suggesting that NKCC1 expression increases on a way down towards lower brain areas. Also Kanaka et al. (2001) found relatively low expression of NKCC1 mRNA in several brain regions, including hippocampal and cerebellar granular neurons.

Results describing the postnatal development and subcellular distribution of NKCC1 have been quite controversial. Plotkin et al. (1997b) reported increasing mRNA levels in cerebral cortex and hippocampus during the first week of postnatal development. The expression of NKCC1 was suggested to peak at around postnatal day (P) 7, after which it decayed towards adulthood. Accordingly, Hubner et al. (2001) reported that during the development of CNS, the expression of NKCC1 shifts gradually from neurons to glial cells, implying a down-regulation of neuronal NKCC1. However, the abovementioned, either biphasic or monophasic, decrease of NKCC1 was not observed by Clayton et al. (1998). In their study the NKCC1 mRNA levels were shown to increase throughout the first three postnatal weeks in the rat neocortex (Clayton et al., 1998). This opinion gained support from studies demonstrating a significant increase in NKCC1 expression after birth in rat hippocampus, especially in the DG region (Wang et al., 2002; Marty et al., 2002). In the work by Marty et al. (2002), the subcellular localization of NKCC1 protein was found to change from mainly somatic to predominantly dendritic, a notion which has been recently challenged by studies describing depolarizing GABA responses in the axonal initial segment, but not in the soma or dendrites of cortical neurones (Szabadics et al., 2006; Khirug et al., 2008).

Studies assessing the efficacy of functional Cl⁻ regulation have shown that the non-uniform expression of NKCC1 (and also other CCCs, Jarolimek et al., 1999) may generate intracellular Cl⁻
gradients (Khirug et al., 2008). As a consequence the [Cl\(^{-}\)]\(_i\) levels vary between subcellular structures and GABA may evoke unique, site-specific responses (for review, see Trigo et al., 2008; Blaesse et al., 2009).

Variable results have been explained with differences in antibody generation and probe sequences used in these studies. The region of mRNA and protein of NKCC1 that was detected by the probes and antibodies of Plotkin et al. (1997) was later found to be involved in alternative splicing (Randall et al., 1997), pointing out that the observed developmental diminution might actually reflect changes in the alternative splicing, not expression, of NKCC1 mRNA (Clayton et al., 1998). Recently a concern on the specificity of NKCC1 antibodies was raised, with a notion that the available antibodies might not be suitable for immunostainings (Zhang et al., 2006).

### 5.18.2 KCC1

In the CNS of adult rat, the expression pattern of KCC1 resembles that of NKCC1. KCC1 mRNA levels were reported to be low in most areas examined, but being strong in the non-neuronal cells of choroid plexus and in cerebellar granule cells (Kanaka et al., 2001). Albeit the KCC1 is mostly non-neuronal isoform, abundant but low expression levels have been detected in the granular and pyramidal cell layers of hippocampal regions from DG to CA3 and CA1 (Rivera et al., 1999), with modest postnatal up-regulation especially in the granule cell layer of DG (Clayton et al., 1998; Wang et al., 2002).

### 5.18.3 KCC2

In the study characterizing KCC2 at the molecular level, its expression was shown to be abundant within the CNS and restricted to neurons only (Payne et al., 1996), an observation that has been confirmed in several reports (Rivera et al., 1999; Lu et al., 1999a; Uvarov et al., 2005). A detailed analysis of KCC2 distribution within hippocampus revealed its localization on the dendritic membranes of pyramidal cells and interneurons, being strongest in the stratum oriens of CA1 and CA3 and in the stratum moleculare of DG (Lu et al., 1999b; Gulyas et al., 2001). A somewhat surprising finding was that the KCC2 is localized heavily on dendritic spines, known to be the main targets of excitatory synaptic inputs to pyramidal cells (Gulyas et al., 2001). This apparent paradox was resolved when the KCC2 was identified as an essential structural protein affecting the spine morphology independent of its ion transport function (Li et al., 2007).
Although the expression of KCC2 is restricted to neurons only, not all neurons in the CNS express KCC2. Apparently the parvalbumin containing neurons of thalamic reticular nucleus and the dopaminergic neurons of substantia nigra pars compacta are devoid of any detectable KCC2 expression (Bartho et al., 2004; Gulacsi et al., 2003). Also the parvalbumin containing neurons of paraventricular and suprachiasmatic nuclei of hypothalamus seem to lack KCC2 expression (Kanaka et al., 2001). The expression of KCC2 increases robustly during the first week after birth, and changes have been reported to be most dramatic in hippocampal and neocortical regions (Rivera et al., 1999; Lu et al., 1999a). Of the recently identified variants KCC2a and KCC2b, the latter is responsible for the “developmental shift” in $E_{\text{GABA}}$ in CNS neurons, but there is little information of the functional role(s) of KCC2a (Uvarov et al., 2007; Blaesse et al., 2009).

### 5.18.4 KCC3

In three articles characterizing the KCC3 ion transporter, two sequence lengths and protein sizes were described (Hiki et al., 1999; Mount et al., 1999; Race et al., 1999). In addition to the size of KCC3 protein, the reported level of expression in the CNS varied a lot. Descriptions written were “very little KCC3 in brain” by Mount et al., (1999), “strong expression was observed in brain” by Hiki et al., (1999), and “most abundant in heart, brain,...” by Race et al., (1999). A partial explanation to this discrepancy came when the two splice variants KCC3a and KCC3b were noticed to have different expression patterns. A larger variant KCC3a was reported to be the form dominantly expressed in brain whereas KCC3b was most abundant in kidney (Pearson et al., 2001; Le Rouzic et al., 2006). KCC3a has also two splice variants and apparently the shorter form seems to have a neuron specific expression pattern (Le Rouzic et al., 2006).

The cell-type expression patterns of KCC3 within CNS are currently inconsistent. In one study the KCC3 was described to be concentrated mainly to the oligodendrocytes of myelinated tracts of the spinal cord. Also white matter tracts in the brain (e.g. corpus callosum) were shown to display strong expression of KCC3 (Pearson et al., 2001), (but see, Howard et al., 2002; Boettger et al., 2003). The KCC3 expression has been detected in somata of large cortical neurons, hippocampal CA1 pyramidal neurons, cerebellar Purkinje neurons, dorsal root ganglion neurons and in the base of the choroid plexus epithelium (Pearson et al., 2001; Howard et al., 2002; Boettger et al., 2003). Like KCC2, the expression of KCC3 is up-regulated during postnatal development (Pearson et al., 2001; Boettger et al., 2003).
5.18.5 KCC4

KCC4 expression seems to be relatively low within CNS (Mount et al., 1999) and is localized mainly to cranial nerves and nuclei and to the suprachiasmatic nucleus of rostral hypothalamus (Karadsheh et al., 2004; Le Rouzic et al., 2006). In general the expression of KCC4 is practically absent in forebrain region, increases toward hindbrain and spinal cord and extends also to peripheral nerves. The large cells of cranial nerves and oligodendrocytes were labelled with KCC4. In peripheral nervous system KCC4 was concentrated in the close vicinity of nodes of Ranvier, indicating a strong expression in Schwann cells (Karadsheh et al., 2004). In clear contrast to the expression profiles of all other CCCs expressed in the CNS, a decrease in KCC4 expression starts already before birth in rodents and continues towards adulthood (Li et al., 2002; Karadsheh et al., 2004).

5.19 Regulation of NKCC and KCC activity

The function of NKCCs and KCCs is affected by kinases and phosphatases. Early work described that increased protein phosphorylation levels correlate positively with enhanced ion transport via NKCC (Lytle & Forbush, III, 1992) and negatively with that via KCC (Jennings & Schulz, 1991). However, the link between increased protein phosphorylation and increased activity of NKCC (or decrease in the activity of KCC) might not to be as straight forward as previously thought. In addition to the transport rate of CCCs, the kinetic regulation mediated by kinases and phosphatases affects both the protein trafficking and stability on the cell membrane (Blaesse et al., 2009). Members of the with-no-lysine kinase family (WNK) and two members of the sterile 20 (STE20) kinase family named STE20-related proline-alanine-rich kinase (SPAK) and oxidative stress responsive kinase 1 (OSR1) have now emerged as candidates regulating the functions of all SLC12 family members (Kahle et al., 2006; Delpire & Gagnon, 2008; Kahle et al., 2008). Currently it is believed that the formation of WNK1/OSR1 and WNK4/SPAK complexes results in a direct phosphorylation of CCCs. WNK3 instead might mediate its effects via inactivation of protein phosphatases. The interactions between regulatory proteins and ion carriers have been resolved in greatest detail for NKCC1. For all other isoforms the information available is more fragmented.

5.19.1 Regulatory kinases of NKCC and KCC

The existence of volume and [Cl⁻]i sensitive kinase(s) reciprocally modulating NKCC and KCC activities have been hypothesised for long. The intense quest for the regulatory proteins of CCCs
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has now resulted in identification of several effectors. Following the method of yeast two hybrid screening, SPAK/OSR1 kinases were shown to interact with KCC3 and NKCC1, but not with KCC1 and KCC4 (Piechotta et al., 2002). The identified SPAK kinase can activate the p38 pathway, known to mediate stress activated signals (Johnston et al., 2000). Expression studies in HEK−293 cells demonstrated that the kinase inactive SPAK mutant dramatically reduced NKCC1 activity, brought about by hypotonicity or low [Cl]$^-$, suggesting a role of SPAK on NKCC1 function. However, the sensitivity of NKCC1 to changes in intracellular chloride was not significantly affected by the expression of wild type SPAK (Dowd & Forbush, 2003). Also in an oocyte expression study, mutations on binding sites preventing the SPAK and NKCC1 interactions were without effect, which calls into question the direct impact of SPAK on NKCC1 function (Piechotta et al., 2003).

The upstream regulators of SPAK and OSR1 were identified as members of the WNK family. Both WNK1 and WNK4 were reported to phosphorylate and activate SPAK and OSR1, suggesting an increase in NKCC1 activity (Moriguchi et al., 2005; Vitari et al., 2005). Supporting this conclusion, expression studies performed in oocytes revealed that the activity of WNK4 and SPAK did lead to the NKCC1 stimulation in isosmotic conditions. In addition the active forms of WNK4 and SPAK reduced the K$^+$ uptake mediated by KCC2 both in iso- and hypo-osmotic conditions (Gagnon et al., 2006).

Contrasting the results above the co-expression of WNK4 with NKCC1 in oocytes led to a dramatic decrease in transport activity due to the reduced surface expression (Kahle et al., 2004). In a subsequent work WNK3 was found to co-localize with NKCC1, KCC1 and KCC2 and promote their phosphorylation. Functional expression studies in oocytes demonstrated that the WNK3 increased NKCC1 and decreased KCC1−2 activity, leading to enhanced Cl$^-$ influx. An inactive WNK3 mutant had the opposite effects, increasing Cl$^-$ efflux by reducing NKCC1 and promoting KCC1−2 activity in hypotonic and isotonic conditions (Kahle et al., 2005). The experiments of Gagnon et al. (2006) and Kahle et al. (2004, 2005) were done using nearly identical protocols and no obvious reasons were found to explain the contrasting results.

Recently Garzón-Muvdi et al. (2007) demonstrated that the WNK4 is a negative regulator of swelling activated KCC1, 3 and 4. The catalytically inactive form of WNK4 was able to activate KCC2 and KCC3, but not KCC1 or KCC4 mediated transport in isotonic conditions. The inhibition of K$^+$/Cl$^-$ cotransport by WNK4 and its activation by inactive WNK4 were independent of SPAK (Garzon-Muvdi et al., 2007).
5.19.2 Regulatory kinases found in CNS

Of the WNK kinases, WNK3 isoform has been shown to be highly present in the mouse brain. Prominent antisense probe staining was detected in hippocampus, dentate gyrus and moderate staining in cortical layers and in thalamic and hypothalamic nuclei. WNK3 was found in neurons but not in glial cells, and its transcription increased gradually after birth, being absent during the first postnatal week and reaching high levels by postnatal day 21 (Kahle et al., 2005). Also both SPAK and OSR1 have been found in brain tissue, where SPAK expression seems to be higher relative to other tissue types (Ushiro et al., 1998; Tamari et al., 1999). Results of more detailed distribution of SPAK have been somewhat controversial; Ushiro et al. (1998) found SPAK immunoreactivity from large midbrain neurons and cerebellar Purkinje cells, whereas Piechotta et al. (2003) showed significant labelling in neurons and glial cells at the brainstem level, but not structures rostral to that. The expression of WNK4 was first reported to be restricted to kidneys only, whereas WNK1 was identified also from skeletal muscle and heart (Wilson et al., 2001a). Later WNK4 was detected also in extrarenal tissues, where it was found almost exclusively in polarized epithelia (Kahle et al., 2004).

5.19.3 Kinetic regulation of NKCC

A high intracellular Cl\(^-\) content has a strong inhibitory effect on the NKCC mediated coupled fluxes of Na\(^+\) and K\(^+\) in the squid giant axon (Russell, 1979; Russell, 1983). The inhibitory effect of high [Cl\(^-\)]\(_i\) is not surprising as such, as the increase in [Cl\(^-\)]\(_i\) reduces the net free energy of NKCC ion uptake. If the changes in ion gradients would be the sole explanation, then a sufficiently high increase in [Cl\(^-\)]\(_i\) or the removal of extracellular Cl\(^-\) should stimulate efflux. However, it turned out that a large increase in [Cl\(^-\)]\(_i\) completely inhibited both influx and efflux (Breitwieser et al., 1996). The removal of extracellular Cl\(^-\) did not increase effluxes mediated by NKCC either. Similar results demonstrating that the high level of [Cl\(^-\)]\(_i\) inhibit the activity of NKCC under conditions, which would otherwise promote ion uptake, have been obtained also in other preparations (Levinson, 1990).

In their pioneering study describing nearly all the basic properties of NKCC in Ehrlich cells, Geck et al. (1980) showed that NKCC mediated ion transport requires the presence of ATP. The thermodynamic considerations of carrier process led to a conclusion that the chemical gradients of translocated ions could not provide enough energy to support ion transport. However, the inhibition of NKCC transport did not have any ATP sparing effect, and thus they could not unequivocally
state whether NKCC is primary or secondary active. Even so, they concluded that although cellular ATP is needed, the hydrolysis of ATP is a very implausible source of energy. The role of cellular ATP in NKCC transport was studied also using internally dialyzed squid giant axons. In conditions where other ion gradients were kept constant, the reduction of ATP below 10µM inhibited the ion transfer in a fully reversible manner (Russell, 1983). Studies describing that the activity of NKCC increases in response to phosphatase inhibitors and decreases in response to broad spectrum kinase inhibitors, (e.g. Pewitt et al., 1990) suggested that ATP is used for NKCC phosphorylation. This was confirmed in a study showing that a direct phosphorylation of the transport protein was associated with the activation of ion transport (Lytle & Forbush, III, 1992). An analysis of $^{32}$P-labeled amino acids and peptide sequences revealed that in response to various activators, the same amino terminal sites of NKCC were phosphorylated (Lytle, 1997). Careful phosphopeptide and phosphorylation stoichiometry analysis of maximally stimulated NKCC1 demonstrated that ~3 phosphates per cotransporter protein were incorporated (Darman & Forbush, 2002) and a heroic experimental effort revealed three amino-terminal threonines, located at amino acid numbers 184, 189 and 202 as phosphoacceptor sites.

### 5.19.4 Kinetic regulation of KCC

The swelling induced erythrocyte KCC transport was first shown to be strongly suppressed by protein phosphatase inhibitor Okadaic acid (Jennings & Schulz, 1991) and the accumulation of data describing that all known KCC isoforms are activated by dephosphorylation has been substantial. Expression studies done in *Xenopus* oocytes or in HEK–293 cells revealed that KCC1 isoform behaves much like K⁺/Cl⁻ transport in erythrocytes (Gillen & Forbush, III, 1999;Su et al., 1999). Hardly any fluxes mediated by KCC1 were detected in isotonic conditions and the activation of transport was brought about by NEM treatment or hypotonic conditions. Protein phosphatase inhibitors prevented the activation of KCC1 transport (Su et al., 1999). In a study comparing the functional properties of KCC1 and KCC4 several inhibitors were tested to identify protein phosphatase (PP) candidates involved in KCC regulation (Mercado et al., 2000). Calyculin A, affecting both PP1 and PP2A, prevented the swelling-induced activation of both KCC1 and KCC4. Okadaic acid, a specific PP2A inhibitor, or cypermethrin blocking only PP2B had no effect, suggesting that the PP1 is most likely responsible for KCC activation (Mercado et al., 2000). As pointed out by Mercado et al. (2000), the efficacy of swelling induced activation seems to be much larger in oocytes, suggesting that HEK–293 cells lack the signalling mechanisms required for full activation. Preparation specific differences have also been described for the neuronal KCC2. In a
study comparing the KCC2 mediated build-up of somatodendritic chloride gradient in cells of acute hippocampal slices and in cultured neurons, it was shown that in the latter preparation the activation of KCC2 was delayed compared to the protein expression and could be released only after protein kinase inhibition (Khirug et al., 2005). However, the general idea that K⁺/Cl⁻ cotransport is invariably promoted by dephosphorylation was challenged by a study showing that the stimulation of IGF–1 receptors led to the phosphorylation and activation of KCC2 in cultured neurons (Kelsch et al., 2001). Manipulations leading to a reduction in the KCC2 protein level and transport rate (BDNF, 0–Mg²⁺ and H₂O₂ incubations) in cultured hippocampal neurons were associated with the reduction of phosphorylated form of KCC2 (Wake et al., 2007). Apparently the membrane stability of functional KCC2 increases in response to a direct phosphorylation (Lee et al., 2007; Watanabe et al., 2009).

5.20 Activity dependent regulation of NKCC and KCC expression

A decrease in the efficacy of GABAAR mediated inhibition is often seen following patophysiological activity (Kapur & Coulter, 1995; Whittington et al., 1995; Kohling et al., 2000). Alterations in the functionality of ion transporters controlling intracellular chloride level are apparently contributing to the diminution of GABAergic inhibition, as a reduced Cl⁻ extrusion capacity of neurons is often associated with the development of epileptic activity (Traub et al., 1999; Huberfeld et al., 2007). However, only few studies have been performed to explain signalling mechanisms controlling CCC expression in adult neurones (Payne et al., 2003).

The expression of KCC2 in hippocampus is regulated by Brain Derived Neurotrophic Factor (BDNF) and tyrosine receptor kinase B (TrkB) engaged signalling cascades (Rivera et al., 2002). Massive release of BDNF evoked by kindling led to a down-regulation of KCC2 mRNA and protein levels in vivo. Comparable results were obtained in hippocampal in vitro slices by using an exogenous application of TrkB agonists BDNF or neurotrophin–4 (NT–4) (Rivera et al., 2002). A more detailed study of activity dependent regulation of KCC2 expression was performed utilizing a mouse strain having point mutations in the docking sites of downstream effectors of TrkB (Rivera et al., 2004). Apparently pathways mediated via phospholipase Cγ/cAMP response element binding protein (PLCγ/CREB) and via src homology 2 domain containing transforming protein/fibroblast growth factor receptor substrate 2 (Shc/FRS–2) are engaged in parallel to down-regulate KCC2, whereas the activation of Shc/FRS–2 cascade leads to up-regulation (Rivera et al., 2004). The effects of BDNF and TrkB signalling on KCC2 expression may depend on the developmental stage as in mice over-expressing BDNF, the perinatal KCC2 levels were higher than in control animals.
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(Aguado et al., 2003), suggesting that the Shc/FRS–2 pathway dominates before birth. Neuronal trauma leading to a down-regulation of KCC2 and depolarizing GABA responses seem to re-establish the juvenile mode of intracellular signalling cascades, as axotomized cells responded to BDNF by increasing KCC2 expression (Shulga et al., 2008).

5.21 Activity dependent changes in chloride and potassium gradients

The intense activation of GABA\(_A\)Rs on mature hippocampal pyramidal neurons can cause biphasic membrane potential responses, which first hyperpolarize and then depolarize cell membrane (Alger & Nicoll, 1979; Andersen et al., 1980; Lambert et al., 1991; Grover et al., 1993; Staley et al., 1995; Kaila et al., 1997; Smirnov et al., 1999). The interdependent shifts of Cl\(^-\) and HCO\(_3^-\) via GABA\(_A\)Rs during prolonged activation were first resolved using a classical crayfish muscle fibre preparation (Kaila & Voipio, 1987; Kaila et al., 1989). Ion selective microelectrode recordings demonstrated that a prolonged GABA application increased intracellular Cl\(^-\) concentration ([Cl\(^-\)]\(_i\)) and decreased intracellular pH (pH\(_i\)). Both phenomena required the presence of HCO\(_3^-\) in the bath solution, revealing the role of bicarbonate conductance in the shaping of intracellular GABA\(_A\)R mediated responses. Due to the relatively stable transmembrane HCO\(_3^-\) gradient \(E_{GABA}\) remained more depolarized than \(E_{Cl}\), favouring the Cl\(^-\) influx as long as GABA\(_A\)Rs were in conducting state and led to a profound increase in intracellular Cl\(^-\) (Kaila et al., 1989). The paradoxical biphasic GABAergic responses encountered in rat hippocampal neurons were subsequently explained analogously by a collapse of Cl\(^-\) gradient, arising from HCO\(_3^-\) dependent intracellular Cl\(^-\) accumulation (Staley et al., 1995; Kaila et al., 1997). The neuronal GABAergic transients are associated with other ionic shifts comparable to those found in crayfish muscle fibre, including an increase in extracellular pH (pH\(_o\)) due to the HCO\(_3^-\) efflux (Kaila et al., 1992; Chen & Chesler, 1992).

In addition to the transmembrane anion shifts, the robust GABA\(_A\)R activation increases extracellular potassium concentration ([K\(^+\)]\(_o\)) in nervous tissue (Morris et al., 1996; Kaila et al., 1997; Smirnov et al., 1999). Both the activity dependent accumulation of intraneuronal chloride (Grover et al., 1993; Kaila et al., 1997; Staley et al., 1995) and the increase in interstitial potassium are dependent on transmembrane HCO\(_3^-\) gradient which is maintained by the intracellular carbonic anhydrases (Kaila et al., 1997; Voipio & Kaila, 2000; Ruusuvuori et al., 2004). As the Cl\(^-\) extrusion of mammalian central neurons is fuelled by a transmembrane K\(^+\) gradient (Thompson et al., 1988; Payne, 1997) the outwardly directed transport is suppressed (or might reverse) when
challenged by an increase in $[K^+]_o$, leading to an increase in $[Cl^-]_i$ and more depolarized GABA$_A$R responses (Jensen et al., 1993; Payne, 1997; Jarolimek et al., 1999). On the contrary, a decrease in $[K^+]_o$ enhances the efflux of $Cl^-$ and hyperpolarizes GABA responses (Thompson & Gahwiler, 1989).

