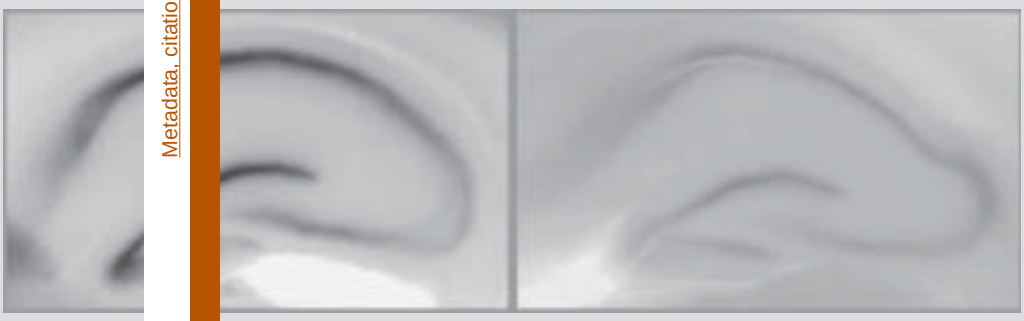

Re ation of the Neuronal Chloride Cot sporter KCC2 by Neurotrophins

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ACADEMIC DISSERTATION

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Gummerus, Helsinki 2004

To my parents, Maria Rosa and Josep LLuís,

and my sister Gemma

TABLE OF CONTENTS

ABBREVIATIONS

AE	Na^+-independent anion exchangers
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
BDNF	Brain-derived neurotrophic factor
CaMKIV	Ca^{2+}-calmodulin-dependent kinase IV
CCC	Cation-chloride cotransporter
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CREB	cAMP response element-binding protein
CSN	Corticospinal neurons
GABA	γ-aminobutyric acid
GAP-43	Growth associated protein 43
GDNF	Glial cell line-derived neurotrophic factor
Glu	Glutamate
GluR	Glutamate receptor
IHC	Immunohistochemistry
ICL	Internal capsule lesion
KCC	K^+-Cl^- cotransporters
LTP	Long-term potentiation
MAP2	Microtubule associated protein 2
MAPK	Mitogen-activated protein kinase
NDAE	Na^+-dependent anion exchangers
NGF	Nerve growth factor
NKCC	Na^+-K^+-$2Cl^-$ cotransporters
NT	Neurotrophin
NT-3	Neurotrophin-3
NT-4/5	Neurotrophin-4/5
$p75^{NTR}$	p75 Neurotrophin receptor
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PI3-K	Phosphatidylinositol 3-kinase
PLCγ	Phospholipase Cγ
PNS	Peripheral nervous system
PODNS	Phosphothionate dideoxy oligonucleotides
RT-PCR	Reverse Transcriptase-Polymerase chain reaction
TfR	Transferrin receptor
TGF	Transforming growth factor
TrkB-Fc	TrkB-soluble receptor bodies
Trk	Tyrosine kinase receptor
TRP	Transient receptor potential
TTX	Tetrodotoxin
WT	Wild type
Y	Tyrosine

ORIGINAL PUBLICATIONS

This thesis is based on the following publications, herein referred to by their Roman numerals (**I-IV**), and in some unpublished results:

I Claudio Rivera, Hong Li, **Judith Thomas-Crusells**, Hannele Lahtinen, Tero Viitanen, Avtandil Nanobashvili, Zaal Kokaia, Matti S. Airaksinen, Juha Voipio, Kai Kaila and Mart Saarma (2002). BDNF-induced TrkB activation down-regulates the K^+/Cl^- cotransporter KCC2 and impairs neuronal Cl^- extrusion. *J Cell Biol 159: 747-752.*[a]

II **Judith Thomas-Crusells**, Amandio Vieira, Mart Saarma and Claudio Rivera (2003). A novel method for monitoring cell surface membrane trafficking on hippocampal acute slice preparation**.** *J Neurosci Methods 125:159-166.*[b]

III Claudio Rivera, Juha Voipio, **Judith Thomas-Crusells**, Hong Li, Zsuzsa Emri, Sampsa Sipilä, John A Payne, Liliana Minichiello, Mart Saarma and Kai Kaila (2004). Mechanism of activity-dependent down-regulation of the neuron-specific K^+-Cl^- cotransporter KCC2. *J Neurosci 24(19):4683-91.*[c]

IV **Judith Thomas-Crusells,** Thomas Sigl, Britta Leiner, Qiao Yan, Andrew A. Welcher, Pedro Mestres, Wolfram Tetzlaff, Mart Saarma, Kai Kaila, Michael Meyer, Claudio Rivera and Klaus M. Giehl. *Adult CNS neurons reactivate embryonic neurotrophin regulation of KCC2 expression and cell survival after pathological damage in vivo* (submitted).