The increase in extracellular $K^+$ level (Somjen & Giacchino, 1985; Traynelis & Dingledine, 1988) and changes in transmembrane $Cl^-$ gradients (Fujiwara-Tsukamoto et al., 2003; Bihi et al., 2005) are intimately involved in the appearance of epileptiform activity patterns. The high frequency stimulation induced, slow GABAergic depolarization of pyramidal cells, augmented by elevated $[K^+]_o$, is thought to participate in the generation of hyper-synchronous field potential oscillations (Kaila et al., 1997; Bracci et al., 1999; Traub et al., 1999), but the mechanisms leading to the increase in $[K^+]_o$ have not been resolved yet.
6 AIMS OF THE STUDY

This Thesis examines the chloride regulation mechanisms and GABAergic inhibition of rat neurons. The dopaminergic and GABAergic neurons of substantia nigra receive extensive GABA_A receptors mediated inputs which evoke hyperpolarizing responses (Lacey et al., 1989; Hajos & Greenfield, 1994). However, the dopaminergic neurons are less inhibited by GABA (Grace & Bunney, 1979; Waszczak et al., 1980; Celada et al., 1999). Our hypothesis was that the chloride extrusion of dopaminergic cells is less efficient than that of GABAergic cells because the chloride regulatory mechanisms differ. The main target was to identify the molecular components and properties of chloride transport processes, which set the GABA_A reversal potential in dopaminergic and in GABAergic neurons (I).

Patophysiological activity induces alterations in the chloride extrusion capacity of central neurons (Traub et al., 1999; Huberfeld et al., 2007). We were set out to reveal the activity engaged molecular cascades, which lead to an increase in intracellular chloride and to a diminution of GABA_A mediated responses in hippocampal pyramidal cells. BDNF/TrkB initiated signalling pathways and their effects on the mRNA and protein levels of KCC2 were addressed. The effects that changes in KCC2 protein levels had on the efficacy of neuronal Cl^- extrusion were studied with electrophysiological means (II).

We then focused on the mechanisms of GABAergic extracellular K^+ responses. The generation of high frequency stimulation induced excitatory K^+ transients were known to depend on HCO_3^- and functional carbonic anhydrase (CAVII) (Kaila et al., 1997; Voipio & Kaila, 2000; Ruusuvuori et al., 2004), but the molecular device(s) extruding potassium to extracellular space were not known. The hypothesis was that KCC2, accelerated by the rapid GABA_A mediated increase in intracellular Cl^-, is responsible for the generation of K^+ responses.

Ion substitution experiments and a various pharmacological treatments were used in combination to isolate the mechanism under study. GABA_A activation was achieved with high frequency stimulation and with pressure, iontophoretic or bath-applied agonist applications. The Cl^- influx rate via GABA_ARs was quantified on the basis of intracellular recordings and the contribution of KCC2 to extracellular K^+ responses was addressed with furosemide (III).
# MATERIALS AND METHODS

## Methods

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**Brain sections prepared for electrophysiology**

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*Table 2.* List of methods used in the parts I–III of the Thesis, in an alphabetical order. Asterisk is used to indicate methods with which most or all of the data presented in the original papers were produced by the author. Detailed descriptions of the methods are listed in the original publications.
7.1 Electrophysiological methods performed by the Author

The methods utilized by the author in Parts I–III of the Thesis are listed in Table 2. More detailed descriptions are given below.

All experiments were performed on rat hippocampal or midbrain slices prepared from animals sacrificed at P15–P35. Rats were anesthetized using an intraperitoneal injection of pentobarbital (40–80mg kg$^{-1}$) or ketamine (100mg kg$^{-1}$). Before preparation of midbrain slices, animals were transcardially perfused with 4–5ml of ice-cold modified Ringer’s solution containing 225mM sucrose, 2.5mM KCl, 0.5mM CaCl$_2$, 7mM MgCl$_2$, 28mM NaHCO$_3$, 1.25mM NaH$_2$PO$_4$, 7mM glucose, 1mM ascorbate, 3mM pyruvate, bubbled with 95% O$_2$/5% CO$_2$. After decapitation the brain was quickly removed and placed into the solution described above (midbrain slices) or to a solution containing 124mM NaCl, 3mM KCl, 2mM CaCl$_2$, 25mM NaHCO$_3$, 1.1mM NaH$_2$PO$_4$, 2mM MgSO$_4$ and 10mM Glucose (hippocampal slices). Slices of 350–400µm thicknesses were cut from a block containing the region of interest and quickly transferred into a nylon mesh container that was submerged to a solution containing 125mM NaCl, 2.5mM KCl, 1.25mM NaH$_2$PO$_4$, 25mM NaHCO$_3$, 1mM MgCl$_2$, 2mM CaCl$_2$, 25mM glucose, 1mM ascorbate, 3mM pyruvate and 0.4mM myo-inositol (midbrain slices) or to a solution containing 124mM NaCl, 3mM KCl, 2mM CaCl$_2$, 25mM NaHCO$_3$, 1.1mM NaH$_2$PO$_4$, 2mM MgSO$_4$ and 10mM Glucose (hippocampal slices). Slices were let to recover for half an hour at 32–34°C (then placed to a room temperature) and continuously bubbled with 95% O$_2$/5% CO$_2$ gas mixture, pH 7.4.

Recordings were performed in a submerged type recording chamber (vol 800µl), heated to 33–34°C, where slices were continuously perfused on both sides with the solutions described above at the flow rate of 2.0–2.4ml min$^{-1}$ (or 4.7ml min$^{-1}$, bath-applied agonist experiments). Measurements were done within pars compacta or pars reticulata of the substantia nigra, or within stratum pyramidale (sp) or stratum radiatum (sr) of the hippocampal CA1 region. Extracellular field potentials were recorded using glass capillary microelectrodes (filling 150mM NaCl, 3mM KCl, R$_e$ 2–5MΩ) and interstitial potassium transients were recorded using valinomycin-based membrane solution (Cocktail B; Fluka) as the ion selective sensor in the tip of the silanized single barrel microelectrode, backfilled with 150mM NaCl, 3mM KCl, (Voipio et al., 1994). The procedure of making tetramethylammonium (TeMA$^+$) selective electrodes was identical with that of K$^+$ electrodes, but the sensor membrane contained 100mg of potassium tetrakis (4-chlorophenyl) borate in 2ml of 1,2-dimethyl-3-nitrobenzene and the backfilling solution was 150mM TeMA–Cl.
A hydraulic double electrode holder (Narishige HDO−20, Tokyo, Japan) was used to position the tips of the reference and ion selective microelectrodes within a nominal distance of 10µm. K⁺ and TMA⁺ selective electrodes were calibrated before and after each experiment using 150mM NaCl solutions containing 3.0mM KCl, 1.5mM TeMA−Cl and 6.0mM KCl, 1.0mM TeMA−Cl. Sharp intracellular electrodes (resistance 90−220MΩ) were filled either with 1M K-Acetate, 5mM KCl solution or with 0.5M K-Acetate, 0.5M KCl solution (pH 6.6−6.8). Whole-cell patch pipettes (5−8MΩ) were filled with a solution containing 130mM K-Gluconate, 8mM KCl, 4mM MgATP, 0.3mM NaGTP, 0.5mM EGTA, 2.1mM KOH, 8mM NaOH and 10mM HEPES (pH 7.3). Osmolarity was adjusted to 285mosm by adding ~17mM sucrose. The liquid junction potential of whole-cell recordings was not corrected.

To elicit potassium transients in the CA1 hippocampal region, four methods were used. In the first method a specific GABAAR agonist isoguvacine hydrochloride was applied via superfusion (2−200µM isoguvacine for 30−60s, interval 15−30min, perfusion flow rate 4.7ml min⁻¹). In the second stimulation paradigm, puffs of GABA (10mM) or isoguvacine hydrochloride (2−10mM), both drugs dissolved in the HEPES buffered physiological solution, were pressure injected (60−150kPa, duration 0.25−3s) every 10−15min via a glass capillary microelectrode (tip Ø 2−4µm) positioned close to the recording site. The third approach utilized an iontophoretic muscimol application (10mM muscimol in 10mM HCl, application current from +50 to +100nA, duration 2−4s and interval 10−15min, on top of a continuous backing current of −1 to −5nA to prevent leakage) via microelectrodes (tip Ø 2−4µm) located near the recording area. The fourth method used a bipolar stimulation electrode, placed in the vicinity of the border between stratum radiatum and stratum pyramidale near (≤500 µm) the recording site. The stimulus intensity was set to elicit a population spike of ~5mV in response to a single pulse of 3−20V/50µs. Trains of stimuli (40 pulses at 100Hz) delivered every 10−15min were used to trigger field potential afterdischarges and extracellular K⁺ transients.

GABAAR responses were isolated pharmacologically using antagonists of AMPA/kainate glutamate receptors, 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt, (NBQX, 10µM), NMDA glutamate receptors, DL-2-Amino-5-phosphonopentanoic acid (DL-AP5, 40µM) and GABAB receptors, (2S)-3-[[1S)-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl] (phenylmethyl) phosphinic acid, (CGP55845, 1µM) (Davies et al., 1990). In the agonist application experiments (GABA, isoguvacine, muscimol) voltage activated Na⁺ channels were blocked with tetrodotoxin (TTX, 0.5−1µM). Furosemide (0.5−1mM) was used to inhibit KCC2 mediated transport, but it blocks also NKCC1 at these concentrations. 3-(aminosulfonyl)-5 (butylamino)-4-
MATERIALS AND METHODS

Phenoxybenzoic acid (bumetanide, 10µM) was applied to selectively inhibit NKCC1 mediated ion fluxes. GABAA receptor antagonists bicuculline methiodide (BMI, 10µM) and picrotoxin (PiTX, 100µM) were routinely applied at the end of each experiment. In those instances when Ba2+ (1–2mM) was added to block K+ channels 2mM MgSO4 was replaced with 2mM MgCl2 to prevent the precipitation of insoluble salts (Alger & Nicoll, 1980). In recordings where tetramethylammonium (TeMA+) sensitive microelectrodes were used, 1.5mM TeMA–Cl was added to the standard physiological solution. Nominally HCO3− free conditions were obtained by replacing 25mM HCO3− with 20mM HEPES (pH 7.4 with NaOH) and the 95% O2/5% CO2 mixture with 100% O2. When 20mM formate was used as GABAAR permeant weak acid anion in nominally HCO3− free conditions, the Cl− concentration was reduced by an equal amount (Lamsa & Kaila, 1997), decreasing the total Cl− concentration from 131mM to 111mM.

The intracellular chloride concentrations ([Cl−]i) and instantaneous rates of chance (d[Cl−]/dt, (mM s−1)) were solved from the Goldman-Hodgkin-Katz (GHK) voltage equation (Hille, 2001), by first arranging it for [Cl−]i and then taking the time derivative of both sides;

\[
E_{\text{GABA}} = \frac{RT}{F} \ln \left( \frac{[C{l}^{-}]_o + p[A^{-}]_o}{[C{l}^{-}]_o + p[A^{-}]_o} \right) \Leftrightarrow [C{l}^{-}]_i = \left( [C{l}^{-}]_o + p[A^{-}]_o \right) e^{\frac{F}{RT}E_{\text{GABA}} - p[A^{-}]} - p[A^{-}]
\]

\[
\frac{d[Cl^{-}]}{dt} = \frac{F}{RT} \left( [Cl^{-}]_o + p[A^{-}]_o \right) e^{\frac{F}{RT}E_{\text{GABA}}} \frac{dE_{\text{GABA}}}{dt}.
\]

The calculations were made using membrane potential values during HFS, from 3rd IPSP (E3rd) to last IPSP (Elast), representing a change in E\text{GABA} (dE\text{GABA}/dt, (mV s−1)), see Fig. 4 a), together with the following concentrations of GABAAR permeable extracellular anions: 131mM [Cl−]o and 25mM [HCO3−]o, 111mM [Cl−]o and 20mM [formate]o. The relative permeability values (p) used were 0.2 for HCO3− and 0.5 for formate (Bormann et al., 1987). R, T and F have their usual meaning.

As the slice interior is slightly more acidic than the perfusate due to the metabolic CO2 production (Voipio & Kaila, 1993), the extracellular pH (pHc) of 7.3 was used in calculations. The 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) fluorescence measurements of intracellular pH (pHi) demonstrated a steady state pH gradient (pHc−pHi) of −0.2 units in the CO2/HCO3− solution (pHi 7.11±0.06, mean±S.D.) and −0.3 units in the HEPES/formate solution (pHi 7.02±0.07, mean±S.D., p<0.001, n=58 cells, 3 slices, not illustrated). The transmembrane distribution of weak acid anions ([A−]i) was expected to obey pH gradient;
$[A^-] = [A^{-}]_{10^{pH-pH_o}}$, ending to $[\text{HCO}_3^-]_i$ of 15.77mM and to $[\text{formate}]_i$ of 10.02mM. The transmembrane $\text{HCO}_3^-$ gradient was assumed to remain constant, although stimulation paradigms comparable to those used in this study have been reported to produce a transient increase in pH_o of about 0.1 units, due to the $\text{HCO}_3^-$ efflux (Kaila et al., 1992; Voipio et al., 1995).

The intracellular voltage and whole-cell current responses were delineated utilizing the Goldman-Hodgkin-Katz constant field theory (Hille, 2001), and the equations written for $\text{CO}_2/\text{HCO}_3^-$ conditions are shown below;

$$E_m = \frac{RT}{F} \ln \left( \frac{P_{\text{Cl}^{-}i} [\text{Cl}^-] + P_{\text{HCO}_3^-i} [\text{HCO}_3^-] + P_{\text{Na}^+i} [\text{Na}^+] + P_{\text{K}^+i} [\text{K}^+]}{P_{\text{Cl}^{-}o} [\text{Cl}^-] + P_{\text{HCO}_3^-o} [\text{HCO}_3^-] + P_{\text{Na}^+o} [\text{Na}^+] + P_{\text{K}^+o} [\text{K}^+]} \right)$$

$$I_{ion} = P_{ion} z_{ion} E F^2 \frac{[ion] - [ion]_0 e^{-z_{ion} F E / R T}}{1 - e^{-z_{ion} F E / R T}}$$

$$I_w = I_K + I_{Na} + I_{Cl} + I_{HCO}_3$$

where $E_m$ is the membrane voltage given by the voltage equation and $E$ is the predefined voltage at which the $I_{ion}$ is calculated. $P_{ion}$ and $z_{ion}$ denote ion permeability (cm s$^{-1}$) and valency, respectively.

At steady state the transmembrane ion gradients were assumed to be as listed in Table 1, $\text{Na}^+$ and $\text{HCO}_3^-$ or formate gradients remained constant. $P_{ion}$ values, $[\text{Cl}^-]_i$ and $[\text{K}^+]_o$ levels were adjusted to obtain $E_m$ and $I_m$ roughly corresponding recorded data. The resulting $E_m$ traces were analysed as $V_m$ recordings. To this end, the $E_{3nd}$, $E_{fast}$ and $E_{\text{GABA}}$ slope ($dE_{\text{GABA}}/dt$, (mV s$^{-1}$)) of each $E_m$ trace was incorporated to the modified GHK voltage equation of $\text{GABA}_A$ responses (see p. 49) to get the $[\text{Cl}^-]_i$ and $d[\text{Cl}^-]_i/dt$ values.

All salts were from Fluka (Buchs, Switzerland). Drugs were from Tocris-Cookson (Bristol, UK), except bumetanide and furosemide were from Sigma (St.Louis, MO). Drugs were prepared as stocks $10^3$ times the final concentration, dissolved in 150mM NaCl 3mM KCl (BMI, CGP55845, NBQX, TTX), or in 40mM NaOH (DL-AP5), or in DMSO (bumetanide, furosemide, PitX). Solutions containing furosemide or PitX were sonicated for 20min prior to use.

Recorded data were low pass filtered at 1.6–2.5kHz (field potential, intra- and whole cell measurements) and 160Hz ($[\text{K}^+]_o$), digitized at 5kHz and stored to a hard disk of personal computer.
using DAQCard-6024E A/D interface (National Instruments Corp.) together with WinEDR V2.7.7 and WinWCP V3.7.8 software (courtesy of Dr. John Dempster, University of Strathclyde, Glasgow, UK). Data were analysed using WinEDR and WinWCP software, graphed and plotted with Sigma Plot 10.0 (Systat Software, Inc.) and Microsoft Office Excel 2003 (Microsoft Corp.) software. Data are expressed as mean±S.E.M., unless otherwise stated and statistical significance was tested with Student’s t-test.

7.2 A comment on pharmacology used on GABA\_ARs and CCCs

In assays based on the isolated protein furosemide and bumetanide have been shown to inhibit carbonic anhydrase (CA) activity (Supuran & Scozzafava, 2000;Temperini et al., 2009). It has also been suggested that at least in red blood cells bumetanide may block Na\(^+\) dependent Cl\(^–\)/HCO\(_3\)\(^–\) exchange as well (Brazy & Gunn, 1976). In addition, GABA\_AR types containing α4 or α6 subunits are significantly inhibited by furosemide in a sub-millimolar range (Korpi et al., 1995;Wafford et al., 1996;Thompson et al., 1999). Furosemide has been shown to reduce GABA\_AR mediated synaptic responses in hippocampal pyramidal neurons (Pearce, 1993), (but see Thompson & Gahwiler, 1989), in neocortical principal neurons (Ing & Poulter, 2007) and in interneurons (Bertrand & Lacaille, 2001). I also noted that furosemide reduced the GABA\_AR conductance of CA1 pyramidal neurons, activated with single pulse electrical stimulation or agonist iontophoresis. However, the quantification of intracellular Cl\(^–\) accumulation rate indicated that the high frequency stimulation induced increase in [Cl\(^–\)]\(_i\) appeared to be relatively insensitive to furosemide.

The pharmacological isolation of a “pure” GABA\_AR dependent component of K\(^+\) transients was obtained by using the GABA\_AR antagonist bicuculline methiodide and/or picrotoxin. I noted that neither alone is sufficient to completely block agonist evoked membrane currents and full block requires the use of both BMI and PiTX. The existence of GABA\_AR subtypes not sensitive to bicuculline in hippocampal CA1 region has been postulated (Rozzo et al., 2002) and they may contribute to the biphasic membrane responses evoked by high frequency stimulation (Alakuijala et al., 2006). In the concentration range used to block GABA\_ARs, the bicuculline-methyl derivatives (but not the free base form of bicuculline) block Ca\(^{2+}\) activated K\(^+\) channels (Khawaled et al., 1999), which are underlying the Ca\(^{2+}\) dependent slow afterhyperpolarization current present in hippocampal CA1 pyramidal cells (Lancaster & Adams, 1986). Thus the BMI sensitive fraction (but not the PiTX sensitive fraction) of [K\(^+\)]\(_o\) responses may contain residuals of K\(^+\) release via K\(_{Ca}\) channels.
8 RESULTS

8.1 Distinct chloride regulatory mechanisms in nigral dopaminergic and GABAergic neurons (I)

Inputs to the dopaminergic neurons of the substantia nigra *pars compacta* (SNc) and to the GABAergic neurons of the substantia nigra *pars reticulata* (SNr) are nearly exclusively mediated by GABA (Grofova, 1975; Ribak *et al*., 1976; Smith & Bolam, 1989). The effects of GABA are mediated mainly via GABA$_A$Rs in both cell types (Grace & Bunney, 1985; Rick & Lacey, 1994; Tepper *et al*., 1995), but the efficacy of inhibition is higher in GABAergic neurons (Grace & Bunney, 1979; Grace & Bunney, 1985; Celada *et al*., 1999).

The light microscopic studies revealed clear differences in the expression patterns of proteins (KCC2, ClC−2) involved in $[\text{Cl}^-]_i$ regulation. Immunocytochemical labelling experiments demonstrated that the SNc dopaminergic neurons (identified as tyrosine hydroxylase positive, TH+) were completely devoid of KCC2 whereas the GABAergic neurons of SNr (parvalbumin positive, PV+) showed clear immunoreactivity for KCC2. Electron microscopic examination of subcellular structures showed that KCC2 was mainly located near the symmetrical, putatively GABAergic synapses targeted to SNr neurons.

Immunocytochemical techniques designed to detect ClC−2 proteins revealed intensively labelled neurons in the SNc region, confirming the previous mRNA results (Smith *et al*., 1995). Double labelling experiments identified ClC−2 positive cells as dopaminergic (TH+) neurons, in which ClC−2 proteins were located throughout the somatic and dendritic compartments.

Electrophysiological recordings utilizing the gramicidin-perforated patch technique showed that the pharmacologically isolated GABA$_A$R mediated responses of dopaminergic and GABAergic neurons had a reversal potential at $-63.5\pm2.0\text{mV}$ and at $-71.6\pm1.4\text{mV}$, the driving force (DF, defined here as $E_{\text{GABA}}-V_m$) of responses being $-3.3\pm2.0\text{mV}$ ($n=10$) and $-9.5\pm2.2\text{mV}$ ($n=11$; $p<0.01$), respectively.

Clearly the dopaminergic neurons can maintain hyperpolarizing inhibition, which therefore necessitates the existence of chloride extrusion mechanism other than KCC2 (Payne *et al*., 2003). The most likely candidate would be the Na$^+$ driven Cl$^-$/HCO$_3^-$ anion exchanger (Romero *et al*., 2000; Grichtchenko *et al*., 2001), the activity of which is suppressed in solutions devoid of HCO$_3^-$.

In line with this hypothesis, a change to nominally HCO$_3^-$ free conditions led to a positive shift in
RESULTS

\( E_{\text{GABA}} \) in dopaminergic neurons \((-49.7\pm3.2\text{mV}, \text{DF} \ 12.1\pm1.9\text{mV}, n=4; p<0.01 \text{ to control})\), but not in GABAergic cells \((-73.41\pm2.3\text{mV}, \text{DF} \ -9.5\pm2.9\text{mV}, n=7)\).

8.2 BDNF induced down-regulation of KCC2 (II)

Several studies have shown that intense neuronal activity down-regulates the efficacy of GABAergic inhibition and chloride extrusion (Kapur & Coulter, 1995; Whittington \textit{et al.}, 1995; Traub \textit{et al.}, 1999; Kohling \textit{et al.}, 2000). The question addressed in this study was whether such effects are mediated by BDNF, frequently reported to reduce GABA mediated responses in hippocampal neurons (Tanaka \textit{et al.}, 1997; Frerking \textit{et al.}, 1998; Brunig \textit{et al.}, 2001).

In line with the previous studies (Binder \textit{et al.}, 2001; Huang & Reichardt, 2001), kindling induced seizures \textit{in vivo} led to an increase in BDNF mRNA expression in the hippocampal region within 2 hours (300–500% over control). Also TrkB mRNA expression was enhanced and peaked in the hippocampal DG granule cell region around 2 hours after kindling (TrkB mRNA 160±5% of control). The up-regulation of BDNF signalling components was followed by a drastic reduction in KCC2 mRNA levels in the DG region, resulting in the lowest value of 30±3% of control after 6 hours. The changes in TrkB mRNA levels were qualitatively similar in the CA3 region (139±3% after 6 hours) and in the CA1 region (138±2% after 6 hours). When compared to control animals, KCC2 mRNA levels remained significantly lower for at least 24 hours after kindling and KCC2 protein levels detected by immunostaining displayed a clear down-regulation of immunoreactivity within all areas of the hippocampus 6 hours after the last seizure. KCC2 immunoreactivity recovered partially within 24 hours. From the \textit{in vivo} kindling experiments, it can be concluded that the activity-induced enhancement of BDNF signalling is correlated with the down-regulation of KCC2 expression.

In organotypic hippocampal slices the exposure to TrkB agonists BDNF and NT–4 (1–100ng ml\(^{-1}\)) for 17–19 hours led to a prominent reduction in KCC2 mRNA and protein levels, independent of neuronal network activity. At concentration of 10ng ml\(^{-1}\) of BDNF, the decrease in KCC2 protein expression was 61±15% and at 100ng ml\(^{-1}\) the reduction was 82±7% when compared to control levels \((n=5, p<0.001)\). NT–4 had a comparable effect. The down-regulation of KCC2 was prevented by scavenging the exogenous and endogenous neurotrophin with a soluble TrkB receptor body (200ng ml\(^{-1}\)) or by inhibiting the tyrosine kinase activity with 1µM K252a. These results demonstrate that the BDNF induced TrkB activation is sufficient to trigger signalling cascades leading to a reduction in KCC2 protein expression.
The application of high doses of exogenous BDNF (100–200ng ml\(^{-1}\), 2–4 h) to acute hippocampal slices, intended to mimic the massive activity dependent release of BDNF evoked by kindling in vivo, led to a rapid down-regulation of both KCC2 mRNA and protein expression within 2 hours, (n=5, p<0.001). To confirm that the reduction in KCC2 protein levels affect intracellular chloride regulation, the Cl\(^-\) extrusion capacity of functional KCC2 present in CA1 pyramidal cell membranes was challenged by a modest Cl\(^-\) load (0.5M KCl) via the intracellular sharp microelectrode. The levels of cytosolic chloride were assessed by reversal potential measurements of pharmacologically isolated GABA\(_A\)R responses, evoked by local electrical stimulation. The synaptic responses were divided into discrete time windows (7–9ms, 20–25ms and 35–40ms from stimulus onset), from which the first is believed to represent mainly perisomatic inputs, followed by responses generated in proximal and distal dendritic regions (cf. Buhl et al., 1994a). The driving force (defined as \(E_{GABA} - V_m\)) of responses within these predefined windows were \(-3.5\pm1.3\) mV, \(-6.3\pm1.1\) mV, \(-7.9\pm1.1\) mV in control (n=9) and \(4.6\pm2.2\) mV, \(1.8\pm1.8\) mV, \(0.03\pm1.8\) mV in BDNF-treated cells (n=10), the difference being statistically significant in all time windows (p<0.01, \(t\)-test). The resting membrane potential of control and BDNF-treated cells was \(-64.7\pm1.9\) mV (n=9) and \(-67.6\pm1.1\) mV (n=10), respectively, (p<0.2). The depolarizing shift in \(E_{GABA}\) caused by the electrode induced chloride load in the BDNF-treated slices indicated that the diminished KCC2 expression is associated with a significantly reduced Cl\(^-\) extrusion capacity.
RESULTS

8.3 KCC2 and GABAergic interstitial potassium transients (III)

Based on the results presented above and elsewhere (Rivera et al., 2002; Rivera et al., 2004), it is evident that the reduced levels of functional KCC2 will have detrimental effects on GABAergic hyperpolarizing inhibition. It is known that the intense activation of GABA$_A$Rs by high frequency stimulation, or by prolonged application of GABA$_A$R specific agonists, generate slow depolarizing responses (Kaila et al., 1997; Bracci et al., 1999) which are promoted by the increase in extracellular [K$^+$]$_o$ (Morris et al., 1996; Kaila et al., 1997). Notably, a massive Cl$^-$ influx via GABA$_A$Rs, driven by the depolarizing action of HCO$_3^-$ efflux, generates thermodynamic conditions favouring K$^+$/Cl$^-$ extrusion via KCC2, which thus could generate a major source of K$^+$ underlying the increase in interstitial [K$^+$]$_o$. This novel idea was opposite to the view assuming a reversal of KCC2 function by transient elevation of [K$^+$]$_o$ under conditions similar to the present ones. The main findings providing evidence for the conclusion that KCC2 promotes GABAergic interstitial K$^+$ transients were published in the original paper (III). In the overview below both published results (III) and substantial amount of unpublished data will be presented.

8.3.1 Bath-applied agonist isoguvacine

8.3.1.1 Whole-cell measurements

Firstly the effects of bath-applied GABA$_A$R agonist isoguvacine were characterized (2–200µM isoguvacine for 60s, interval 10min, perfusion flow rate 4.7ml min$^{-1}$). Whole-cell measurements from CA1 pyramidal cells, clamped close to the resting membrane potential (V$_{\text{hold}}$ 61.3±1.0mV, I$_{\text{hold}}$ 1.67±3.09pA, R$_{\text{in}}$ 180.25±21.38MΩ, n=3) revealed that in the CO$_2$/HCO$_3^-$ buffered solution, 2µM isoguvacine evoked monophasic membrane currents with a peak amplitude of 24.77±4.05pA, which was reached with a relatively slow time course, rise time from 10% to 90% level (t$_{10\%-90\%}$) being 43.1±8.1s. Under similar conditions and with similar cells (V$_{\text{hold}}$ 62.5±0.8mV, I$_{\text{hold}}$ −0.62±0.64pA, R$_{\text{in}}$ 197.36±16.1MΩ, n=8), the currents generated by 200µM isoguvacine had a first outward peak of 59.75±5.7pA, t$_{10\%-90\%}$ 6.03±0.48s, which recovered to zero in 14.74±1.26s and turned to an inward peak of −110.59±16.09pA, t$_{10\%-90\%}$ 12.11±0.65s (Fig. 1). Thus, the membrane currents during 200µM isoguvacine perfusion had the typical biphasic nature, so often associated with other forms of stimuli producing strong GABA$_A$R activation (Alger & Nicoll, 1979; Staley et al., 1995; Kaila et al., 1997).
RESULTS

Figure 1. Bath-applied isoguvacine evokes biphasic membrane currents in CA1 pyramidal cell. (a; upper panel) Initially monophasic current was gradually shifted to biphasic with increasing concentrations of isoguvacine (2µM, 20µM, 200µM). The outward current peak is marked with an arrow to show the increase in amplitude and the shift in time upon an increase in the concentration of isoguvacine. (a; lower panel) The fast outward component of 200µM isoguvacine response was suppressed near the predicted reversal potential of GABAAR mediated currents. The application period of isoguvacine (60s) is marked with a horizontal bar below current traces. (b) Summary of the peak amplitude (±S.E.M.) of bath applied isoguvacine generated currents (2µM, n=3 and 200µM, n=8). The appearance of late inward phase is evident in the 200µM group.

8.3.1.2 Extracellular ion selective- and field potential recordings

Bath-applied isoguvacine, dissolved in the CO₂/HCO₃⁻ buffered solution, produced a transient increase in extracellular K⁺ concentration (Δ[K⁺]₀) to 1.93±0.18mM above the baseline of 3mM, with a t₁₀%−₉₀% of 23.78±1.44s (n=5 slices), when measured from the CA1 stratum pyramidale (sp) region. Change from the CO₂/HCO₃⁻ buffered to the nominally HCO₃⁻ free, HEPES buffered solution damped the responses (Δ[K⁺]₀, 0.56±0.04mM, p<0.01, t₁₀%−₉₀% 26.75±1.52s, n=4 slices) and the switch to HEPES/formate perfusate enhanced ion shifts again (Δ[K⁺]₀ 1.49±0.24mM, p=0.12 to CO₂/HCO₃⁻, t₁₀%−₉₀% 23.70±0.68s, n=4 slices), (Fig. 2 a, c) confirming that the GABAAR permeant weak acid anions HCO₃⁻ or formate (Bormann et al., 1987) are promoting the generation of interstitial K⁺ transients (Lamsa & Kaila, 1997; Kaila et al., 1997).

In the CO₂/HCO₃⁻ buffered solution (supplemented with TTX, NBQX, DL-AP5 and CGP55845) the Δ[K⁺]₀ induced by 200µM isoguvacine had an amplitude of 0.99±0.16mM and t₁₀%−₉₀% 23.40±0.73s (n=4 slices). The wash in of 2mM Ba²⁺ facilitated isoguvacine induced ion shifts (Δ[K⁺]₀ 0.171±0.17mM, p<0.05, t₁₀%−₉₀% 25.52±1.34s, n=4 slices), (Fig. 2 b, d). Responses were blocked by BMI (Δ[K⁺]₀ 0.53±0.09mM, p<0.01, t₁₀%−₉₀% 22.50±3.38s, n=4 slices, data not shown).
RESULTS

In the presence of TTX, NBQX, DL-AP5 and CGP55845 in the HEPES/formate perfusate ($\Delta[K^+]_o$ induced by 200µM isoguvacine 1.12±0.23mM, $t_{(10\%-90\%)}$ 30.64±2.08s) the wash-in of KCC2 inhibitor furosemide (1mM) reduced the transients slightly ($\Delta[K^+]_o$ 0.71±0.09mM, p<0.05, $t_{(10\%-90\%)}$ 32.20±3.36s, n=6 slices) (Fig. 2 e). The effect of furosemide was reversible and a perfusion period of 30min with the CO$_2$/HCO$_3^-$ solution (devoid of any blockers) led to an increase in responses ($\Delta[K^+]_o$ 1.18±0.15mM, $t_{(10\%-90\%)}$ 30.63±5.66s, n=6 slices), which were nearly fully blocked by BMI ($\Delta[K^+]_o$ 0.29±0.05mM, p<0.05, $t_{(10\%-90\%)}$ 29.86±4.88s, n=6 slices), (not illustrated).

**Figure 2.** The characterization of [K+]$_o$ responses evoked with bath-applied isoguvacine. (a) Ion selective microelectrode recordings of interstitial K$^+$ responses in the CA1 stratum pyramidale region showed that both the GABA$_A$R permeant weak acid anions HCO$_3^-$ and formate promoted the accumulation of K$^+$ to interstitial space. (b) Under blockade of iGluR, GABA$_A$R and voltage activated Na$^+$ channels, the application of 2mM Ba$^{2+}$ (a non-specific K$^+$ channel inhibitor) enhanced the [K$^+$]$_o$ transients. The application period of isoguvacine (200µM for 30–35s, interval 15–30min) is marked with a line below ISM traces in figures (a) and (b). The experiments illustrated in (a, b) are summarized in (c, d), respectively, as mean±S.E.M. (**p<0.01, *p<0.05; n=4 for both sets). (e) In the HEPES/formate solution, supplemented with iGluR, GABA$_A$R and voltage sensitive Na$^+$ channel blockers, the KCC2 inhibitor furosemide produced a slight decrease in [K$^+$]$_o$ responses (*p<0.05; n=6). Statistical significance tested with paired t-test.
8.3.2 High frequency stimulation

8.3.2.1 Extracellular recordings in the CO₂/HCO₃⁻ solution

Due to the slow kinetics of bath-applied K⁺ responses, the contribution of KCC2 to the generation of [K⁺]₀ transients was studied with high frequency stimulation (HFS) method. The stimulation trains (40 pulses/100Hz, stimulus interval 10–15min) were delivered to the CA1 stratum radiatum (sr) region. When recorded from CA1 sp, the HFS reliably produced [K⁺]₀ shifts of 4.25±0.64mM (t(10%–90%) 1.435±0.099s, n=12 slices) which were associated with field potential afterdischarges. iGluR and GABABR blockers had no discernible effect on responses (Δ[K⁺]₀ 3.69±0.47mM, p=0.48, t(10%–90%) 1.815±0.121s, n=15 slices). The pharmacologically isolated GABAₐR dependent [K⁺]₀ transients were frequently reduced with PiTX or BMI (Δ[K⁺]₀ 1.26±0.13mM, p<0.01, t(10%–90%) 1.027±0.159s, n=15 slices), (Fig. 3 a).

The KCC2 inhibitor furosemide (1mM), in the presence of NBQX, DL-AP5 and CGP55845, inhibited fully the HFS evoked field potential afterdischarges and reduced the Δ[K⁺]₀ transients of 6.09±0.47mM by 72.5±2.8% (p<0.005 to control, n=4 slices), t(10%–90%) 2.033±0.206s. The application of 10µM BMI on top of furosemide had an inhibitory effect of 2.1±2.6% only (p=0.4 to furosemide, n=4 slices), suggesting that the GABAₐR dependent fraction of [K⁺]₀ transients was nearly fully blocked by furosemide (data not shown, cf. Fig. 2 of part III). In the presence of iGluR- and GABABR blockers, the amplitude of [K⁺]₀ transients tended to increase with more intense HFS trains. Interestingly, the responses which remained after the wash in of GABAₐR antagonists seemed to be less sensitive to changes in stimulation intensity, suggesting that only the GABAₐR mediated component was increased in parallel with stimulation intensity. This is illustrated in (Fig. 3 b) showing that although the amplitude of control transients increases, the BMI insensitive fraction remains relatively small. In order to quantify how the GABAₐR dependent and the BMI insensitive components were affected by the HFS intensity, the author studied the properties of [K⁺]₀ responses further. Also the furosemide effects on the GABAₐR mediated (BMI sensitive) fraction were inspected in more details.

Occasionally, a small increase in Δ[K⁺]₀ was noted when BMI was applied on top of furosemide. To obtain a conservative estimate of furosemide efficacy, the experiments in which the application of GABAₐR antagonists led to an increase in [K⁺]₀ transients were discarded from further analysis (2 out of 15 slices), (Fig. 3 b). The selected experiments were then divided into two groups,
RESULTS

constituted of responses having a control $\Delta[K^+]_o$ either above or below the mean of 3.71±0.53mM. The GABA$_A$R independent fraction covered only 22.1±2.4% of the transients in the HFS$^{\text{high}}$ group (n=5) but 49.4±8.4% in the HFS$^{\text{low}}$ group (n=8, p<0.05, non-paired $t$-test), showing that the GABA$_A$R mediated component was selectively enhanced upon the increase in stimulation intensity (Fig. 3 c). The $\Delta[K^+]_o$ of control responses in the HFS$^{\text{high}}$ group was 6.09±0.48mM, $t_{(10\%-90\%)}$ 2.028±0.160s and 2.42±0.35mM, $t_{(10\%-90\%)}$ 1.602±0.317s in the HFS$^{\text{low}}$ group (Fig. 3 d, e). Furosemide suppressed the extracellular $K^+$ shifts in both groups (HFS$^{\text{high}}$: $\Delta[K^+]_o$ furosemide 1.72±0.12mM, p<0.005 to control, $t_{(10\%-90\%)}$ 1.195±0.096s, $\Delta[K^+]_o$ BMI 1.45±0.10mM, p<0.05 to furosemide, $t_{(10\%-90\%)}$ 0.797±0.302s, n=4 slices, HFS$^{\text{low}}$: $\Delta[K^+]_o$ furosemide 1.42±0.17mM, p<0.05 to control, $t_{(10\%-90\%)}$ 1.327±0.271s, $\Delta[K^+]_o$ BMI 1.01±0.23mM, p=0.08 to furosemide, $t_{(10\%-90\%)}$ 0.835±0.160s, n=5 slices). The 30min application of 1mM furosemide produced a near complete inhibition of the GABA$_A$R dependent component (93.3±1.7%, $\tau$~13.9min, tested on the HFS$^{\text{high}}$ set, n=4 slices) and at 0.5mM level the reduction was 69.1±9.8%, ($\tau$~17.6min, tested on the HFS$^{\text{low}}$ set, n=5 slices), (Fig. 3 d, e, insets). 1mM L-octanol reduced the BMI insensitive residual responses (HFS$^{\text{high}}$: $\Delta[K^+]_o$ 1mM L-octanol 0.20±0.12mM, n=4 slices, p<0.005 to BMI), suggesting that the electrical synapses of inhibitory interneuron networks promote the HFS evoked $K^+$ transients, or that the octanol sensitive T-type $Ca^{2+}$ channels contribute to the non-synaptic fraction of $K^+$ transients (Heady et al., 2001) via $Ca^{2+}$ activated BK type $K^+$ channels (Sun et al., 2003).

The modest effect of 10µM bumetanide exposure (45min) on $\Delta[K^+]_o$ induced by the HFS$^{\text{low}}$ suggested that NKCC1 does not contribute to the clearance of $K^+$ from interstitial space ($\Delta[K^+]_o$ control 2.37±0.23mM, $t_{(10\%-90\%)}$ 1.810±0.080s, $\Delta[K^+]_o$ bumetanide 2.74±0.25mM, p<0.05, $t_{(10\%-90\%)}$ 1.905±0.107, n=4 slices, data not shown). The $\Delta[K^+]_o$ remaining after GABA$_A$R antagonist BMI was 1.19±0.31mM, $t_{(10\%-90\%)}$ 1.569±0.337s (n=4 slices), resulting in a BMI insensitive fraction of 42.1±11.5%. This is well in line with the other HFS$^{\text{low}}$ experiments (BMI insensitive fraction 49.4±8.4%, p=0.62, non-paired $t$-test), indicating no change in slice excitability during a long bumetanide application. Parallel recordings of $K^+$ and TMA$^+$ activities (the latter used as an interstitial volume indicator, baseline 1.5mM) showed that the HFS$^{\text{low}}$ evoked $[K^+]_o$ responses of 2.81±0.36mM, $t_{(10\%-90\%)}$ 1.919±0.098s, were associated with an increase in TMA$^+$ activity of only 0.124±0.035mM, p<0.001, $t_{(10\%-90\%)}$ 1.630±0.168s (n=9 slices, not illustrated). The small 8.3±2.4% decrease in extracellular volume does not contribute significantly to the 93.6±11.9% increase in interstitial $K^+$ activity.
Figure 3. GABAergic interstitial responses evoked with the high frequency stimulation (HFS). (a) Simultaneous K⁺ selective microelectrode and field potential recordings showed that the [K⁺]o transients and afterdischarges were well maintained after blockade of iGluR and GABA_A_R (p=0.8), but were suppressed after inhibition of GABA_A_R. The changes in [K⁺]o responses are shown in the inset as mean+S.E.M. (**p<0.005; n=12−15). (b) The amplitude of control responses ([K⁺]CTRL) increased in parallel with stimulation intensity, whereas the residual BMI insensitive component ([K⁺]BMI) remained relatively constant. In two occasions the wash-in of GABA_A_R antagonist BMI on top of furosemide led to a small increase in K⁺ responses. Such responses were rejected from further analysis (expelled [K⁺]o transients are marked with white dots). (c) Accepted experiments (grey dots) were divided to two pools either above (HFS_high) or below (HFS_low) the mean [K⁺]o amplitude of 6.71mM. The [K⁺]BMI component covered ~22% of [K⁺]CTRL transients in the HFS_high group (n=5) and ~50% of [K⁺]CTRL responses in the HFS_low group (*p<0.05; n=8), indicating that the GABA_A_R mediated K⁺ component was specifically enhanced by the high intensity HFS trains. (d) In the HFS_high group 1mM furosemide produced a near complete block of GABA_A_R dependent [K⁺]o responses (**p<0.005; n=4) after which BMI had only a modest effect (*p<0.05 to furosemide; n=4). The remaining [K⁺]BMI component was abolished with a putative gap-junction blocker L-octanol (**p<0.005 to BMI; n=4). (e) In the HFS_low group 0.5mM furosemide had qualitatively similar effects on [K⁺]o responses (**p<0.05; n=5). The development of ∆[K⁺]o inhibition induced by 1mM furosemide (τ~13.9min) (d; inset) and 0.5mM furosemide (τ~17.6min) (e; inset) show roughly similar time courses. Statistical significance was tested with paired (a, d, e) or unpaired t-test (c).
RESULTS

8.3.2.2 Intracellular recordings in the CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{−} solution

The intracellular sharp microelectrode recordings of CA1 pyramidal cells ($V_m$ $-67.0\pm3.1\text{mV}$, $R_{in}$ $42.0\pm4.1\text{M}\Omega$, n=4) revealed the typical biphasic form of pharmacologically isolated GABAergic responses. The HFS evoked fast hyperpolarization shifted quickly to a prolonged depolarization of $25.3\pm1.8\text{mV}$ above the resting $V_m$ (Fig. 4 a). The late part of depolarization is enhanced by the increase in [K\textsuperscript{+}]\textsubscript{o} (Kaila et al., 1997; Smirnov et al., 1999). The characterization of HFS responses showed that during stimulation the membrane potential is mainly governed by a GABA\textsubscript{A}R conductance (equivalent to $E_{GABA}$) and that the initial depolarizing shift is due to an increase in intracellular chloride. The changes in [Cl\textsuperscript{−}]\textsubscript{i} concentration were calculated using $E_{GABA}$ values measured after 3\textsuperscript{rd} pulse ($E_{3rd}$) and after last pulse ($E_{last}$). The points corresponding $E_{3rd}$ and $E_{last}$ values are connected with a grey line in (Fig. 4 a). During the train membrane voltage shifted to a depolarizing direction ($E_{3rd}$ $-77.3\pm2.2\text{mV}$, $E_{last}$ $-59.4\pm2.2\text{mV}$, n=4) indicating an increase in chloride from $4.20\pm0.62\text{mM}$ to $11.33\pm1.12\text{mM}$ with an initial chloride influx rate of $13.19\pm1.43\text{mM s}^{-1}$. Interestingly, the action potentials evoked by the HFS train emerged at more depolarized potentials ($-46.9\pm1.2\text{mV}$) than those evoked by depolarizing current injections ($-57.0\pm0.8\text{mV}$, $p<0.01$, n=10), (inset of Fig. 4 a), suggesting that the robust HFS induced GABA\textsubscript{A}R conductance prevented the appearance of spikes even at membrane potential levels more positive than the action potential threshold.