Cover image
Free-floating *in situ* hybridization of KCC2 mRNA expression on acute hippocampal slices before (left) and after application of 100ng/ml BDNF for 3h (right).

“ …Always look at the bright side of life…*fifuuuuufifuuuuufifufifuuuuuuuuuu*…”

from Monty Phyton´s *Life of Brian (1979)*

ABSTRACT

The mammalian neurotrophins: Nerve growth factor (NGF), Brain-derived neurotrophic factor (BDNF), Neurotrophin-3 (NT-3) and Neurotrophin-4/5 (NT-4/5), comprise a group of proteins that are essential for differentiation, growth and survival of various populations of neurons as well as some non-neuronal cells. They signal via ligand specific Trk receptor tyrosine kinases A, B and C, as well as the common neurotrophin receptor $p75^{NTR}$, which belongs to the family of tumor necrosis factor receptors.

The effects of BDNF on neurotransmission in developing and mature neurons have been partly associated with the regulation of GABAergic transmission, the main inhibitory system in the brain, and in particular with $GABA_A$-mediated responses. The polarity of these GABAergic responses is dependent on the concentration of intracellular chloride, which is controlled by the activity of the K^+/Cl^- cotransporter KCC2, a member of the cation chloride cotransporter family (CCC).

The expression of neurotrophins is affected by both physiological (e.g. developmental changes, functional state) and pathophysiological (e.g. epileptogenesis, trauma, etc.) conditions. KCC2 expression is also modulated under these conditions. The time course of theses changes, e.g. along the transition from an immature to a mature neuronal phenotype, or after axotomy of adult neurons, often coincides with the respective changes in the regulation of neurotrophins. Therefore, in this study, we investigated whether neurotrophins regulate KCC2 during development and under pathological situations.

We observed that KCC2 and BDNF expressions are regulated in a mirror fashion in hippocampal neurons after kindling, a widely studied *in vivo* model of epileptogenesis and neuronal plasticity (I). This pointed us to investigate whether BDNF directly regulates KCC2 expression. Under Mg^{2+}-free conditions, an *in vitro* model of sustained interictal-like activity induced by the absence of Mg^{2+}, we demonstrated that BDNF and NT-4/5 cause a TrkB-mediated down-regulation of KCC2 mRNA and protein in adult hippocampal neurons in acute organotypic slice cultures. Consistently, this treatment also resulted in a reduced capacity for neuronal Cl^- extrusion (I, III). Furthermore, after developing a new technical approach to study surface membrane trafficking on hippocampal slices, we demonstrated that altered activity affects the stability of KCC2 under 0-Mg^{2+} conditions increasing the turnover of plasmalemmal KCC2 (II, III). Studies using transgenic mice demonstrated that the molecular mechanism behind the activity-dependent regulation of KCC2, involves BDNF/TrkB down-stream cascades of both Shc/FRS-2 and PLCγ-CREB signalling (III).

Finally, we studied the effect of neurotrophins on KCC2 under pathological conditions. To this end, we used an *in vivo* axotomy model of adult corticospinal neurons (CSNs) combined with intracortical neurotrophin application. Here, we demonstrated that after axotomy, adult CSN undergo a temporary switch to a developmental mode in their BDNF-mediated KCC2 regulation as well as in their

trophic dependencies. This ontogenetic reactivation implies an up-regulation of KCC2 under the effect of BDNF and a BDNF dependency, which appears to be restricted to survival regulation (IV).

Overall, the results presented in this thesis reveal a novel mechanism whereby BDNF/TrkB signaling suppresses chloride-dependent fast $GABA_A$-mediated responses by modulating KCC2. This is most likely contributing to the well-known role of TrkB-activated signaling cascades in the induction and establishment of epileptic activity, and provides a novel role for changes in KCC2 expression in diverse manifestations of neuronal plasticity. Additionally, the results demonstrate that injury in the adult brain induces a switch in neurotrophin-mediated survival and KCC2-regulation back to a mode normally seen in immature neurons, which are probably interacting to regulate neuronal survival and to promote recovery in a critical post-injury phase.

1. REVIEW OF THE LITERATURE

1.1 NEUROTROPHINS

1.1.1 *Neurotrophins: Family ligands and receptors*

The neurotrophin family of growth factors (NTs), belong to the functionally related neurotrophic factor family (see Table 1). This is a broad family of proteins that after being secreted bind to cell surface receptors initiating intracellular signaling cascades that result in an extensive variety of biological effects.