Following application of 1mM furosemide the fast hyperpolarizing component was slightly suppressed ($E_{3rd}$ in furosemide $-71.9\pm3.6\text{mV}$, n=4, $p<0.05$). However, the base line [Cl\textsuperscript{−}]\textsubscript{o} was not affected ($6.03\pm1.23\text{mM}$, n=4, $p=0.069$), (Fig. 4 b) suggesting that the Cl\textsuperscript{−} extrusion was not fully blocked. The shift in $E_{GABA}$ during stimulation ($E_{last}$ furosemide $-59.3\pm1.7\text{mV}$) indicated that the initial influx rate of Cl\textsuperscript{−} is not affected by furosemide ($11.08\pm0.82\text{mM s}^{-1}$, n=4, $p=0.13$), (Fig. 4 c). The [Cl\textsuperscript{−}]\textsubscript{i} level at the end of HFS train ($11.34\pm0.94\text{mM}$, n=4, $p=0.99$) and the late depolarization of $24.1\pm2.3\text{mV}$ (n=4, $p=0.69$) in the presence of furosemide were comparable to control values. Also the $V_m$ of $-69.4\pm3.0\text{mV}$ was equal with control, but the input resistance was decreased in furosemide ($R_{in}$ $26.4\pm2.6\text{M}\Omega$, $p<0.01$ to control, n=4).
Figure 4. The properties of GABAergic intracellular responses induced with the high frequency stimulation. (a) The rapid membrane hyperpolarization was followed by a prolonged depolarization which often promoted long trains of action potentials (action potentials truncated). The depolarizing shift during the HFS is thought to arise from GABA_AR mediated Cl\(^{-}\) accumulation (cf. Fig. 1 in Part III). The steady state levels and accumulation rates of [Cl\(^{-}\)]_i were quantified on the basis of the E_GABA shift, starting from the 3\(^{rd}\) stimulation pulse (E_{3rd}, the start point of grey line) and ending just after the last pulse (E_{last}, the end point of grey line), (see materials and methods for details). (a; inset) The action potentials appeared at less negative levels (p<0.005; n=10 cells) during the depolarizing phase of HFS response (black trace) than during the V_m deflections produced by positive current steps (grey traces). This suggests that the stimulus train induced strong GABA_A conductance may prevent the full-blown action potentials despite the depolarization. The mean V_m (dashed lines) ± S.E.M. (dotted lines) from which the action potentials were triggered after the stimulation train (black) or during current steps (grey) is marked above traces. (b, c) Furosemide (1mM) did not affect either the steady state intracellular chloride level (b) or the initial influx rate of Cl\(^{-}\) (c) within the time window used in experiments. Data from four cells are shown in (b, c).

8.3.2.3 Extracellular recordings in the HEPES/formate solution

In the continuous presence of NBQX, DL-AP5 and CGP55845, the HFS induced field potential afterdischarges and K\(^{+}\) transients were preserved following a change from the CO\(_2\)/HCO\(_3\)\(^{-}\) (Δ[K\(^{+}\)]_o 2.56±0.24mM, t_{(10\%-90\%)} 1.516±0.106s) to the HEPES/formate (Δ[K\(^{+}\)]_o 3.05±0.28mM, p=0.26, t_{(10\%-90\%)} 1.486±0.123s, n=11–15 slices, p=0.11), (Fig. 5 a, b). In line with the results obtained under CO\(_2\)/HCO\(_3\)\(^{-}\) conditions, the KCC2 inhibitor furosemide (0.5mM) reduced the Δ[K\(^{+}\)]_o responses of 2.21±0.21mM by 28.55±5.48% (p<0.001 to control, n=15 slices), t_{(10\%-90\%)} 0.875±0.076s. When added on top of furosemide, the GABA\(_A\)R antagonist BMI and PiTX had no
discernible effect (p=0.1 to furosemide, n=15 slices), suggesting a prominent inhibition of GABAergic [K+]o transients by furosemide (data not shown, cf. Fig. 5 in Part III).

However, the effect of 0.5−1mM furosemide on K+ transients was clearly smaller in the HEPES/formate solution (28.6±5.5%, n=15) than in the CO2/HCO3− solution (53.9±6.4%, p<0.05, n=9, and in HFS_{high} subset 72.5±2.8%, p<0.05, n=4, non-paired t-test). Under HEPES/formate conditions the amplitudes of control responses and BMI insensitive fractions increased in parallel (Fig. 5 d), contrary to the results obtained in the CO2/HCO3− solutions (cf. Fig. 3 b). Furthermore, the application of GABAAR antagonists on top of furosemide led frequently to an increase in the amplitude of K+ responses, which occasionally reached levels that were higher than the control responses. To resolve the relative fractions of synaptic and non-synaptic components of the GABAergic [K+]o transients under HEPES/formate conditions, the selection criterion reasoned previously by the author (Δ[K+]o in furosemide ≥ Δ[K+]o in furosemide+BMI and/or PiTX) led to a rejection of 15 responses out of 28 (Fig. 5 d).

As previously, the selected experiments were then divided to two pools, containing the control responses of amplitudes either below or above the mean Δ[K+]o of 1.74±0.29mM. The contribution of GABAAR independent K+ release was 45.0±12.8% in the Formate-HFS_{high} group (n=5 responses) and 21.6±12.8% in the Formate-HFS_{low} group (n=7 responses, p=0.15), (Fig. 5 e). Under HEPES/formate conditions only modest stimulation intensities provided K+ responses that were dominated by the GABAergic component, indicating that the practice of applying high intensity HFS trains to evoke large GABAergic K+ transients may work only under CO2/HCO3− conditions.

The Formate-HFS_{high} group had a control Δ[K+]o 2.73±0.40mM, t(10%−90%) 1.201±0.272s, (Fig. 5 f) and the Formate-HFS_{low} group had a control Δ[K+]o 0.96±0.16mM, t(10%−90%) 0.580±0.059s (Fig. 5 g). 0.5mM furosemide decreased the extracellular K+ transients in the Formate-HFS_{high} set to 1.63±0.40mM (p<0.05), t(10%−90%) 1.277±0.361s, and in the Formate-HFS_{low} set to 0.39±0.09mM (p<0.01), t(10%−90%) 0.723±0.135s, (Fig. 5 f, g). The inhibitory effect of 0.5mM furosemide on GABAAR dependent component was 72.0±3.0% in the Formate-HFS_{high} group and 75.6±6.6% in the Formate-HFS_{low} group, in good agreement with the results obtained in CO2/HCO3− solutions (0.5mM furosemide effect 73.5±8.2%, p=0.84 to Formate-HFS_{high} and p=0.85 to Formate-HFS_{low}, non-paired t-test). The amplitude of Δ[K+]o responses in the presence of GABAAR antagonists BMI and PiTX within the Formate-HFS_{high} group was 1.26±0.41mM, t(10%−90%) 1.000±0.255s and 0.17±0.07mM, t(10%−90%) 1.212±0.502s within the Formate-HFS_{low} group.
Figure 5. The GABAAR mediated $[\text{K}^+]_o$ transients, field potential afterdischarges and intracellular responses induced by high frequency stimulation under HEPES/formate conditions. (a) The $[\text{K}^+]_o$ selective microelectrode (upper panel) and field potential recordings (lower panel) showing nearly identical HFS responses under CO$_2$/HCO$_3^-$ and HEPES/formate conditions (in the presence of iGluR- and GABAAR blockers). However, the intracellular recordings revealed that the E3rd values were markedly depolarized under HEPES/formate conditions. The Vm levels during the initial part of HFS responses in the CO$_2$/HCO$_3^-$ and in the HEPES/formate solutions are shown in the insets (calibration labels of 0.5mV/5s apply to field potential traces and labels of 40mV/1s to Vm traces). (b) The amplitudes of $[\text{K}^+]_o$ transients ($n=11\sim15$) and (c) the voltage levels of E3rd responses (**p<0.05; $n=5$) are summarized as mean±S.E.M. (d) In the HEPES/formate solution both the control ([$[\text{K}^+]_{CTRL}$] and GABAAR independent ([$[\text{K}^+]_{BMI+PiTX}$]) responses increased markedly with increasing stimulation intensities, unlike in the CO$_2$/HCO$_3^-$ solution (compare with Fig. 3b). Also the $[\text{K}^+]_o$ transients suppressed by furosemide were more often increased in response to the application of BMI and PiTX. Such responses were discarded from analysis (rejected transients marked with white dots). Accepted experiments (grey dots) were split into groups containing $[\text{K}^+]_{CTRL}$ responses either above (HFS$^{\text{high}}$, $n=5$) or below (HFS$^{\text{low}}$, $n=9$) the mean amplitude of 4.60mM. (e) The $[\text{K}^+]_{BMI+PiTX}$ component of $[\text{K}^+]_{CTRL}$ responses was not different between the HFS$^{\text{high}}$ and the HFS$^{\text{low}}$ groups (p=0.33), contrary to the results obtained under CO$_2$/HCO$_3^-$ conditions (cf. Fig. 3e). (f, g) The fraction of $[\text{K}^+]_o$ transients sensitive to GABAAR antagonists was reduced by 0.5mM furosemide both in the HFS$^{\text{high}}$ data set (**p<0.05; $n=5$) and in the HFS$^{\text{low}}$ data set (**p<0.005; $n=9$). The co-application of BMI and PiTX on top of furosemide led to a further reduction of $[\text{K}^+]_o$ shifts in the HFS$^{\text{high}}$ group (**p<0.05 to furosemide; $n=5$) and in the HFS$^{\text{low}}$ group (**p<0.05 to furosemide; $n=9$). Statistical significance was tested with paired (b, c, f, g) or unpaired t-test (e).
8.3.2.4 Intracellular recordings in the HEPES/formate solution

Upon a change from the CO₂/HCO₃⁻ solution to the HEPES/formate solution (both solutions supplemented with NBQX, DL-AP5 and CGP55845) the membrane potential of CA1 pyramidal neurons underwent a hyperpolarization from the \( V_m \) of \(-66.2\pm2.7 \text{mV} \), \( R_{in} \) 41.52\(\pm5.76 \text{M}Ω \), (n=5) to the \( V_m \) of \(-72.5\pm2.3 \text{mV} \) (p<0.05), \( R_{in} \) 47.89\(\pm7.91 \text{M}Ω \), (n=5). An even more dramatic change was a prominent, an unexpectedly large and instantaneous depolarizing shift in the HFS train responses. The hyperpolarizing \( E_{3rd} \) of \(-74.7\pm2.5 \text{mV} \) present in the CO₂/HCO₃⁻ solution, turned into strongly depolarizing in the HEPES/formate (\( E_{3rd} \) \(-55.6\pm1.4 \text{mV} \), p<0.005, n=5), (Fig. 5 c).

The \( E_{3rd} \) values correspond to cytosolic Cl⁻ levels of 5.00\(\pm0.77 \text{mM} \) in CO₂/HCO₃⁻ and 9.83\(\pm0.76 \text{mM} \) in HEPES/formate (n=5, p<0.01). The \( E_{last} \) values at the end of HFS train (\(-56.4\pm3.0 \text{mV} \) in CO₂/HCO₃⁻ and \(-43.8\pm1.9 \text{mV} \) in HEPES/formate) were reflecting a \([\text{Cl}^-]_i\) accumulation up to 13.55\(\pm1.78 \text{mM} \) under CO₂/HCO₃⁻ and 18.22\(\pm1.54 \text{mM} \) under HEPES/formate conditions. The initial rate of increase was 15.34\(\pm2.02 \text{mM s}^{-1} \) in CO₂/HCO₃⁻ and 17.90\(\pm1.75 \text{mM s}^{-1} \) in HEPES/formate (n=5, p=0.37) indicating that despite the increased steady state level, a substantial influx of chloride is maintained under HEPES/formate conditions. The slow depolarization of pyramidal cells was unaltered, being 26.3\(\pm3.4 \text{mV} \) in the CO₂/HCO₃⁻ solution and 32.5\(\pm2.9 \text{mV} \) in the HEPES/formate (n=5, p=0.32), (data not illustrated).

The KCC2 inhibition had no effect on the intracellular parameters of HFS responses. In the presence of 1mM furosemide \( E_{3rd} \) of \(-53.9\pm1.8 \text{mV} \) (p=0.7, n=5) and \( E_{last} \) of \(-40.3\pm1.9 \text{mV} \) (p=0.24, n=5) resulted in a base line \([\text{Cl}^-]_i\), of 10.85\(\pm1.08 \text{mM} \), (p=0.66, n=5), which increased upon stimulation up to 21.51\(\pm1.82 \text{mM} \) (p=0.20, n=5) with an initial chloride accumulation rate of 21.98\(\pm1.73 \text{mM s}^{-1} \) (p=0.09, n=5). Also the late depolarization reaching a level of 31.5\(\pm2.1 \text{mV} \) (p=0.65, n=5) above the resting \( V_m \) of \(-69.5\pm2.3 \text{mV} \) remained intact. Unlike under CO₂/HCO₃⁻ conditions, the membrane input resistance was not sensitive to furosemide (\( R_{in} \) 47.35\(\pm8.28 \text{M}Ω \), n=5, p=0.63 to control), (data not shown).
8.3.3 Pressure applied agonists

8.3.3.1 GABA

Clearly the HFS evoked $K^+$ transients had both $\text{GABA}_A$R dependent and $\text{GABA}_A$R independent components, the relative fractions of which were different under $\text{CO}_2/\text{HCO}_3^-$ and HEPES/formate conditions. To isolate the $\text{GABA}_A$R mediated component of $[K^+]_o$ transients, responses were studied by local pressure microinjections of agonists to the CA1 sp region. Agonist application experiments were started with puffs of 10mM GABA (duration of pulses 0.5–3s, pressure 60kPa and interval 5–10min, pipette tip $\varnothing$ 2–4µm).

In the HEPES/formate solution supplemented with DL-AP5, NBQX, CGP55845 and TTX (perfusion flow rate 2.5ml min$^{-1}$) the GABA puff evoked $\Delta[K^+]_o$ had an amplitude of 1.23±0.15mM, $t_{(10\%-90\%)}$ 2.94±0.77s (n=7 slices), which was reduced by 0.5mM furosemide to 0.81±0.12mM, $t_{(10\%-90\%)}$ 5.20±2.02s (n=2 slices), the latter being not different from responses remaining after wash in of BMI ($\Delta[K^+]_o$ 0.71±0.09mM, $p=0.54$, $t_{(10\%-90\%)}$ 3.68±0.82s, n=7 slices, data not shown). The contribution of GABA uptake mechanisms to the $\text{GABA}_A$R independent component was tested in two experiments. In the presence of PiTX, GABA injections produced a $\Delta[K^+]_o$ of 0.36±0.13mM, $t_{(10\%-90\%)}$ 2.84±0.18s, to which the GABA uptake inhibitor NO-711 (2.5µM) did not have any effect ($\Delta[K^+]_o$ 0.40±0.15mM, $p=0.86$, $t_{(10\%-90\%)}$ 4.03±1.01s), (data not shown).

Switching from the HEPES/formate to the $\text{CO}_2/\text{HCO}_3^-$ solution led to an increase in $[K^+]_o$ responses. The $\Delta[K^+]_o$ of 0.31±0.01mM, $t_{(10\%-90\%)}$ 3.26±0.14s was first potentiated up to 0.79±0.73mM, ($p<0.001$), $t_{(10\%-90\%)}$ 3.33±0.06s, (n=4 slices) and then damped by the $\text{GABA}_A$R antagonists to 0.18±0.02mM ($p<0.001$), $t_{(10\%-90\%)}$ 3.20±0.16s (n=4 slices). The fraction of $\text{GABA}_A$R blocker insensitive component of 55.9±6.7% (n=7 slices) present in HEPES/formate was smaller in $\text{CO}_2/\text{HCO}_3^-$ solution (24.7±5.0%, $p<0.01$, n=4 slices), in agreement with previous HFS experiments (data not illustrated).

8.3.3.2 Isoguvacine

Surprisingly, the interstitial $K^+$ shifts generated by GABA puffs contained still both $\text{GABA}_A$R dependent and $\text{GABA}_A$R independent fractions. Therefore the microinjections of $\text{GABA}_A$R specific agonist isoguvacine were used to evoke $[K^+]_o$ transients (2–10mM, pulse duration 1–3s, pressure 100–150kPa, interval 10min, pipette tip $\varnothing$ 2–4µm). In the $\text{CO}_2/\text{HCO}_3^-$ solution puffs of
RESULTS

isoguvacine (10mM) into CA1 sp region evoked $\Delta[K^+]_o$ with a peak of 1.65±0.57mM, $t_{(10\%-90\%)}$ 5.40±0.16s, (n=7 slices). As with GABA microinjections, changing from the CO$_2$/HCO$_3^-$ solution to the HEPES/formate solution reduced the responses to a $\Delta[K^+]_o$ of 0.88±0.09mM (p<0.05 to CO$_2$/HCO$_3^-$), $t_{(10\%-90\%)}$ 5.38±0.32s (n=5 slices), (Fig. 6 b).

In the HEPES/formate solution containing DL-AP5, NBQX, CGP55845 and TTX, the control transients had a $\Delta[K^+]_o$ of 0.61±0.08mM ($t_{(10\%-90\%)}$ 4.60±1.43s, n=7 slices). Well in line with previous results, furosemide (1mM) suppressed the responses to a $\Delta[K^+]_o$ of 0.25±0.05mM (p<0.01 to control), $t_{(10\%-90\%)}$ 5.86±2.69s (n=7 slices), (Fig. 6 a, c). The wash-in of GABA$_A$R antagonist left a residual $\Delta[K^+]_o$ of 0.16±0.04mM, $t_{(10\%-90\%)}$ 4.69±2.78s (n=7 slices), which constituted 28.2±5.8% of the $K^+$ transients. Thus, furosemide reduced the GABA$_A$R antagonist sensitive fraction by 75.5±4.3% and 61.2±3.7% of the whole $\Delta[K^+]_o$. Lower concentration of furosemide (0.5mM) suppressed the isoguvacine (2mM) evoked responses of 0.45±0.05mM, $t_{(10\%-90\%)}$ 4.41±0.26s, down to 0.30±0.03mM (p<0.01), $t_{(10\%-90\%)}$ 8.00±1.24s (n=4 slices), (Fig. 6 d). The GABA$_A$R dependent component was reduced by 46.9±8.9% (for 32.5±3.0% of the whole $\Delta[K^+]_o$) upon wash-in of 0.5mM furosemide. The remaining antagonist insensitive $\Delta[K^+]_o$ of 0.13±0.03mM, $t_{(10\%-90\%)}$ 1.28±0.81s (n=4 slices) corresponded to 28.1±6.0% of the responses.

In the CA1 sr region, 2mM isoguvacine puffs produced a $\Delta[K^+]_o$ of 0.58±0.16mM, $t_{(10\%-90\%)}$ 6.10±1.57s, which after 0.5mM furosemide application had an amplitude of 0.29±0.06mM (p=0.13), $t_{(10\%-90\%)}$ 11.67±0.06s (n=3 slices), (Fig. 6 e). The effect of furosemide reflects a 61.2±14.3% block of the antagonist sensitive fraction (for 47.6±7.6% of the whole $\Delta[K^+]_o$) and the residual $\Delta[K^+]_o$ of 0.12±0.06mM covered 19.9±5.8% of the responses.

The application of the $K^+$ channel blocker Ba$^{2+}$ (1–2mM) to the HEPES/formate solution (supplemented with DL-AP5, NBQX, CGP55845 and TTX) enhanced the isoguvacine (2mM) evoked shifts recorded in CA1 sp region, from $\Delta[K^+]_o$ of 0.51±0.05mM, $t_{(10\%-90\%)}$ 4.85±0.52s, to 1.12±0.16mM (p<0.05), $t_{(10\%-90\%)}$ 20.78±2.63s (p<0.01), (n=7 slices). The component of Ba$^{2+}$ boosted $K^+$ transients remaining in PiTX had an amplitude of 0.27±0.06mM (n=3 slices). The $\Delta[K^+]_o$ evoked by pressure applied isoguvacine was dramatically prolonged upon wash in of Ba$^{2+}$ (Fig. 7 c), an effect that was not noticed in considerably slower $\Delta[K^+]_o$ produced by the bath-applied isoguvacine (cf. Fig. 2 b). In another set of experiments, made in the presence of 1mM Ba$^{2+}$, the prolonged $\Delta[K^+]_o$ of 2.29±0.19mM, $t_{(10\%-90\%)}$ 29.9±2.89s, was reduced by 0.5mM furosemide to the level of 1.03±0.17 (p<0.01), $t_{(10\%-90\%)}$ 37.02±1.83s (n=2 slices), (data not illustrated).
RESULTS

Figure 6. The GABA<sub>A</sub>R agonist isoguvacine evoked [K<sup>+</sup>]<sub>o</sub> transients. (a) Representative ISM recordings of interstitial K<sup>+</sup> responses in HEPES/formate evoked with pressure-applied isoguvacine (10mM). The beginning of puff is marked with an arrow below each trace (puff interval 10−15min). The amplitude of [K<sup>+</sup>]<sub>o</sub> responses was suppressed upon furosemide (1mM) application, which produced a near complete block of GABA<sub>A</sub>R dependent component. (b) Unlike the HFS evoked responses, the isoguvacine induced potassium transients were reduced upon a change from the CO<sub>2</sub>/HCO<sub>3</sub>− to the HEPES/formate (*p<0.05; n=5). (c) Under HEPES/formate conditions furosemide (1mM) produced a marked inhibition of responses (**p<0.005; n=7), after which the application of PiTX (100µM) did not induce discernible effects (p=0.16 to 1mM furosemide; n=7), suggesting that 1mM furosemide inhibited fully the GABA<sub>A</sub>R dependent component. (d) The inhibitory effect of furosemide on K<sup>+</sup> transients was already evident at the concentration level of 0.5mM (*p<0.05; n=4), but the following application of PiTX led to a further reduction in the amplitude (*p<0.05 to furosemide; n=4). Qualitatively similar results were obtained both in s. pyramidale (d) and in s. radiatum (e, n=3). Excluding the data shown in (b) all experiments were performed in the HEPES/formate solution and in the presence of NBQX, DL-AP5, CGP55845 and TTX. Statistical significance was tested with paired t-test.
8.3.3.3 Intracellular recordings in the CO$_2$/HCO$_3^-$ solution

Recordings of CA1 pyramids ($V_m$ $-69.2\pm4.5$ mV, $R_{in}$ 37.9 M$\Omega$) confirmed that in the presence of DL-AP5, NBQX, CGP55845 and TTX, the brief puffs of isoguvacine (10mM, pulse duration 200–250ms, pressure 100–150kPa, interval 2–3min, pipette tip $\Phi$ 2–4µm) produced classical biphasic responses. The initial fast hyperpolarization of $-74.6\pm3.3$ mV, $t_{(10\%\sim90\%)}$ 91.9$\pm$91.8ms was followed by a prolonged depolarization of $11.8\pm5.0$ mV, $t_{(10\%\sim90\%)}$ 3.44$\pm$0.08s (see Fig. 7 a). After furosemide (1mM) application neither the initial response ($-65.9\pm4.8$ mV, n=3, p=0.14) nor the late depolarization (9.2$\pm$2.5mV, n=3, p=0.42, $t_{(10\%\sim90\%)}$ 2.73$\pm$0.16s) were affected (data not illustrated). Also the $V_m$ of $-69.0\pm6.0$ mV remained intact, but the $R_{in}$ of cells decreased to 22.9$\pm$4.6M$\Omega$, (n=3, p<0.05, data not shown). However, the steady state [Cl$^-$]$_i$ of 5.05$\pm$1.07mM increased to 8.41$\pm$1.93mM in furosemide (n=3, p<0.05). The slope of slow depolarization (ascending from the peak of initial fast response to the $V_m$ level 800ms after the initial starting point) was 9.23$\pm$3.16mV s$^{-1}$ in control and 4.13$\pm$1.78mV s$^{-1}$ in furosemide, which results in the initial [Cl$^-$]$_i$ influx rate of 2.83$\pm$0.87mM s$^{-1}$ in control and 1.62$\pm$0.55mM s$^{-1}$ in furosemide (n=3, p=0.3, data not illustrated). The role of K$^+$ conductance on isoguvacine puff induced intracellular responses was studied with Ba$^{2+}$. At the concentration of 1mM Ba$^{2+}$ did not affect the initial fast hyperpolarizing component, which was $-71.3\pm1.3$ mV, $t_{(10\%\sim90\%)}$ 120$\pm$62ms in control conditions and $-70.7\pm2.2$ mV (p=0.59), $t_{(10\%\sim90\%)}$ 120$\pm$72ms in 1mM Ba$^{2+}$. Also the late depolarization, being 11.5$\pm$1.8mV, $t_{(10\%\sim90\%)}$ 3.31$\pm$0.3s in control and 8.0$\pm$1.4mV (p=0.14), $t_{(10\%\sim90\%)}$ 2.45$\pm$0.47s in 1mM Ba$^{2+}$ was not altered. The $V_m$ of $-60.1\pm2.1$ mV remained stable upon wash in of Ba$^{2+}$ ($-59.1\pm1.2$ mV, p=0.88) despite the marked increase in $R_{in}$ from 50.74$\pm$3.30M$\Omega$ to 90.45$\pm$8.48M$\Omega$ (n=4, p<0.05). When the Ba$^{2+}$ concentration was increased to 2mM, cells depolarized quickly to the $V_m$ of $-48.2\pm1.1$ mV and $R_{in}$ increased further to 117.93$\pm$0.43M$\Omega$ (n=2). The initial brief hyperpolarization to $-67.0\pm1.6$ mV, $t_{(10\%\sim90\%)}$ 53$\pm$2.3ms remained largely intact but the late depolarization was fully blocked (to $-0.2\pm3.1$ mV, n=2), (Fig. 7 a). The exposure to 2mM Ba$^{2+}$ often led to an appearance of slow continuous cycles of spikes (Fig. 7 b) perhaps generated by voltage activated Ca$^{2+}$ channels present in the dendrites of CA1 pyramids (Magee & Johnston, 1995).

8.3.3.4 Intracellular recordings in the HEPES/formate solution

In the presence of DL-AP5, NBQX, CGP55845 and TTX the intracellular responses evoked by isoguvacine microinjections (2mM isoguvacine, puff duration 1–3s, pressure 100–150kPa, interval
10 min, pipette tip Ø 2–4 µm) exhibited mainly monophasic responses, which depolarized the cell by 9.1 ± 1.9 mV, \( t_{(10\%-90\%)} \) 2.24 ± 0.41 s, above the resting membrane potential of −65.6 ± 1.0 mV (n=3). 1 mM Ba\(^{2+}\) introduced a positive shift in the membrane potential (\( V_m \) −57.3 ± 1.1 mV, \( p<0.01, n=2 \)) but left the isoguvacine evoked depolarizations unaltered (9.8 ± 1.6 mV, \( p=0.79, t_{(10\%-90\%)} \) 6.94 ± 1.83 s, n=2). In two cells tested (\( V_m \) −64.6 ± 1.8 mV) the wash in of 2 mM Ba\(^{2+}\) depolarized the \( V_m \) to −36.3 ± 3.6 mV, which often promoted sustained spiking. The isoguvacine induced depolarization of 9.5 ± 2.0 mV, \( t_{(10\%-90\%)} \) 3.17 ± 0.10 s (n=2) changed to a hyperpolarization of −13.3 ± 1.5 mV, \( t_{(10\%-90\%)} \) 1.11 ± 0.21 s (n=2), (Fig. 7 c). Following the application of PiTX on top of 1 mM Ba\(^{2+}\) cells were depolarized further and begun to spike continuously (\( V_m \) −38.7 ± 2.0 mV, n=3). Surprisingly, in the presence of PiTX the puffs of isoguvacine evoked marked hyperpolarizations (−13.6 ± 3.1 mV), which inhibited spiking (data not shown).
RESULTS

Figure 7. The effect of K⁺ channel blocker Ba²⁺ on the isoguvacine induced membrane potential responses of pyramidal cells and extracellular K⁺ transients in the CA1 region. (a) A typical biphasic responses evoked by brief isoguvacine puffs (duration 200–250ms) in the CO₂/HCO₃⁻ solution supplemented with NBQX, DL-AP5, CGP55845 and TTX (black traces). Following the addition of 2mM Ba²⁺ the initial hyperpolarization remained largely intact but the $V_m$ was depolarized and $R_m$ increased (grey traces). (b) Despite the continuous presence of TTX (and DL-AP5, NBQX, CGP55845) the pyramidal cells often exhibited slow spikes in 2mM Ba²⁺ (grey trace). The pyramidal cell response to a depolarizing current injection of 100ms, in the CO₂/HCO₃⁻ solution devoid of any blockers is shown as a reference (black trace). (c; upper panel) The time course of K⁺ transients evoked by isoguvacine (puff duration 1–3s) was roughly similar under CO₂/HCO₃⁻ (black trace) and HEPES/formate (dark grey trace) conditions, but noticeably prolonged in response to 2mM Ba²⁺ (grey trace). (c; lower panel) Intracellular recordings done in parallel with extracellular K⁺ measurements described the characteristics of membrane potential responses in the CO₂/HCO₃⁻ solution (black trace) and in the HEPES/formate solution (dark grey trace), both supplemented with NBQX, DL-AP5, CGP55845 and TTX. Note the disappearance of the initial hyperpolarizing component upon change to HEPES/formate. Addition of 2mM Ba²⁺ to the HEPES/formate solution led to a substantial depolarization of membrane potential, during which the isoguvacine puffs evoked long hyperpolarizing responses (grey trace). The timing of isoguvacine puffs is marked with arrows above traces in (a, c).
8.3.4 Muscimol iontophoresis

The results obtained with isoguvacine microinjections suggested that the GABA$_A$R antagonists used (PiTX or BMI) were not efficiently blocking the GABA$_A$R mediated intracellular responses, which thus would explain the existence of antagonist insensitive fraction of K$^+$ transients evoked with HFS, GABA and isoguvacine. As the optical measurements of intracellular Cl$^-$ activity (based on 6-methoxy-N-ethylquinolinium iodide (MEQ) fluorescence) have also revealed an incomplete block of Cl$^-$ accumulation by 50µM PiTX or 25µM bicuculline (Isomura et al., 2003), an iontophoretic application method was established (10mM muscimol in 10mM HCl, application current from +50nA to +100nA, duration 2−4s, interval 10min, continuous backing current of −1nA to −5nA, pipette tip Ø 2−4µm) and the inhibitory effects of BMI and/or PiTX on muscimol evoked whole cell currents were characterized.

CA1 pyramidal cells recorded under CO$_2$/HCO$_3^-$ conditions (devoid of any blockers) were clamped to a holding potential of −60mV. Iontophoretic application of muscimol evoked classical biphasic membrane currents (n=6) on which the effects of BMI and PiTX were tested. The recordings revealed clear differences in the inhibitory effects of the above-mentioned antagonists. BMI had a more profound effect on the late part of the response (n=3), whereas PiTX inhibited the initial part more efficiently (n=3). The co-application of both antagonists was required to obtain a near complete block (n=6), (Fig. 8 a, inset).

In the CO$_2$/HCO$_3^-$ solution, devoid of any blockers, muscimol induced [K$^+$]$_o$ responses of 2.01±0.26mM, t$_{(10\%-90\%)}$ 6.12±0.32s were reduced upon a change to the HEPES/formate solution (∆[K$^+$]$_o$ 0.97±0.06mM, t$_{(10\%-90\%)}$ 6.03±0.48s, n=2−4 slices, p<0.01), (Fig. 8 b). In HEPES/formate the wash-in of iGluR-, GABA$_B$R- and voltage activated sodium channel blockers diminished the ∆[K$^+$]$_o$ of 1.37±0.13mM further to ∆[K$^+$]$_o$ 0.71±0.05mM (n=7 slices, p<0.001) and the following application of furosemide (0.5−1mM) damped the responses to 0.50±0.04mM (n=6 slices, p<0.01) inhibiting the ∆[K$^+$]$_o$ fraction sensitive to GABA$_A$R antagonist by 39.3±5.0% (for 29.4±3.0% of the whole ∆[K$^+$]$_o$), (Fig. 8 a, c). The residual responses after wash-in of both PiTX and BMI had an amplitude of 0.18±0.07mM (n=6 slices, p<0.01), which corresponded 26.7±8.3% of the original K$^+$ transients.
Figure 8. The properties of GABA₉R agonist muscimol evoked responses. (a) In the HEPES/formate solution, supplemented with NBQX, DL-AP5, CGP55845 and TTX, the iontophoretic application of muscimol (10mM) produced a transient increase in extracellular potassium concentration, the amplitude of which was suppressed upon wash-in of furosemide (1mM). The onset of iontophoresis pulse of is noted with an arrow beneath each trace (pulse interval 10−15min). (a; inset) Under CO₂/HCO₃⁻ conditions the muscimol evoked whole-cell currents (white trace; n=14) showed classical biphasic characteristics. The effects of GABA₉R antagonists BMI (grey trace; n=5) and PiTX (grey trace; n=3) had clearly different properties and both antagonists were needed to provide a near complete block (black trace; n=8). (b) The muscimol evoked interstitial K⁺ responses diminished when the CO₂/HCO₃⁻ buffered solution was replaced with the HEPES/formate solution (⁎⁎p<0.05; n=2−4) (c) In the HEPES/formate solution and under pharmacological blockade of iGluR, GABA₉R and voltage activated Na⁺ channels, the application of furosemide (0.5−1.0mM) reduced the amplitude of [K⁺]ᵽ responses (⁎⁎⁎p<0.0005; n=7). The wash in of both PiTX and BMI led to a further inhibition (⁎⁎p<0.005 to furosemide; n=7). Statistical significance tested with unpaired (b) or paired (c) t-test.
8.3.5 The effect of furosemide on GABA$_A$R conductance

During the course of intracellular recordings of CA1 pyramids ($V_m$ $-65.90 \pm 1.13$ mV, $R_{in}$ 41.82 $\pm$ 3.68 M$\Omega$, $n=6$) done in the CO$_2$/HCO$_3^-$ solution, the author noticed that upon wash-in of furosemide, the mean rise time of single-pulse stimulation induced inhibitory postsynaptic potentials (IPSP) prolonged, from 10.05 $\pm$ 0.27 ms ($n=386$ events) to 14.41 $\pm$ 0.40 ms ($n=393$, $p<0.001$), (Fig. 9 a). IPSPs were recorded at various $V_m$ levels below and above $E_{GABA}$ and all responses $< \pm 1.0$ mV were discarded from rise time analysis. The prolonged rise time of IPSPs in furosemide suggested that the furosemide sensitive, somatic GABA$_A$R sub-population (Pearce, 1993; Banks et al., 1998) was inhibited. Therefore, the alterations in GABA$_A$R conductance ($G_{GABA}$) and $E_{GABA}$ were studied in more detail with the single-pulse stimulation method.

The furosemide evoked rapid shift ($\tau$ $\sim$ 6.25 min) in $E_{GABA}$ (control $-81.5 \pm 2.4$ mV, $n=6$) started immediately upon wash-in and the new level of $-75.7 \pm 2.0$ mV was attained within 20 min ($p<0.05$ to control) and maintained for at least 40 min ($-75.5 \pm 1.60$ mV, $p<0.05$ to control, $p=0.4$ to 20 min furosemide), (Fig. 9 b). These baseline $E_{GABA}$ values correspond to a [Cl$^-]$ level of 3.20 $\pm$ 0.54 mM in control and of 4.75 $\pm$ 0.49 mM after 20 min in furosemide ($p<0.05$ to control). The control $G_{GABA}$ of 27.2 $\pm$ 4.6 nS ($n=6$) activated by a single-pulse stimulation decreased gradually in the presence of furosemide and was 17.8 $\pm$ 2.41 nS after 20 min ($p=0.08$ to control) and 11.6 $\pm$ 1.90 nS after 40 min period ($p<0.05$ to control). As a whole the drop in $G_{GABA}$ was 45.6 $\pm$ 9.4% in 40 min and developed slowly ($\tau$ $\sim$ 47 min), (Fig. 9 c). Compared to the changes in [Cl$^-]$ regulation the reduction in GABA$_A$R conductance was slow and did not reach a clear steady state level during the time of observation. As previously noted, the $R_{in}$ was decreased by furosemide (control $R_{in}$ 41.82 $\pm$ 3.68 M$\Omega$, furosemide $R_{in}$ 30.51 $\pm$ 2.81 M$\Omega$, $n=6$, $p<0.05$). Due to the profound depolarizing shift in the GABA$_A$R mediated responses (Fig. 9 d, see also Fig. 5 c) the effects of furosemide on $E_{GABA}$ or $G_{GABA}$ of single-pulse responses were not quantified under HEPES/formate conditions. However, the amplitude of responses arising from hyperpolarized $V_m$ levels became smaller and slower upon prolonged furosemide application (Fig. 9 e). As the inhibition of Cl$^-$ extrusion by furosemide should promote an increase in IPSP amplitudes evoked at $V_m$ levels below $E_{GABA}$, the decrease in IPSP amplitudes suggests a reduction in the GABA$_A$R conductance.
RESULTS

Figure 9. Furosemide effects on the intracellular GABA$_A$R mediated postsynaptic potentials evoked with single-pulse stimulation. (a) In the CO$_2$/HCO$_3^-$ solution (containing NBQX, DL-AP5, CGP55845) the rise time of inhibitory postsynaptic potentials (IPSPs) was prolonged upon 1mM furosemide application (p<0.001; n=386–393 events). The Vm level was varied during recordings to change the polarity of IPSPs and the rise time of IPSPs is shown as a function of amplitude (control; white circles, +1mM furosemide; grey circles). All responses near the E$_{GABA}$ (amplitude < ±1.0mV) were rejected from rise time analysis. (b, c) The depolarizing shift in E$_{GABA}$ (*p<0.05; n=6) induced by furosemide was relatively fast and was associated with a reduction in GABA$_A$R conductance (G$_{GABA}$) (*p<0.05; n=6). The new steady state E$_{GABA}$, being indicative of higher [Cl$^-$], was reached within 20 minutes (τ~9.2min), whereas the decrease in G$_{GABA}$ was markedly slower (τ~47min). (d) Prominent depolarizing switch in the GABA$_A$R mediated postsynaptic potentials in response to change from the CO$_2$/HCO$_3^-$ buffered solution to the HEPES/formate solution was also evident in single pulse responses (cf. Fig. 5 e). (e) Under HEPES/formate conditions the rise time of GABA$_A$R mediated responses was increased whereas the amplitude was reduced by furosemide, suggesting that the G$_{GABA}$ is decreased. The Vm is hyperpolarized with a current injection by the experimenter to show the reduction in response amplitude and lack of spiking in furosemide. The timing of stimuli is indicated with arrows above and below traces in (d, e). Statistical significance was tested with paired t-test.
The furosemide induced, slowly progressing reduction in the single-pulse stimulation evoked $G_{\text{GABA}}$, may be either due to the inhibition of GABA$_{\text{A}}$R receptor subtypes on postsynaptic membranes or due to the reduced GABA release from presynaptic terminals. Whole-cell voltage clamp recordings, combined with somatic muscimol iontophoresis (10mM, pulse duration 20ms, current 50–150nA, continuous backing current from $-1nA$ to $-5nA$, pipette tip $\varnothing$ 2–4µm), (Fig. 10 a) revealed that the furosemide induced rapid ($\tau$~3.7min) shift in $E_{\text{GABA}}$ (control $-71.5\pm1.7mV$, furo 5min $-66.6\pm1.8mV$, p<0.01 to control; n=5, furo 15min $-64.9\pm1.5mV$, p<0.01 to control, p<0.05 to 5min in furo; n=5), was associated with an equally rapid ($\tau$~3.9min) shift in the maximal membrane conductance (Fig. 10 b, c). The steady state intracellular [Cl$^-$] was increased from $6.00\pm0.54mM$ to $8.57\pm0.66mM$ (p<0.001; n=5) in 15 minutes in response to furosemide. The parallel decrease in control conductance of $22.1\pm2.6pS$ proceeded in 5 minutes to the level of $15.9\pm1.7pS$ (p<0.05 to control; n=5) and was reduced further to $13.7\pm2.2pS$ in 15 minutes (p<0.05 to control, p=0.7 to 5min; n=5), (Fig. 10 b, d). These results clearly show that the 38.2% reduction in GABA$_{\text{A}}$R conductance was of postsynaptic origin.

The results presented above suggested that the Cl$^-$ accumulation during HFS might be suppressed in furosemide. However, the HFS induced initial influx rate of Cl$^-$ was well maintained in furosemide (85.9±15.4% of control, n=4, p=0.15) and also the steady state [Cl$^-$] levels were largely unaffected (4.2mM in control and 6.03mM in furosemide, n=4, p=0.069).
RESULTS

**Figure 10.** The inhibitory action of furosemide on GABA<sub>A</sub> conductance is of postsynaptic origin. (a) GABA<sub>A</sub>R mediated whole-cell currents of CA1 pyramidal cell, evoked with 10mM muscimol iontophoresis, recorded at various voltage levels before (grey traces) and after 1mM furosemide application (black traces). (b) Current-voltage plot of muscimol responses before (grey) and 5 min and 15 min after the wash-in of 1mM furosemide (black). Note the furosemide induced rapid change in the reversal potential and chord conductance. (c, d) The furosemide induced positive shift in $E_{\text{GABA}}$ (**p<0.01; n=5) proceeded in a roughly similar time course ($\tau \sim 3.7\text{min}$) as the reduction in $G_{\text{GABA}}$ (*p<0.05; n=5) that had a $\tau \sim 3.9\text{min}$, suggesting that GABA<sub>A</sub>R sub-types located close the somatic area (activated by muscimol applied to soma) are more prone to furosemide blockade than receptor isoforms at the distal dendritic region (contributing to responses activated by single pulse stimulation, see Fig. 9 c). The experiments were done in the CO$_2$/HCO$_3^-$ solution and in the presence of DL-AP5, NBQX, CGP55845 and TTX. Statistical significance was tested with paired t-test.
8.3.6 *Furosemide does not inhibit carbonic anhydrases present in CA1 pyramidal region*

Furosemide has been shown to inhibit intracellular carbonic anhydrases within a nanomolar concentration range (Supuran & Scozzafava, 2000; Temperini *et al.*, 2009). As the generation of K⁺ transients is dependent on the stable [HCO₃⁻]ᵢ level maintained by intracellular carbonic anhydrases (Ruusuvuori *et al.*, 2004), the effect of furosemide on K⁺ responses could arise from reduced HCO₃⁻ production. To test this possibility, the intracellular pH (pHi) measurements of CA1 pyramidal neurons (based on BCECF fluorescence) were used to assess the functional efficacy of carbonic anhydrases (Ruusuvuori *et al.*, 2004). The alkaline pHi shift in response to extracellular CO₂/HCO₃⁻ withdrawal was not affected by 30–40min application of 1mM furosemide (control 0.063±0.003min⁻¹, furosemide 0.063±0.008 min⁻¹ (mean±S.D.), p=0.76, n=18 cells in four slices), whereas the membrane permeant carbonic anhydrase inhibitor ethoxyzolamide (EZA, 100µM) reduced the rate of pHi changes (control 0.067±0.009min⁻¹, EZA 0.046±0.007min⁻¹ (mean±S.D.), p<0.001, n=6 cells in one slice, data not shown). The effect of a poorly permeant carbonic anhydrase inhibitor benzolamide (10µM) was tested in two out of four slice experiments with furosemide. Benzolamide was without any effect, suggesting that extracellular carbonic anhydrase activity (Chesler, 2003) has no role on the pHi shifts generated by CO₂/HCO₃⁻ withdrawal. Similar results were obtained with acute isolated CA1 pyramidal neurons. Superfusion for 20min with 0.5mM furosemide did not affect the pHi transients induced with CO₂/HCO₃⁻ removal, but the following EZA application reduced the rate and amplitude of pHi shifts clearly (n=4), (see Fig 3. of Part III). The pHi measurements indicate that the inhibitory effect of furosemide on [K⁺]ₒ transients is not due to the inhibition of intracellular carbonic anhydrase activity.
RESULTS

<table>
<thead>
<tr>
<th>CO₂/HCO₃⁻</th>
<th>Control</th>
<th>1mM Furosemide</th>
<th>Furo effect</th>
<th>τ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>∆[K⁺]₀ (mM)</td>
<td>6.09±0.48</td>
<td>1.72±0.12</td>
<td>−72%</td>
<td>~14</td>
</tr>
<tr>
<td>∆[Cl⁻]₀ (mM s⁻¹)</td>
<td>13.19±1.43</td>
<td>11.08±0.82</td>
<td>−16%</td>
<td>−</td>
</tr>
<tr>
<td>G_GABA (nS)</td>
<td>26.9±3.4</td>
<td>12.9±1.8</td>
<td>−45%</td>
<td>~45</td>
</tr>
<tr>
<td>G_MUSC (nS)</td>
<td>22.1±2.6</td>
<td>13.7±2.2</td>
<td>−40%</td>
<td>~4</td>
</tr>
<tr>
<td>E_GABA (mV)</td>
<td>−81.5±2.3</td>
<td>−75.5±1.6</td>
<td>+6.0mV</td>
<td>~6.5</td>
</tr>
<tr>
<td>E_MUSC (mV)</td>
<td>−71.5±1.7</td>
<td>−66.6±1.8</td>
<td>+4.9mV</td>
<td>~4</td>
</tr>
</tbody>
</table>

Table 3. Summary of the furosemide (1mM) effects on parameters affecting GABAₐR mediated responses and KCC2 transport. All experiments were done in the CO₂/HCO₃⁻ buffered solution and in the presence of iGluRB and GABAₐR blockers, using either HFS trains (∆[K⁺]₀, ∆[Cl⁻]₀), single-pulse synaptic simulation (G_GABA and E_GABA) or muscimol iontophoresis (G_MUSC and E_MUSC, recorded in the presence of TTX).
RESULTS

8.3.7 Modelling of GABAergic responses based on Goldman-Hodgkin-Katz formalism

The biphasic responses evoked by strong GABA<sub>A</sub>R activation can be dissected to two components. The initial part of depolarizing shift in $V_m$ immediately after the rapid hyperpolarization is thought to arise from intraneuronal Cl<sup>-</sup> accumulation (Staley et al., 1995; Kaila et al., 1997) and the pronounced depolarization, peaking ~1.5–2.0s after the onset of HFS train, has been suggested to be caused by the increase in $[K^+]_o$ (Kaila et al., 1997; Smirnov et al., 1999). Previous quantification of the effects of extracellular potassium transients unequivocally demonstrated that the increase in $[K^+]_o$ results in a net current that is inward at any $V_m$ (Kaila et al., 1997). As the increase in $[K^+]_o$ increases the availability of K<sup>+</sup> ions for an unidirectional influx of K<sup>+</sup>, an inward current component that adds to the net K<sup>+</sup> current is generated. At membrane potential levels above $E_K$, where the net K<sup>+</sup> current is outward, the potassium-induced inward K<sup>+</sup> current component is observed as a decrease in the net outward K<sup>+</sup> current. Also the depolarizing effect of non-selective K<sup>+</sup> channel blocker Ba<sup>2+</sup> (see p. 67–69) supports the conclusion that reduced net K<sup>+</sup> efflux, either due to the increased unidirectional influx (caused by the increase in $[K^+]_o$) or due to the reduced K<sup>-</sup> permeability (caused by the Ba<sup>2+</sup> application) has a depolarizing effect on $V_m$. However, the results presented in this study clearly demonstrated that the KCC2 inhibitor furosemide markedly suppressed the HFS induced interstitial K<sup>+</sup> responses, but did not inhibit the late depolarization of CA1 pyramids. Such observation suggests that the peak depolarization of CA1 pyramids is not caused by the increase in $[K^+]_o$.

In an attempt to estimate the effects that $[K^+]_o$ responses have on the neuronal membrane, the author created two simplified models of membrane voltage ($E_m$) and membrane current ($I_m$). The model equations (see p. 50 for details) were based on the GHK constant-field theory, developed by (Goldman, 1943) and (Hodgkin & Katz, 1949), (see also Hille, 2001).

The following reasoning was used to adjust the ion permeability values of GHK equations. The first assumption was that the CA1 membrane is mainly permeable to potassium. If the membrane is exclusively selective for K<sup>+</sup> it will have a resting membrane potential that is equal with potassium equilibrium potential. As the resting $V_m$ of CA1 pyramidal cells is clearly above the $E_K$ of $-95$ mV, at least one other ion permeability having an equilibrium potential above the resting $V_m$ must contribute to this deviation. I assumed that this other ion is Na<sup>+</sup>, which has equilibrium potential around +70 mV under the ion gradients listed in Table 1. Then, the values of Na<sup>+</sup> and K<sup>-</sup> permeabilities ($P_{Na}$ and $P_K$) were selected to give resting $E_m$, $I_m$ and $R_{in}$ figures that were
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comparable to the recorded values. As the KCC2 mediated K⁺/Cl⁻ efflux leading to an increase in [K⁺]o does not predict changes in P_K or P_Na, the values were assumed to stay constant.

The GABA_A R mediated responses were assumed to arise from a rapid increase in the permeability of Cl⁻ (P_Cl) and HCO₃⁻ (P_HCO) that led to a Cl⁻ accumulation. The anion permeability values and Cl⁻ accumulation rates of each model were adjusted to reproduce the GABA_A R mediated part of recorded traces. Averaged HFS induced intracellular responses under CO₂/HCO₃⁻ condition were used as a reference and modelled with GHK voltage equation (to obtain E_m) and the GABA_A R agonist muscimol and isoguvacine generated whole-cell currents recorded in the CO₂/HCO₃⁻ solution were reproduced with GHK current equations (to obtain I_m).

8.3.7.1 High frequency stimulation induced intracellular responses

The GHK voltage equation was written for four ions under CO₂/HCO₃⁻ conditions (K⁺, Na⁺, Cl⁻ and HCO₃⁻) and the effect of [K⁺]₀ increase on E_m was tested both in the absence (Fig. 11; left panel) and in the presence of transient increase in anion permeation (Fig. 11; right panel). The resting permeability values (cm s⁻¹) of ions were selected to give a resting E_m of −66.7mV (Fig. 11 a, b; P_K=8.0×10⁻⁵ (blue), P_Na=3.04×10⁻⁶ (dark blue), P_Cl=2.0×10⁻⁷ (red), P_HCO=4.0×10⁻⁸ (grey)). During the GABA_A R like robust increase in anion permeability, the maximum P_Cl of 3.0×10⁻⁴ cm s⁻¹ (and P_HCO of 0.6×10⁻⁴ cm s⁻¹) was set to occur 30ms after the onset (Fig. 11 b). In the absence of anion permeation the [Cl⁻]i remains at the steady state level of 3.6mM (Fig. 11 c; red dots), whereas the increase in Cl⁻ permeability resulted in a rapid increase in [Cl⁻]i, from the steady state of 3.6mM to 16.4mM (Fig. 11 d; red dots). The initial hyperpolarization and the early part of depolarizing shift occurring during the stimulation train are likely represent changes in E_GABA (and [Cl⁻]) rather accurately, but the late pronounced depolarization following the stimulation train may itself produce small, voltage activated changes in membrane permeability. Therefore, the decay of anion permeability was chosen to be slightly faster than that of HFS evoked conductance (Kaila et al., 1997).

For simplicity, the changes in intracellular Cl⁻ and extracellular K⁺ levels were considered independent at this stage (see p. 79 for an interdependent K⁺/Cl⁻ flux model). As the stimulation artefacts often compromised the early phase of K⁺ electrode signal, the modelling of HFS responses was done with hypothetical responses arising linearly from 3mM baseline to Δ[K⁺]₀ of 3mM, 9mM and 22mM within 2 seconds (Fig. 11 c, d; indicated with blue dots). The range of 3mM–22mM covers well or exceeds markedly the amplitudes of recorded Δ[K⁺]₀ responses, ranging from
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~2.4mM to ~6.1mM with a mean of ~3.7mM (n=12–15), (see Fig. 3 a). To illustrate the time course of \([K^+]_o\) recordings, an averaged trace (mean; dark grey line, ±S.E.M grey line) is shown as a reference (peak \([K^+]_o\) 7.69mM, n=8) (Fig. 11 c, d).

The \(E_m\) profiles given by the GHK model are presented in (Fig. 11 e, f; black dots), superimposed on the averaged recording trace (mean; dark grey, ±S.E.M.; grey, n=4). The initial fast hyperpolarization and the early depolarizing shift were well reproduced with increased anion permeation and \(Cl^-\) accumulation. The increase in anion permeation did not affect the late depolarization markedly, but promoted the early depolarizing phase associated with 3mM and 9mM \([K^+]_o\) transients. Surprisingly, the assumed levels of constant \(P_K\) and \(P_{Na}\) resulted in a model \(E_m\) having rather low sensitivity to \([K^+]_o\). The \(Δ[K^+]_o\) of 3mM, 9mM and 22mM produced a late depolarization of 7.8mV, 18.5mV and 32.9mV, respectively, suggesting that the recorded \([K^+]_o\) transients of ~6mM may not evoke \(V_m\) depolarizations of ~20–25mV. This may indicate that the recorded ISM signals reflect underestimations of actual \([K^+]_o\) responses, or that during the peak depolarization voltage sensitive conductances are altered, reducing the \(P_K P_{Na}^{-1}\) (or \(P_K P_{Ca}^{-1}\)) permeability ratio.

The GHK models described above, all constructed using a known \(Cl^-\) accumulation rate, provided an opportunity to test the accuracy of [\(Cl^-\)] \(i\) and \(d[Cl^-]/dt\) calculations (based on the assumption that during the HFS train \(V_m\) equals \(E_{GABA}\)) and compare the results with those obtained from actual recordings. The \(E_{3rd}\) of ~74.6mV of all \(E_m\) models resulted in a [\(Cl^-\)] \(i\) of 4.89mM that was only slightly higher that the pre-set level of 4.32mM. The \(E_{last}\) values associated with 3mM, 9mM and 22mM \(Δ[K^+]_o\) responses were ~59.0mV, ~58.2mV and ~56.6mV, corresponding [\(Cl^-\)] levels of 11.4mM, 11.8 and 12.8mM, respectively, all quite close to the defined value of 12.7mM. Thus the GHK based calculations of Part III, which neglected all other permeabilities except those associated with \(GABA_A R\) activation, give relatively accurate estimates of [\(Cl^-\)] \(i\) levels. For comparison of the HFS parameters of modelled \(E_m\) and recorded \(V_m\) responses, see Table 4.
Figure 11. The effects of $[K^+]_o$ and HFS evoked GABA$_A$R like anion permeation on membrane potential modelled with the Goldman-Hodgkin-Katz formalism. The voltage equations were written for CO$_2$/HCO$_3^-$ conditions in the absence (left panel) or in the presence (right panel) of anion permeation and Cl$^-$ accumulation. (a, b) The dominant ion permeabilities ($P_{ion}$) setting the membrane potential of model ($E_m$) at rest were suggested to be $P_K$ (blue) and $P_{Na}$ (dark blue), the values of which were selected to give an $E_m$ of $-66.7$ mV ($P_{Na}/P_K^{-1}=0.038$). The permeability profile of Cl$^-$ and HCO$_3^-$ intended to mimic GABAergic HFS responses is shown in (b) ($P_{Cl}$ (red), $P_{HCO}$ (grey); $P_{HCO}/P_{Cl}^{-1}=0.2$ throughout the response). (c, d) The intracellular Cl$^-$ of 3.6mM increased up to 16.4mM ([Cl$^-$]$_i$ levels marked with red dots) during the anion permeation period. The effects of $[K^+]_o$ on $E_m$ was evaluated using linearly increasing K$^+$ levels reaching values of 6mM, 12mM and 25mM (illustrated with blue dots, baseline 3mM). The $[K^+]_o$ accumulation profile of recorded traces (n=8) having an amplitude close to $\sim$7mM is shown as a reference (mean; dark grey, ±S.E.M; grey). (e, f) The $E_m$ values given by the GHK model are presented with black dots, superimposed on recorded $V_m$ trace (mean; dark grey, ±S.E.M.; grey, n=4). The combination of increased anion permeation and Cl$^-$ accumulation replicated well the initial part of biphasic responses, irrespectively of the amplitude of K$^+$ responses. Notably, after the brief hyperpolarization, the Cl$^-$ accumulation promoted the early depolarization phase of 6mM and 12mM [K$^+$]$_o$ responses. The effect of 6mM [K$^+$]$_o$ on late depolarization was rather modest, predicting a depolarization of 7.8mV, whereas the [K$^+$]$_o$ transients of 12mM and 25mM depolarized the $E_m$ for 18.5mV and 32.9mV, respectively, thus approaching, or exceeding, the mean level of $\sim$25mV observed in this study. Assuming that the $P_K/P_{Na}^{-1}$ ratio selected to give the recorded resting $V_m$ level remains constant, the resulting $E_m$ model appears to be rather insensitive to an increase in $[K^+]_o$. 

\[
\begin{align*}
\text{(a)} &
\text{(b)} \\
0 &
4e-4 \\
3e-4 &
1e-4 \\
0 &
1e-4 \\
\text{(c)} &
\text{(d)} \\
24 &
21 \\
18 &
15 \\
12 &
9 \\
6 &
3 \\
\text{(e)} &
\text{(f)} \\
-30 &
-60 \\
-90 &
-60 \\
-60 &
-90 \\
0 &
1 &
2 &
3 \\
0 &
1 &
2 &
3 \\
\text{Time (s)} &
\text{Time (s)}
\end{align*}
\]
8.3.7.2 GABA<sub>A</sub>R agonist evoked current responses

The modelling of HFS responses with GHK voltage equation suggested that the V<sub>m</sub> shifts during the stimulation train could be described relatively well with an increase in anion permeability and an intracellular Cl<sup>−</sup> accumulation. However, the recorded V<sub>m</sub> levels after the HFS train deviated markedly from modelled E<sub>m</sub> levels, indicating that either the [K<sup>+</sup>]<sub>i</sub> recordings are inaccurate or that the voltage dependent changes in P<sub>Na</sub> or P<sub>K</sub> contribute to the depolarizing phase. If the latter alternative is true, then the whole-cell recordings of GABA<sub>A</sub>R agonist responses should obey the GHK current equations, as the voltage clamp used in those recordings should exclude all the voltage dependent changes in membrane permeability.

To this end, the GHK equations were written for four ions and the resulting currents carried by K<sup>+</sup>, Na<sup>+</sup>, Cl<sup>−</sup> and HCO<sub>3</sub><sup>−</sup> were summed to obtain a net membrane current I<sub>m</sub>. The resting permeability values of ions used in I<sub>m</sub> calculations were otherwise the same as in voltage equation, but the P<sub>Na</sub> was set to 3.2×10<sup>−6</sup> cm s<sup>−1</sup> (Fig. 12 d), which gave an E<sub>m</sub> of −66.0mV and a resting input resistance of 134MΩ. As the [K<sup>+</sup>]<sub>i</sub> measurements of agonist responses often contained tolerable artefacts, the I<sub>m</sub> models (unlike the E<sub>m</sub> models) were constructed using recorded [K<sup>+</sup>]<sub>i</sub> transients. The averaged [K<sup>+</sup>]<sub>i</sub> responses (mean; dark grey, ±S.E.M.; grey) evoked with muscimol iontophoresis (left panel) or with bath-applied isoguvacine (right panel) are presented in (Fig. 12 b) and the predicted profiles of P<sub>HCO</sub>, P<sub>Cl</sub> and [Cl<sup>−</sup>]<sub>i</sub> accumulation are shown in (Fig. 12 c, d). In the insets of (Fig. 12 a) the IV profile of resting I<sub>m</sub> (grey) is shown together with the modelled muscimol evoked peak conductance of ~16.7nS (left panel; black) or with the modelled isoguvacine evoked peak conductance of ~5.3nS (right panel; black).

For the calculations of I<sub>m</sub> produced by the muscimol iontophoresis, a holding voltage (E) of −63.0mV was selected to obtain the recorded small positive holding current. The assumed maximal P<sub>Cl</sub> of 1.6×10<sup>−4</sup> cm s<sup>−1</sup> and P<sub>HCO</sub> of 0.32×10<sup>−4</sup> cm s<sup>−1</sup> was reached 250ms after the response onset. The intracellular [Cl<sup>−</sup>] was set to increase from 3.6mM to 22.4mM while the recorded [K<sup>+</sup>]<sub>i</sub> increased from 3mM to 3.85mM. Isoguvacine induced I<sub>m</sub> responses were calculated at an E of −66.0mV. The predicted increase in P<sub>Cl</sub> and P<sub>HCO</sub> was set to be smaller and slower, the peak values of 0.5×10<sup>−4</sup> cm s<sup>−1</sup> and 0.1×10<sup>−4</sup> cm s<sup>−1</sup>, respectively, were attained in 10s. The [Cl<sup>−</sup>]<sub>i</sub> was assumed to increase from 3.6mM to 14.4mM and the recorded [K<sup>+</sup>]<sub>i</sub> increased from 3mM to 4.4mM, both peaking roughly ~35s after the response onset.

The I<sub>m</sub> results of muscimol (left panel) and isoguvacine (right panel) responses are shown in (Fig. 12 a; black dots), superimposed on recorded whole cell currents traces (mean; dark grey, ±S.E.M.;
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grey). Both the muscimol $I_m$ and the isoguvacine $I_m$ appear essentially identical with recorded currents, indicating that under voltage clamp the currents generated by the robust GABA$_A$R activation can be modelled assuming a constant $P_K$ and $P_{Na}$. This suggests that the HFS evoked late depolarization is likely to contain voltage sensitive changes in cation permeability. The small net inward current due the increase in $[K^+]_o$ only is marked with blue dots in (Fig. 12 a).

![Graph](image_url)

**Figure 12.** The effects of $[K^+]_o$ and GABA$_A$R agonists evoked anion permeation on membrane currents modelled with Goldman-Hodgkin-Katz formalism. (a) The models of muscimol iontophoresis (left panel) and bath-applied isoguvacine (right panel) currents ($I_m$) are illustrated with black dots, superimposed on whole-cell voltage clamp traces recorded under CO$_2$/HCO$_3^−$ conditions (mean; dark grey, S.E.M.; grey, n=6–13). Note the nearly perfect fit to the recorded data. The recorded $[K^+]_o$ transients (shown in b, mean; dark grey, ±S.E.M.; grey, n=4–6) induced a small inward current in the absence of anion permeation (marked with blue dots in (a)). The $I_m$ models of muscimol and isoguvacine responses were constructed using the ion concentration and ion permeability ($P_{ion}$) profiles summarized in (c, d). The resting permeability values ($P_{HCO}$ (grey) / $P_{Cl}$ (red) =0.2, $P_{Na}$ (dark blue) / $P_{K}$ (blue) =0.04) were selected to give a resting $V_m$ of −66.0mV and a resting input resistance of 134MΩ. The IV curves of modelled resting membrane (grey) and muscimol and isoguvacine responses (black) are shown in the insets of (a) and the application periods of agonists are marked with horizontal lines below traces in (a, b).
8.3.8 Ionic environment of outward KCC transport

8.3.8.1 The effects of experimental conditions

Previous modelling work based on the GHK constant-field theory provided further support for the conclusion that a strong GABA_A R activation leads to an increase in intracellular Cl^- level, which then may promote the thermodynamic activation of secondary active K^+/Cl^- extrusion. To resolve the thermodynamic equilibrium conditions of KCC2 mediated outward transport, the author estimated the free energy levels during a hypothetical HFS response under CO_2/HCO_3^- and HEPES/formate conditions. Both [Cl^-]_o and [K^+]_i were assumed to stay constant and the rate of change in [Cl^-]_i and [K^+]_o were considered independent. The averaged [K^+]_o transient profile (cf. Fig. 11 c, d) arising from 3.0mM to 7.03mM (E_K shift from −95.3mV to −72.8mV) was used in calculations both under CO_2/HCO_3^- and HEPES/formate conditions to emphasize the effects of intracellular Cl^- on KCC2 transport (Fig. 13 a).

A change in free energy was estimated as previously described (see p. 31) during a transient increase in [Cl^-]_i that was in parallel with a [K^+]_o accumulation. In line with the results of GHK voltage model, the [Cl^-]_i accumulation was set to proceed from 3.6mM to 16.4mM in CO_2/HCO_3^- (E_Cl change from −94.5mV to −54.6mV) and from 10.0mM to 22.8mM in HEPES/formate (E_Cl shift from −63.3mV to −41.6mV). Notably, under the resting CO_2/HCO_3^- conditions the KCC2 seem to be very close to equilibrium, and the rapid [Cl^-]_i accumulation markedly increased the free energy of outward transport indicating that the thermodynamic activation of KCC2 is indeed a plausible scenario. However, due to the higher baseline level of [Cl^-], the outward KCC2 was well fuelled in HEPES/formate and the further increase in [Cl^-]_i had only a modest effect (Fig. 13 b).
**RESULTS**

**Figure 13.** Changes in the Nernst equilibrium potentials of potassium and chloride ions and the thermodynamic conditions of KCC2 mediated outward transport in response to a HFS train. (a) The shifts in reversal potentials of Cl\(^-\) (black lines) and K\(^+\) (grey line) in the HEPES/formate and in the CO\(_2\)/HCO\(_3\)^- buffered solution. The intracellular Cl\(^-\) (black lines) and extracellular K\(^+\) (grey line) concentrations used in E\(_K\) and E\(_{Cl}\) calculations are shown in the inset. (b) Under a steady state conditions the KCC2 is very close to thermodynamic equilibrium in the CO\(_2\)/HCO\(_3\)^- and clear outward direction is revealed only after the HFS induced Cl\(^-\) accumulation, but due to the higher steady state level of [Cl\(^-\)], the K\(^+\)/Cl\(^-\) transport is constantly outward in the HEPES/formate. The duration of HFS stimulation train is marked with a black horizontal line below traces in (a, b).
8.3.8.2 The extrusion efficacy of KCC2

The preceding GHK models of Cl\(^-\) accumulation and the studies of KCC2 energetics suggested that the increase in \([\text{Cl}^-]_i\) under CO\(_2/\text{HCO}_3^-\) conditions rapidly fuels the outward K\(^+\)/Cl\(^-\) transport. The obvious drawback of all previous examinations is that the changes in \([\text{Cl}^-]_i\) and \([\text{K}^+]_o\) were treated as independent phenomena, which fails to describe the hallmark feature of KCC2 transport, the 1:1 coupling. The somatic recordings of CA1 pyramids suggested that the Cl\(^-\) influx via GABA\(_A\)Rs can be as high as 13–15mM s\(^{-1}\), which is at least twice faster than the maximum Cl\(^-\) extrusion rate of 5–7mM s\(^{-1}\) encountered in the dendrites of CA1 pyramidal neurons (Staley & Proctor, 1999). Assuming that the HFS evoked increase in \([\text{K}^+]_o\) is exclusively mediated by the K\(^+\)/Cl\(^-\) cotransport having 1:1 kinetics, the \([\text{K}^+]_o\) should increase at the same rate as the \([\text{Cl}^-]_i\) decreases, if the intracellular volume fraction (\(V_{\text{ol}}\)) is equal with the extracellular volume fraction (\(V_{\text{o}}\)).

As the extracellular volume fraction has been estimated to cover ~20% of neuronal tissue volume (McBain et al., 1990; Nicholson & Phillips, 1981), (for review, see Sykova & Nicholson, 2008) the author created two models that should define the ends in between the actual volume relation should exist. In the lower end the \(V_{\text{ol}}\) was set to be equal with the \(V_{\text{o}}\) (\(V_{\text{ol}}=V_{\text{o}}\)) (Fig. 14 a, c, e) and the higher end expressed the theoretical upper limit, having the intracellular space that was four times larger than the extracellular space (\(4V_{\text{ol}}=V_{\text{o}}\)) (Fig. 14 b, d, f).

Within the intracellular compartment of both models, the transient Cl\(^-\) influx was assumed to be analogous with the GHK voltage model results, arising from the baseline of 3.6mM to 16.4mM, with a \(d[\text{Cl}^-]/dt\) of 12.8mM s\(^{-1}\). As the investigation of KCC2 energetics suggested a near-equilibrium resting state (cf. Fig. 13 b) even a slight increase in \([\text{Cl}^-]_i\) may lead to an enhancement of outward transport. This rapid activation of KCC2 was described by applying a constant 1:1 extrusion of K\(^+\) and Cl\(^-\) (\(\Delta\mu_\text{n}; \text{grey and black upward arrows, respectively}\)) in parallel with the transient Cl\(^-\) influx (\(\Delta\gamma\); black downward arrow). Three K\(^+\)/Cl\(^-\) extrusion rates were used (\(\Delta\mu_1=7\text{mM s}^{-1}\), \(\Delta\mu_2=1\text{mM s}^{-1}\) and \(\Delta\mu_3=0.14\text{mM s}^{-1}\)), the largest figure being the fastest rate estimation given by Staley & Proctor (1999). Each step down reduced the K\(^+\)/Cl\(^-\) extrusion rate for ~85% in an attempt to mimic the effect of furosemide. The net change in intracellular chloride content (\(\Delta[\text{Cl}^-]_i\)) was the sum of influx and efflux (\(\Delta\gamma-\Delta\mu_\text{n}\)). Despite the K\(^+\) efflux the \([\text{K}^+]_i\) of 110mM was assumed to stay constant.

Within the extracellular space of models the ion shifts were identical (\(V_{\text{ol}}=V_{\text{o}}\)) or four times larger (\(4V_{\text{ol}}=V_{\text{o}}\)) than the intracellular shifts. The net change in extracellular chloride (\(\Delta[\text{Cl}^-]_o\))...
was a sum of Cl\(^-\) influx and Cl\(^-\) efflux (\(\Delta \mu_n-\Delta \gamma\)), and the net K\(^+\) accumulation to extracellular space (\(\Delta [K^+]_o\)) was determined by the rate of K\(^+\)/Cl\(^-\) transport (\(\Delta \mu_n\)). Briefly, if Vol\(_o\)=Vol\(_i\), then interstitial ions shifts were; \(\Delta [Cl^-]_o=\Delta \mu_n-\Delta \gamma\) and \(\Delta [K^+]_o=\Delta \mu_n\), or, if 4Vol\(_o\)=Vol\(_i\), then interstitial shifts were; \(\Delta [Cl^-]_o=4(\Delta \mu_n-\Delta \gamma)\) and \(\Delta [K^+]_o=4\Delta \mu_n\) (Fig. 14 a, b).

The time courses of extra (\(-\Delta \gamma\), \(-4\Delta \gamma\)) and intracellular Cl\(^-\) shifts (\(\Delta \gamma\)) in the absence of K\(^+\)/Cl\(^-\) transport are marked with dotted lines and the effect of ion extrusion on Cl\(^-\) and K\(^+\) shifts are depicted with black and grey lines, respectively (Fig. 14 e, d). The fastest \(\Delta \mu_1\) suppressed the intracellular Cl\(^-\) accumulation and led to a rapid (~1.8s) recovery from transient [Cl\(^-\)]\(_i\) load (\(\Delta \gamma-\Delta \mu_1\)). The \(\Delta \mu_2\) did not affect the channel mediated Cl\(^-\) accumulation and introduced a full recovery of Cl\(^-\) gradient in ~13s (\(\Delta \gamma-\Delta \mu_2\)) and with \(\Delta \mu_3\) the recovery was accomplished in ~90 seconds (\(\Delta \gamma-\Delta \mu_3\)).

As the extrusion of K\(^+\)/Cl\(^-\) also reduced the transmembrane K\(^+\) gradient needed to fuel outward transport, within the Vol\(_o\)=Vol\(_i\) compartment model the extrusion rate of 7mM s\(^{-1}\) produced a near instant (0.9s) exhaust of free energy (\(\Delta \gamma-\Delta \mu_1\)). With the slower rate of 1mM s\(^{-1}\) the equilibrium conditions were attained in 6.7s (\(\Delta \gamma-\Delta \mu_2\)) and at the speed of 0.14mM s\(^{-1}\) the transport remained outward for ~44s (\(\Delta \gamma-\Delta \mu_3\)) (Fig. 14 e). Within the 4Vol\(_o\)=Vol\(_i\) model the extrusion rates of 1.0mM s\(^{-1}\) (\(\Delta \gamma-\Delta \mu_2\)) and 0.14mM s\(^{-1}\) (\(\Delta \gamma-\Delta \mu_3\)) reached equilibrium conditions in 3.2s and ~23s, respectively (Fig. 14 f). The change in free energy introduced by the Cl\(^-\) influx only is shown as a reference (\(\Delta \gamma\); dotted line) (Fig. 14 e, f).
RESULTS

Figure 14. The properties of compartment models in which potassium and chloride fluxes were studied. (a, b) Net fluxes between intracellular (white area) and extracellular (grey area) volume fraction (left panel; Vol_o=Vol_i, right panel; 4Vol_o=Vol_i) were comprised of transient influx of Cl\(^-\) that was in parallel with 1:1 efflux of Cl\(^-\) (black arrows) and K\(^+\) (grey arrow). Three K\(^+\)/Cl\(^-\) efflux rates of 7mM s\(^{-1}\) (\(\Delta\mu_1\)), 1mM s\(^{-1}\) (\(\Delta\mu_2\)) and 0.14mM s\(^{-1}\) (\(\Delta\mu_3\)) are presented in the left panel (Vol_o=Vol_i) and only the latter two in the right panel (4Vol_o=Vol_i). The transient Cl\(^-\) influx led to a net increase in [Cl\(^-\)]\(_i\) (\(\Delta\gamma\)) and an equal (-\(\Delta\gamma\)) or four time larger (-4\(\Delta\gamma\)) reduction in a [Cl\(^-\)]\(_o\) (c, d; black dotted lines). The constant K\(^+\)/Cl\(^-\) efflux (\(\Delta\mu_n\)) suppressed the \(\Delta\gamma\) generated Cl\(^-\) transients in both compartments, resulting a net \(\Delta[\text{Cl}^-]\) of \(\Delta\gamma-\Delta\mu_n\) and a net \(\Delta[\text{Cl}^-]\) of 4(\(\Delta\mu_n-\Delta\gamma\)) (c, d; black lines). The K\(^+\)/Cl\(^-\) efflux led also to an increase in [K\(^+\)]\(_o\) at the rate of \(\Delta\mu_n\) in the Vol_o=Vol_i model and at the rate of 4\(\Delta\mu_n\) in the 4Vol_o=Vol_i model (e, d; grey lines). The changes in the free energy of outward K\(^+\)/Cl\(^-\) transport during the ion shifts described in (c, d) are shown in (e, f).
The predicted changes in extracellular $[K^+]_o$ (grey lines) within Vol_o=Vol_i model (left panel) and 4Vol_o=Vol_i model (right panel) are shown superimposed on the recorded $[K^+]_o$ traces (Fig. 15 a, b; upper panel). The endpoint of each grey line (if within the region of interest) describes the $[K^+]_o$ level at the equilibrium of $K^+/\text{Cl}^-\text{transport (cf. Fig. 14 e, f).}$ Irrespective of the transport rate, the maximal $[K^+]_o$ reached within the Vol_o=Vol_i model was between 9.1–9.3mM (Fig. 15 a) and within the 4Vol_o=Vol_i model around 15.8mM (Fig. 15 b). The reference $[K^+]_o$ recordings represent the mean ($\pm$S.E.M) of selected HFS\textsuperscript{high} and HFS\textsubscript{low} transients.

In the lower panel of (Fig. 15 a, b) the changes in $E_{\text{GABA}}$ (black lines) during intra- and extracellular Cl$^-\text{shifts are shown, each visible endpoint marking the equilibrium of }K^+/\text{Cl}^-\text{transport. Within the Vol_o=Vol_i model the recovery from Cl}^-\text{influx ended to an }E_{\text{GABA}}\text{value that corresponded a }[\text{Cl}^-]_i\text{level of 9.74mM } (\Delta\gamma-\Delta\mu_1), 10.21mM (\Delta\gamma-\Delta\mu_2) \text{ or } 10.3mM (\Delta\gamma-\Delta\mu_3). The }\Delta[\text{Cl}^-]_i\text{, rates (}\Delta\gamma-\Delta\mu_2, \Delta\gamma-\Delta\mu_3\text{) applied to the 4Vol_o=Vol_i model gave a }[\text{Cl}^-]_i\text{ level of } \sim13.2mM \text{ at the equilibrium.}

The intracellular Cl$^-\text{(}\Delta\gamma-\Delta\mu_n\text{) and the corresponding extracellular Cl}^-\text{(}\Delta\mu_n-\Delta\gamma \text{ and } 4(\Delta\mu_n-\Delta\gamma))\text{ and } K^+(\Delta\mu_n \text{ and } 4\Delta\mu_n)\text{ transients were then applied to the previously described GHK voltage model (see p. 74). The results are illustrated in (Fig. 15 c, d) with black lines, representing the }E_m\text{ values when the }K^+/\text{Cl}^-\text{ extrusion rates (determined on the intracellular side) of } 7mM \text{ s}^{-1}, 1mM \text{ s}^{-1} \text{ and } 0.14mM \text{ s}^{-1}\text{ were applied to the Vol_o=Vol_i model (c), and when the rates of } 1mM \text{ s}^{-1} \text{ and } 0.14mM \text{ s}^{-1}\text{ were incorporated to the 4Vol_o=Vol_i model (d). As previously, the resulting }E_m\text{ traces were analysed using the modified GHK equation to obtain } d[\text{Cl}^-]/dt\text{ values (and other parameters, see Table 4. for details). The predicted } \sim85\%\text{ inhibition of fastest }K^+/\text{Cl}^-\text{ extrusion rate in the Vol_o=Vol_i model (7mM s}^{-1} \text{ to 1mM s}^{-1}\text{) increased the initial Cl}^-\text{accumulation from 11.94mM s}^{-1} \text{ to 13.33mM s}^{-1}\text{, but the further } \sim85\%\text{ reduction of 1mM s}^{-1}\text{ extrusion to 0.14mM s}^{-1}\text{ had only a minor effect on Cl}^-\text{accumulation rate, ending to a value of 13.52mM s}^{-1}\text{. However, the } \sim85\%\text{ reduction in }K^+/\text{Cl}^-\text{ efflux (1mM s}^{-1} \text{ to 0.14mM s}^{-1}\text{) in the 4Vol_o=Vol_i model predicted a slight decrease in the Cl}^-\text{accumulation, from 17.25mM s}^{-1} \text{ to 17.03mM s}^{-1}\text{. This indicates that under the 4Vol_o=Vol_i conditions the inhibition of KCC2 does not enhance the Cl}^-\text{ accumulation, although the }K^+\text{ extrusion is largely blocked. The averaged intracellular recordings of HFS responses (grey lines) are shown as reference (Fig. 15 a, b, c, d).}
RESULTS

Figure 15. The effect of intracellular $\text{K}^+/\text{Cl}^-$ extrusion rate on the extracellular potassium, $E_{\text{GABA}}$ and membrane potential levels of GHK model (a; upper panel) In the $\text{Vol}=\text{Vol}_1$ model the two fastest $\text{K}^+/\text{Cl}^-$ rates of $\Delta\mu_1$: 7mM s$^{-1}$ and $\Delta\mu_2$: 1mM s$^{-1}$ on the intracellular side predicted a $[K^+]_o$ transients which had a comparable rate of rise with the recorded high and low amplitude $[K^+]_o$ responses (mean±SEM illustrated as a reference), whereas the $\Delta\mu_3$ of 0.14mM s$^{-1}$ appeared to be too slow. The maximal amplitude of predicted $[K^+]_o$ shifts was slightly above 9mM when a new equilibrium state was reached (endpoints of $\Delta\mu_1$ and $\Delta\mu_2$ lines). (a; lower panel) As the fast $\text{K}^+/\text{Cl}^-$ extrusion rate also reduced the net intracellular $\text{Cl}^-$ accumulation ($\Delta\gamma-\Delta\mu_1$) the depolarizing shift in $E_{\text{GABA}}$ was clearly suppressed when compared to the averaged $V_m$ recordings (grey line). The net $[\text{Cl}^-]$ changes of $\Delta\gamma-\Delta\mu_2$ and $\Delta\gamma-\Delta\mu_3$ predicted $E_{\text{GABA}}$ shifts that were equal with recorded $V_m$ values. (b; upper panel) When applied to the $4\text{Vol}=\text{Vol}_1$ model also the slower rates of intracellular $\text{K}^+/\text{Cl}^-$ extrusion predicted $[K^+]_o$ shifts with reasonable rate of rise ($4\Delta\mu_2$: 4mM s$^{-1}$, $4\Delta\mu_3$: 0.56mM s$^{-1}$), and equilibrium conditions at $[K^+]_o$ levels above 15mM (endpoint of $4\Delta\mu_2$). (b; lower panel) Within the $4\text{Vol}=\text{Vol}_1$ model the net $[\text{Cl}^-]$ shifts of $\Delta\gamma-\Delta\mu_2$ and $\Delta\gamma-\Delta\mu_3$ were associated with a marked reduction in $[\text{Cl}^-]_o$ and predicted markedly faster and larger changes in $E_{\text{GABA}}$. (c, d) The abovementioned changes of $\text{Cl}^-$ and $\text{K}^+$ concentrations in intra- and extracellular compartments of $\text{Vol}=\text{Vol}_1$ and $4\text{Vol}=\text{Vol}_1$ models were then applied to the previously described GHK model of membrane potential. The predicted $E_m$ levels of GHK model are presented with black lines and averaged $V_m$ trace is shown as a reference (grey line).
### RESULTS

<table>
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<th>Recordings</th>
<th>$V_m$ (mV)</th>
<th>$E_{\text{GABA}}$ (mV)</th>
<th>$\Delta[\text{Cl}^-]_i$ (mM s$^{-1}$)</th>
<th>$\Delta V_{\text{depol}}$ (mV)</th>
<th>Peak $\Delta[\text{K}^+]_o$ (mM)</th>
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<td><strong>This study</strong></td>
<td>$-67.0\pm3.1$ (n=4)</td>
<td>$-77.3\pm2.2$ (n=4)</td>
<td>$13.19\pm1.43$ (n=4)</td>
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<td>$3.69\pm0.47$ (sp; n=15)</td>
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<td>$-66.2\pm2.7$ (n=5)</td>
<td>$-74.7\pm2.5$ (n=5)</td>
<td>$15.34\pm2.02$ (n=5)</td>
<td>$26.3\pm3.4$ (n=5)</td>
<td>$6.09\pm0.48$ (sp; n=4)</td>
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<td><strong>Kaila et al. (1997)</strong></td>
<td>$-74.2\pm1.4$ (n=19)</td>
<td>$-74.2\pm1.4$ (n=19)</td>
<td>$20.2\pm0.6$ (n=46)</td>
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<td>$1.9\pm0.3$ (sp; n=4)</td>
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<td>$\Delta[K^+]_o$ (mM s$^{-1}$)</td>
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<td>$E_{\text{GABA}}$ (mV)</td>
<td>$\Delta[\text{Cl}^-]_i$ (mM s$^{-1}$)</td>
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<td>$E_{\text{GABA}}$ (mV)</td>
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Table 4. The properties of recorded and modelled GABAergic HFS responses. The first two sets of recorded parameters of CA1 pyramidal cells (V_m, E_GABA, ∆[Cl]_i, and ∆V_{depol}) are from separate experiments. The interstitial ∆[K]_o of 6.09±0.48mM represents a subset of transients with high amplitude (n=4) selected from recordings with an average ∆[K]_o of 3.69±0.47mM (n=15), measured from CA1 stratum pyramidale (sp). The third set is adopted from Kaila et al. (1997), showing the E_GABA and ∆V_{depol} values of CA1 pyramids and compares the ∆[K]_o recordings from sp and stratum radiatum (sr) regions. The first GHK E_m model was combined with four [K^-]_o transients with peak values of 3.0mM, 9mM and 22mM, from which the latter two gave depolarizations that approached or exceeded, respectively, the recorded levels. Note that if the extracellular Cl^- level is assumed to stay constant, the model also predicts that a rapid increase in [K^-]_o will affect the results of ∆[Cl^-]_i calculations, giving faster Cl^- influx rates with increasing [K^-]_o transients. The GHK models 2 and 3 describe the effects of three (model 2) or two (model 3) interdependent [K^+/Cl^-]_o accumulation rates on model E_m, under conditions in which the intracellular volume is equal (model 2; Vol_o=Vol_i) or four times larger than the extracellular space (model 3; 4Vol_o=Vol_i). The GHK model 2 indicated that the reduction in [K^+/Cl^-]_o accumulation from 7mM s^{-1} to 1mM s^{-1} had a marked effect on the [Cl^-]_i accumulation. This implies that if the KCC2 mediated transport is instantly activated under Vol_o=Vol_i conditions, the application of furosemide should both increase the [Cl^-]_i accumulation and reduce the K^+ efflux. On the other hand, the GHK model 3 predicted that if the intracellular space is significantly larger than the extracellular space, the inhibition of [K^+/Cl^-]_o accumulation may not affect the [Cl^-]_i accumulation, despite the K^+ efflux is inhibited. Note that the late depolarizations (∆V_{depol}) of all E_m models are sensitive to [K^-]_o levels. The depolarization and [K^-]_o value of E_m model marked with an asterisk represent 1.8s points, otherwise 2s points are presented.
DISCUSSION

9 DISCUSSION

9.1 Main conclusions

The central finding of the part I was that the dopaminergic neurons of the substantia nigra \textit{pars compacta} express Cl\textsuperscript{−}2 chloride channels but are devoid of KCC2 and exhibit less hyperpolarizing GABA\textsubscript{A}R responses than the GABAergic cells of the substantia nigra \textit{pars reticulata} which contain KCC2 (Gulacsi \textit{et al.}, 2003). Thus the Cl\textsuperscript{−} regulation of dopaminergic neurons differs from the classical scheme, according to which an intraneuronal Cl\textsuperscript{−} level is in a site specific manner controlled by the Cl\textsuperscript{−} accumulation via NKCC1 and the Cl\textsuperscript{−} extrusion via KCC2 (Payne \textit{et al.}, 2003; Farrant & Kaila, 2007; Blaesse \textit{et al.}, 2009). The molecular machinery of intracellular chloride regulation in dopaminergic neurons was, at least partly, comprised of inward rectifying Cl\textsuperscript{−}2 channels (Smith \textit{et al.}, 1995) and a HCO\textsubscript{3}\textsuperscript{−} dependent Cl\textsuperscript{−} extrusion mechanism (most likely Na\textsuperscript{+} driven Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} anion exchanger). From the functional point of view, the chloride regulation of dopaminergic cells has some interesting properties. Due to the HCO\textsubscript{3}\textsuperscript{−} dependency and stoichiometry (exchange ratio of 1 Cl\textsuperscript{−} to 2 acid-base equivalents) the Cl\textsuperscript{−} extrusion via the Na\textsuperscript{+} driven Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchanger requires an intracellular acid load, and even in its presence, is rather limited (Kaila, 1994; Payne \textit{et al.}, 2003). In line with this, the intracellular chloride content was higher in the dopaminergic cells than in the GABAergic cells (~8mM vs. ~5mM, respectively). The difference in [Cl\textsuperscript{−}], became even larger in the nominal absence of bicarbonate (~17mM in the dopaminergic cells and ~5mM in the GABAergic cells) confirming that the Cl\textsuperscript{−} extrusion present in dopaminergic neurons is strongly dependent on the HCO\textsubscript{3}\textsuperscript{−} gradient. This suggests that the (KCC2 dependent) Cl\textsuperscript{−} extrusion of GABAergic cells is indeed considerably more efficient than that of dopaminergic cells.

In the work II of the Thesis a novel BDNF/TrkB mediated signalling pathway modulating the intraneuronal [Cl\textsuperscript{−}] regulation was described. The KCC2 mRNA and protein levels were shown to be down-regulated in response to an initiation of TrkB mediated signalling cascades, leading to a prolonged reduction in the Cl\textsuperscript{−} extrusion efficacy of hippocampal CA1 pyramidal neurons (Rivera \textit{et al.}, 2002). The TrkB receptor activation was accomplished either by exogenous agonist applications (BDNF or NT–4) or by evoking patophysiological activity patterns \textit{in vivo} and \textit{in vitro}, which in turn led to a massive release of BDNF (Rivera \textit{et al.}, 2002; Rivera \textit{et al.}, 2004). This seemingly paradoxical reduction of functional KCC2 by intense neuronal activity has been
DISCUSSION

associated with a reduced synaptic inhibition and seizures (Jin et al., 2005; Pathak et al., 2007; Ben Ari, 2006).

The work III was designed to address unresolved questions of dynamic ion shifts during an intense GABA_A receptor (GABA_A) activation, with particular emphasis on the generators of interstitial $[K^+]_o$ transients. The working hypothesis implicated a role for KCC2 as a significant K$^+$ extruder. The rapid HCO$_3^-$ (or formate) driven increase in $[Cl^-]_i$ would “thermodynamically” activate the electroneutral $1K^+:1Cl^-$ extrusion mediated by the KCC2, leading to an accumulation of $[K^+]_o$. If the KCC2 is to have a significant contribution to the $[K^+]_o$ transients, then the previously described activity induced down-regulation of KCC2 (Rivera et al., 2002; Rivera et al., 2004) might after all have stabilizing effects on neuronal network activity by reducing the K$^+$ load to interstitial space.

In the standard CO$_2$/HCO$_3^-$ buffered solution an intense GABA_A receptor (GABA_A) activation (achieved by synaptic high-frequency stimulation (HFS) or GABA_A receptor agonist application via perfusion, microinjection or iontophoresis) invariably produced $[K^+]_o$ transients, which were associated with the well described biphasic membrane potential or membrane current responses (Alger & Nicoll, 1979; Andersen et al., 1980; Lambert et al., 1991; Grover et al., 1993; Staley et al., 1995; Kaila et al., 1997; Smirnov et al., 1999). The experiments done under CO$_2$/HCO$_3^-$ conditions and nominally HCO$_3^-$ free, HEPES buffered conditions recapitulated the previous findings that a stable transmembrane gradient of GABA_A receptor permeable weak-acid anions (either HCO$_3^-$ or formate) promote the K$^+$ responses and HFS evoked afterdischarges substantially (Kaila et al., 1997; Lamsa & Kaila, 1997; Ruusuvuori et al., 2004).

The clear, inhibitory effect of furosemide on GABA_A receptor dependent extracellular K$^+$ responses was taken as evidence for the role of KCC2 as the prime K$^+$ release mechanism. By far the largest $[K^+]_o$ responses (~9mM) were evoked by a strong HFS stimulation. Under CO$_2$/HCO$_3^-$ conditions furosemide suppressed the K$^+$ transients by ~70% indicating a marked KCC2 contribution. The inhibitory effect of furosemide on HFS evoked K$^+$ transient was also evident in the HEPES/formate solution. The HFS induced initial Cl$^-$ accumulation rates and the steady state Cl$^-$ levels were derived from intracellular recordings of CA1 pyramidal neurons and both values remained largely intact in furosemide. In line with the HFS experiments, the $[K^+]_o$ responses evoked with GABA_A receptor agonists under HEPES/formate conditions were inhibited by furosemide (30–60%) but not completely abolished by GABA_A receptor antagonists, leaving a residual $\Delta [K^+]_o$ of 20–30%. When assessed relative to the K$^+$ transient component sensitive to GABA_A receptor antagonists furosemide (0.5–1.0mM) reduced the responses by 40–75% in a dose dependent manner, again indicating the role of KCC2 in the generation of GABA_A receptor dependent $[K^+]_o$ transients. Furosemide did not inhibit carbonic anhydrase activity either and the profound increase in GABA_A receptor agonist evoked $[K^+]_o$
transients observed in the presence of Ba\(^{2+}\) suggested that the contribution of Ba\(^{2+}\) sensitive K\(^+\) channels to the K\(^+\) release is likely to be negligible.

### 9.2 Ion gradients and the effect of furosemide

Formate is more permeant to GABA\(_A\)Rs than HCO\(_3^-\) (Bormann et al., 1987), predicting a depolarizing shift in \(E_{\text{GABA}}\). However, the larger permeability of formate was not enough to explain fully the positive shifts in initial \(E_{\text{GABA}}\) values, suggesting changes in \([\text{Cl}^-]\) regulation. Indeed, the calculations of baseline \(\text{Cl}^-\) levels indicated significantly higher concentrations under HEPES/formate (~10mM) than CO\(_2\)/HCO\(_3^-\) conditions (~5mM). Due to the marked depolarizing shift in \(E_{\text{GABA}}\) in the HEPES/formate, both the number of excited cells and the spiking rate of neurons may increase leading to a relatively larger portion of non-synaptically released K\(^+\), which may explain, at least partly, the smaller inhibitory effect of furosemide on \([K^+]_o\) shifts in the HEPES/formate (~30%) than in the CO\(_2\)/HCO\(_3^-\) solution (50%–70%).

Regarding the suggested dual role of KCC2 as a \([\text{Cl}^-]\) regulator and K\(^+\) extruder, the HFS results are somehow confusing. It seems that when assessed with HFS, the KCC2 plays no role in the steady state \([\text{Cl}^-]\) regulation but is involved in the K\(^+\) extrusion only. However, the expected increase in \([\text{Cl}^-]\) level following the KCC2 inhibition was readily detected both in single-pulse stimulation (increase ~1.6mM) and muscimol iontophoresis (increase ~2.6mM) experiments done in CO\(_2\)/HCO\(_3^-\) solution, confirming that the furosemide sensitive K\(^+\)/Cl\(^-\) transport has an effect on \([\text{Cl}^-]\) level, in line with the experiments done two decades ago (Misgeld et al., 1986; Thompson & Gahwiler, 1989). It was also noted that furosemide reduced the GABA\(_A\)R conductance slightly, probably due to the inhibition of somatic sub-population of receptors (Pearce, 1993), (see Table 3.).

### 9.3 The effects of potassium and chloride gradients on neuronal membrane

The GHK based \(E_m\) model introduced by the author is in line with a previous report showing that CA1 pyramidal neurons are slightly depolarized (~7mV) in response to an increase in \([K^+]_o\) from 3.5mM to 7.5mM (Jensen et al., 1993) and with the former GHK based description explaining the depolarizing effects of \([K^+]_o\) increase (Kaila et al., 1997). The rather surprising outcome of the \(E_m\) model was that the recorded \([K^+]_o\) transients appeared to be too small to support the late HFS depolarization. Nearly twice larger \([K^+]_o\) shifts were needed to bring the \(E_m\) close to the recorded \(V_m\) levels. When the recorded large amplitude K\(^+\) transients were combined with \(\Delta[\text{Cl}^-]_o\) and \(\Delta[\text{Cl}^-]_i\) profiles used in \(\text{Vol}_o=\text{Vol}_i\) and \(4\text{Vol}_o=\text{Vol}_i\) compartment models the predicted \(E_m\) levels
approached the recorded $V_m$ trace levels but did not reach them. One explanation might be that the insertion of ISM to a slice interior artificially extends the extracellular space. Such recording conditions would low-pass filter the $[K^+]_o$ transients, giving responses with smaller amplitudes slower kinetics. If so, then the true $[K^+]_o$ response amplitudes might actually be as large as predicted by the GHK model of $E_m$. However, the furosemide results showing that the HFS evoked $[K^+]_o$ transients were markedly suppressed without any effect on the late depolarization do not support this interpretation.

Another explanation may arise from the notion that action potentials evoked by the HFS emerged at voltages $\sim 10$ mV more positive than action potentials triggered by a direct current injection (cf. Fig. 4). Rather than being an indicative of actual shift in threshold, the author believes that Na$^+$ channels became activated at the same $V_m$ level irrespective of the stimulation method. The massive HFS induced $G_{GABA}$ dissipates the $Cl^-$ gradient and drives the $V_m$ towards and beyond action potential threshold, but at the same time prevents the fast regenerative spikes efficiently. The partial activation of Na$^+$ channels generates a small inward current that has a marked depolarizing effect on the $V_m$. Supporting this conclusion, the GHK equation of $E_m$ gives values which compare well to recorded $V_m$ traces, if $P_{Na}$ is increased from $3.04 \times 10^{-6}$ cm s$^{-1}$ to $1.22 \times 10^{-5}$ cm s$^{-1}$ after the hypothetical HFS train. Notably, in real recordings this putative 4$\times$ increase in $P_{Na}$ would have practically undetectable effects on total membrane conductance. Also the GHK $I_m$ models of GABA$\alpha$R agonist currents lend support for the conclusion that voltage activated conductance(s) is contributing to the late depolarization produced by HFS, as the currents recorded under whole-cell voltage clamp were well reproduced with appropriate ionic shifts and anion permeability profiles.

9.4 Potassium and chloride gradients and the efficacy of KCC2 transport

The $Cl^-$ influx rates obtained from intracellular recordings gave similar values under CO$_2$/HCO$_3^-$ and HEPES/formate conditions, although the furosemide sensitive fractions of HFS induced K$^+$ responses were clearly of different amplitudes ($\sim 4.2$ mM in CO$_2$/HCO$_3^-$ and $\sim 1.6$ mM in HEPES/formate). As the tight 1:1 coupling is the hallmark feature of KCC2 mediated ion transport and as the working hypothesis implies a direct causal relationship from the rapid increase in $[Cl^-]_i$ to the increase in $[K^+]_o$ via KCC2, one might expect a $[K^+]_o$ transients of equal amplitude in response to a nearly equal $[Cl^-]_i$ influx. Also the GHK based $E_m$ and $I_m$ models predicted that irrespectively of the GABA$\alpha$R stimulation method, the recorded increase in $[K^+]_o$ was always smaller than the increase in $[Cl^-]_i$, despite that the amplitude of $[K^+]_o$ responses was measured from
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extracellular space, which covers ~20% of total neuronal tissue volume (Nicholson & Phillips, 1981; Mc Bain et al., 1990), (for review, see Sykova & Nicholson, 2008). In addition the lack of effect of furosemide on the HFS induced changes in [Cl\(^-\)]\(_i\) became as surprise to the author, as the reduction in a Cl\(^-\) extrusion efficacy should be more easily detected with approaches that induce intracellular Cl\(^-\) load (Blaesse et al., 2009).

This motivated the author to consider the energetic of KCC2 transport and related ionic shifts in greater details. The thermodynamic inspections suggested that under CO\(_2\)/HCO\(_3\)\(^-\) conditions the outward KCC2 was at or very close to equilibrium, whereas under HEPES/formate conditions the transport was well fuelled throughout, due to the increased [Cl\(^-\)]\(_i\) level. The type of thermodynamic consideration used in this study give predictions of the direction, but not the rate, of transport (Kaila, 1994; Payne et al., 2003; Blaesse et al., 2009). As the increase in [Cl\(^-\)]\(_i\) under CO\(_2\)/HCO\(_3\)\(^-\) conditions may shift the KCC2 from equilibrium towards the outward state, it is in line with the interpretation of “accelerated” K\(^+\)/Cl\(^-\) extrusion (Voipio & Kaila, 2000; Payne et al., 2003). However, the shift of KCC2 from “outward” to “more outward” condition in the HEPES/formate solution cannot be used to predict an increase in the [K\(^+\)]\(_o\) accumulation. This notion, together with the more depolarized E\(_{GABA}\) responses that may increase the K\(^+\) release via voltage dependent conductances might further explain the reduction of GABA\(_A\)R dependent and furosemide sensitive K\(^+\) components brought about by the HEPES/formate solution.

To obtain a gross estimation of the effects of K\(^+\)/Cl\(^-\) extrusion rate on depolarizing E\(_{GABA}\) shifts and [K\(^+\)]\(_o\) transient amplitudes, the ionic shifts during hypothetical HFS train were estimated using two compartment models. Within the V\(_{o}=V_{li}\) model the fastest K\(^+\)/Cl\(^-\) extrusion rate of 7mM s\(^{-1}\) gave [K\(^+\)]\(_o\) shifts with an initial accumulation rate comparable to the recorded high-amplitude [K\(^+\)]\(_o\) traces. Such a high extrusion rate had also a profound effect on the [Cl\(^-\)]\(_i\) accumulation. As the actual intracellular recordings demonstrated that furosemide was without any measurable effect on the initial [Cl\(^-\)]\(_i\) accumulation, the realistic rate of KCC2 must be lower than 7mM s\(^{-1}\) (but see Staley & Proctor, 1999). The other evident conclusion is that the fast [K\(^+\)]\(_o\) shifts generated by KCC2 are unlikely in structures that have larger extracellular than intracellular space. The K\(^+\)/Cl\(^-\) extrusion of 1mM s\(^{-1}\) increased the [K\(^+\)]\(_o\) at rate that was close to that of measured low amplitude responses, and 0.14mM s\(^{-1}\) gave the [K\(^+\)]\(_o\) increase of too slow slope.

Due to the 1:1 coupling of K\(^+\)/Cl\(^-\) transport the amplitude of extracellular transients should become increasingly larger than the intracellular Cl\(^-\) shifts, if the former space gets smaller. This was well exemplified with the 4V\(_{o}=V_{li}\) model, which suggested that the high and low amplitude [K\(^+\)]\(_o\) responses can be produced with the extrusion rate of 1mM s\(^{-1}\) and 0.14mM s\(^{-1}\) (or with slightly
faster rate, as the volume relation is likely to be lower). Both tested K\(^+\)/Cl\(^-\) extrusion rates had only negligible effects on the depolarizing \(E_{\text{GABA}}\) shifts, suggesting that under a conditions close to the 4Vol\(_o\)=Vol\(_i\) model even the complete inhibition of fast KCC2 mediated [K\(^+\)]\(_o\) transients can be achieved without a detectable effect on the slope of \(E_{\text{GABA}}\).

However, as the extrusion rates capable of reproducing the high amplitude [K\(^+\)]\(_o\) responses were applied either to the Vol\(_o\)=Vol\(_i\) or to the 4Vol\(_o\)=Vol\(_i\) model, the free energy of outward transport became exhausted in few seconds, ending to a new post-stimulus [Cl\(^-\)]\(_i\) equilibrium level that was much higher than the previous baseline level. This contrasts the recording results, in which the recovery of [Cl\(^-\)]\(_i\) was evident, both in the absence and presence of furosemide.

As demonstrated by the Ba\(^{2+}\) experiments, the K\(^+\) buffering mechanisms may reduce the [K\(^+\)]\(_o\) accumulation. To maintain the conditions for outward KCC2 transport, the K\(^+\) buffering mechanisms (activated after a certain threshold or delay to allow the initial K\(^+\) accumulation) may expel the K\(^+\) from interstitial space at rate faster than the K\(^+\)/Cl\(^-\) extrusion via KCC2. The delayed but efficient K\(^-\) buffering might also have a role in explaining the [K\(^+\)]\(_o\)<[Cl\(^-\)]\(_i\) behavior of responses under conditions (Vol\(_o\)<Vol\(_i\)) required for the fast KCC2 mediated [K\(^+\)]\(_o\) transients.

9.5 How well the shifts in \(E_{\text{GABA}}\) represent intracellular chloride accumulation?

The GABA\(_A\)R mediated and HCO\(_3^\-) dependent Cl\(^-\) accumulation was originally described with intracellular ion selective recordings of crayfish muscle fibre (Kaila & Voipio, 1987;Kaila et al., 1989), but the depolarizing shift in neuronal \(E_{\text{GABA}}\) might as well arise from a reduction in [Cl\(^-\)]\(_o\) and an increase in [Cl\(^-\)]\(_i\). The compartment models created by the author assumed that the rapid increase in [Cl\(^-\)]\(_i\) reduced the corresponding ion concentration also on the extracellular side. Previous studies have shown that an exogenous GABA application rapidly reduced the [Cl\(^-\)]\(_o\) level in CA1 sp (and CA3 sp) region and increased slowly the [Cl\(^-\)]\(_o\) level in sr region (Muller et al., 1989). Thus the assumption of constant [Cl\(^-\)]\(_o\) often used in calculations determining the fast changes in [Cl\(^-\)]\(_i\) levels may not hold (Staley et al., 1995;Kaila et al., 1997;Smirnov et al., 1999;Staley & Proctor, 1999;Viitanen et al., 2010). This would predict that the [Cl\(^-\)]\(_i\) calculations based on somatic \(E_{\text{GABA}}\) recordings over-estimate the initial rate and the amplitude of [Cl\(^-\)]\(_i\) shift. In line with this interpretation, despite the biphasic GABAergic membrane current and voltage responses are fast and robust (Alger & Nicoll, 1979;Andersen et al., 1980;Lambert et al., 1991;Grover et al., 1993;Staley et al., 1995;Kaila et al., 1997;Smirnov et al., 1999), the [Cl\(^-\)]\(_i\) transients measured with imaging techniques appear to be modest and slow (Isomura et al.,
DISCUSSION

2003;Marandi et al., 2002;Berglund et al., 2008). The Cl$^-\,$ accumulation profile used in thermodynamic calculations was constructed assuming a constant [Cl$^-\,$]_o and may thus over-estimate the changes in [Cl$^-\,$]_i. If the intracellular Cl$^-\,$ accumulation is not as robust as expected, the post-stimulus equilibrium conditions of outward KCC2 are met earlier. In the case of 1mM s$^{-1}$ applied to 4Vol_o=Vol_i model, the time is less than 3.2s.

9.6 CCCs as regulators of neuronal computation modes

Neocortical principal neurons have been suggested to behave as a coincidence detectors, generating output only in response to precisely timed, simultaneous excitation, or as an integrator, combining arriving inputs over long time periods (König et al., 1996). Recent data on hippocampal pyramids suggest that both modes of operation can be applied to the single cell, the somatic region behaving as a coincidence detector, capable of discriminating and conveying signals in millisecond precision, whereas the dendritic region works as an integrator, combining and modifying both excitatory and inhibitory inputs (Pouille & Scanziani, 2001). Taking into an account the functional specifications of these intracellular compartments, it appears feasible that the inhibition mediated by the sub-classes of interneurons targeting to anatomically specific locations have also target specific properties (Buhl et al., 1994a;Miles et al., 1996;Freund & Katona, 2007;Klausberger & Somogyi, 2008). The various interneuron types have been shown to contribute differently to the generation and maintenance of rhythmic activity states, evoked or endogenous, present in hippocampal microcircuits. The rhythmic activity patterns cover a considerable wide range in frequency domain (from 4Hz to 200Hz) (Klausberger et al., 2003;Somogyi & Klausberger, 2005). Recent findings have demonstrated that the structure of feedback inhibitory circuitry has frequency dependent properties, shifting towards the dendritic region with increasing frequencies (Pouille & Scanziani, 2004). This means that for a given pyramidal cell, the recruitment of inhibitory synapses depends on the stimulation frequency. Thus in addition to site-specific features, the structure of inhibitory circuitry has also frequency dependent properties.

What about IPSPs? Do they change according to the input frequency? Compared to the underlying conductance, hyperpolarizing inhibition spreads longer both in time and in space, and thus has detrimental effects on the precision of integration in both domains (Farrant & Kaila, 2007). Due to the frequency (or activity) dependent collapse of Cl$^-\,$ gradient, the largely hyperpolarization based inhibition would gradually change to predominantly shunting inhibition, increasing the spatial precision of inhibition and shortening the integration window, allowing higher spike rates and better spike timing (Mann & Paulsen, 2007). In some modelling studies the robust shunting inhibition has
been shown to homogenize neuronal firing rates, leading to a robust $\gamma$-oscillations (Vida et al., 2006). The shunting may have also other beneficial consequences; the dendritic computations will be divided to smaller units, each of which is capable of reliably separating inputs with shorter intervals (London & Hausser, 2005; Magee & Johnston, 2005). This is to say, the information processing capacity of dendritic tree increases dramatically in time, ideal adaptation to meet the needs of increased information flow.

However, the information flow through neuronal circuits will cease at some point. The KCC2 would be essential in re-establishing the “idling” mode of neuron, defined by hyperpolarizing inhibition and dendritic properties suitable for integration and boosting of spatially and temporally distant inputs. Taking into account that the KCC2 is contributing to the increase in external $K^+$, together with GABA$_\alpha$R activity, the prevailing level of $[K^+]_o$ would represent the recent intensities of both inhibitory and excitatory synaptic activity. The activity induced alterations in ion gradients might lead to a situation where $E_{GABA}$ equals $V_m$, the latter of which is driven closer to the action potential threshold due to the increase in $[K^+]_o$. Thus the net excitation needed to trigger a spike would be smaller in the “processing” mode than in the “idling” mode. The external $K^+$ is assumed to modulate the intrinsic properties of neurons, affecting their spiking modes (Fröhlich et al., 2006) and being very sensitive to the changes in $[K^+]_o$, the KCC2 would be ideally tuned for adjusting the dendritic computation capacity according to the ongoing activity states. The postanl developmental pattern of KCC2 (Rivera et al., 1999) and its absence from midbrain dopaminergic neurons (Gulacsi et al., 2003) suggests that KCC2 is needed only in those neurons and networks in which the input frequency (or information flow) can vary considerably.

9.7 Putative conductive sources of extracellular potassium

The developed GHK models of $E_m$ hinted that modest voltage dependent Na$^+$ conductances might have a role in the late depolarization of pyramidal neurons. Then, naturally, the $[K^+]_o$ responses induced by HFS might contain components that are due to the activation of voltage sensitive $K^+$ conductances, masked by the large GABA$_\alpha$ conductance. $K^+$ efflux via leak conductances may also play a role, as the driving force for $K^+$ efflux increases markedly during the large depolarisations. The $[K^+]_o$ transients are suppressed by quinidine and quinine (Smirnov et al., 1999), known to have multitude inhibitory effects on e.g. gap junctions and voltage- and ATP sensitive $K^+$ channels (Juszczak & Swiergiel, 2009). Also the two-pore-domain potassium channels that contribute to the stimulation induced release of potassium from neurons are sensitive to quinidine and quinine (Päsler et al., 2007). The profound depolarizing shift during GABA$_\alpha$R responses might facilitate the
opening of low voltage sensitive Ca$^{2+}$ channels (Magee & Johnston, 1995) that activate Ca$^{2+}$ sensitive K$^+$ channels (Alger & Nicoll, 1980; Lancaster & Adams, 1986). As the non-synaptic GABA$_A$R antagonist insensitive fraction was nearly fully abolished by L-octanol, the octanol sensitive T-type Ca$^{2+}$ channels may contribute to the non-synaptic fraction of K$^+$ transients (Heady et al., 2001) via the Ca$^{2+}$ activated BK type K$^+$ channels (Sun et al., 2003). Among the K$^+$ channel types relatively insensitive to Ba$^{2+}$ (Lesage, 2003), the strongly outward rectifying TASK channel is activated by an extracellular alkalosis (Duprat et al., 1997) and might thus respond to the increase in cell surface pH evoked by formate or HCO$_3^-$ efflux via GABA$_A$R (Mason et al., 1990). Also the Na$^+$ activated K$^+$ channels may play a role (Brown et al., 2008; Bhattacharjee & Kaczmarek, 2005).
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ACKNOWLEDGEMENTS

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The experimental part of this study was carried out at the Department of Biosciences at the University of Helsinki during the years 2000–2006. The analysis and writing was initiated at the beginning of 2009 and then continued and accomplished at the Minerva Institute of Medical Research at the late 2009 and early 2010.

I owe gratitude to my supervisors prof. Juha Voipio and prof. Kai Kaila, who introduced the fascinating art of electrophysiology and neuroscience to me. I want to emphasize the importance of rigorous methodological training given by prof. Voipio; the biophysical understanding of his is truly phenomenal and under his excellent guidance I grow up to appreciate the electrophysiological techniques. The journey to scientific writing with my supervisors greatly extended my knowledge of the literature and improved the written argumentation. To write the Thesis in my way certainly did not make things any easier and brought up constrictions that I was completely unaware of.

It has been a great privilege to have prof. Kristian Donner and docent Jouni Sirviö as follow-up group members. They accurately pointed out the caveats of my Thesis project several years ahead and the conversations with them eased the frustration that I felt when the foreseeable problems were realized. Without their support, I may have crumbled even worst during the unbearable difficult periods of this project.

The instrumental comments and suggestions of prof. Staffan Johansson and senior assistant Vesa Paajanen improved the Thesis substantially and their expertise greatly helped me to shape the Thesis to its final form.

I want to thank all those who have provided help and guidance and have shared the moments of good and bad during these years. Of those persons that I have worked with, Tiina Huttu, accompanying me since 1997, has been by far the closest friend and colleague. Her openness towards the world have initiated such a numerous conversations, ranging from physics to behaviour, which have greatly extended the understanding of mine and provided cheerful moments both on and off duty. Without you, Tiina, this journey would have been far less interesting.

Special thanks to the Old School of Arkadiankatu; Eva Ruusuvuori, Marylka Uusisaari, Katri Wegelius, Pekka Paalasmaa, Sampsa Sipilä, Sergei Smirnov, Pekka Tallgren, and to the technicians of laboratory and animal facilities. The efficient but still joyful working atmosphere and enthusiastic scientific attitude encouraged me to initiate this journey.

I owe a great debt to prof. Kid Törnquist, whose truly altruistic aid was crucial during the late part of this project. The financial support provided by the Finnish Graduate School of Neuroscience, the
ACKNOWLEDGEMENTS

Academy of Finland and Sigrid Juselius Foundation was of outmost importance without which this study would not exist.

I am most grateful to my mother, father and sister, who have always provided help without asking and have encouraged me during these years.

And finally great thanks to Mari; this project of mine has been a huge burden to you. Words are not enough to describe the gratitude that I feel for the love and care that I have received from you.

Helsinki, June 2010

Tero