The neurotrophins were initially described as target-derived trophic factors required for the survival of specific neuronal populations. Secreted at limiting amounts, these factors are thought to ensure a balance between the size of a target organ and the number of innervating neurons. According to this concept, during the development, those neurons that are sufficiently supplied

Table 1. Families of neurotrophic factors*

- Neurotrophins
  - Nerve Growth Factor (NGF)
  - Brain-derived Neurotrophic factor (BDNF)
  - Neurotrophin-3 (NT-3)
  - Neurotrophin-4/5 (NT-4/5)
  - Neurotrophin-6 (NT-6)
  - Neurotrophin-7 (NT-7)
- Neuropoietins
  - Ciliary Neurotrophic Factor (CNTF)
  - Interleukin-6 (IL-6)
  - Leukemia Inhibitory Factor (LIF)
- Transforming Growth Factors
  - Transforming Growth Factor α (TGFα)
  - Transforming Growth Factor β(TGFβ1, TGFβ2, TGFβ3, Bone Morphogenetic proteins (BMPs))
    - Glial Cell Line -Derived Neurotrophic Factor (GDNF)
    - Neurturin (NRTN)
    - Persephin (PSPN)
    - Artemin (ARTN)
- Fibroblast Growth Factors
  - Acidic Fibroblast Growth Factor (FGF-1)
  - Basic Fibroblast Growth Factor (FGF-2)
  - Fibroblast Growth Factor-5 (FGF-5)
- Other factors
  - Platelet-Derived Growth Factor (PDGF)
  - Stem Cell Factor (SCF)
  - Vascular Endothelial Growth Factor (VEGF)
  - Insulin-like Growth Factors 1-2 (IGF1, IGF-2)
  - Epidermal Growth Factors (EGF)

* Adapted from Huang and Reichardt, 2001; Airaksinen and Saarma, 2002; de Caestecker, 2004; Wong and Guillaud, 2004.

with NTs survive, while the others die (reviewed in Huang and Reichardt, 2001). Since this initial definition, a great advance in the study of these molecules has been made revealing a family of growth factors of outstanding importance in the regulation of many functions for developing and mature cells of the central and peripheral nervous system. To name a few functions, NTs regulate neuronal and glial survival, axonal and dendritic growth and guidance, synaptic structure and function, neurotransmitter release, and synaptic plasticity (reviewed in McAllister *et al.*, 1999; Huang and Reichardt, 2001; Poo, 2001). During early stages of development, neurotrophins predominantly regulate cell fate decisions and neuronal survival, whereas during maturation, their regulatory repertoire shifts towards functions in neuronal plasticity, synaptic transmission, or the maintenance of a differentiated neuronal phenotype (Huang and Reichardt, 2001).

1.1.1.1 Ligands

The first neurotrophin reported, Nerve growth factor (NGF), was identified more than 50 years ago by Rita Levi-Montalcini, Viktor Hamburger and Stanley Cohen, when searching for target-derived factors that support the survival and growth of motor and sensory neurons (reviewed in Levi-Montalcini, 1987). Since then, more molecules have been incorporated to the family: Brain-derived neurotrophic factor (BDNF), being the first molecule of this kind found in the CNS (Barde *et al.*, 1982; Leibrock *et al.*, 1989), Neurotrophin-3 (NT-3; Ernfors *et al.*, 1990; Maisonpierre *et al.*, 1990); Neurotrophin-4/5 (NT-4/5; Berkemeier *et al.*, 1991; Hallböök *et al.*, 1991), and neurotrophin-6 (NT-6) and neurotrophin-7 (NT-7); (Gotz *et al.*, 1994; Lai *et al.*, 1998). So far, the two latter have been found only in fish.

The four mammalian neurotrophins are stable, basic dimeric proteins of about 12kDa, and are expressed at very low levels during development. They share about 50% amino acid sequence identity and their three dimensional structure is similar to NGF (McDonald *et al.*, 1991; Ibañez, 1998). The NTs are all synthesized as prepro-proteins. The proNTs that result after the signal peptide is cleaved off, accumulate in prevesicles en route to be secreted in the constitutive (spontaneous, independent of intracellular Ca^{2+}) or regulated (response to external stimuli, Ca^{2+}- dependent and lipid environment specific) secretory pathway (reviewed in Lessmann *et al.*, 2003). Cleavage of the pro-domain by proteases results in mature forms of NT. Once the NTs are released, their biologically active homodimeric forms bind to their receptors (see below). These ligand-receptor complexes are subsequently incorporated as signaling endosomes at pre- and/or postsynaptic compartments (i.e. according to autocrine or paracrine NT action), which are then retrogradely or anterogradely transported to perikaryal sites (Fig. 1; for review see Lessmann *et al.*, 2003). The process of maturation seems to play a major role in the control of neurotrophin activity as revealed recently by Lee and collaborators (Lee *et al.*, 2001) when studying NGF, BDNF, and their non-processed pro-forms. These studies suggest that the pro-form and mature forms bind different neurotrophin receptors, with the pro-form displaying

high affinity binding to the common neurotrophin receptor $p75^{NTR}$ (see below), and the mature form preferentially activating Trk receptors. Theses findings could be physiologically significant because the neurotrophin precursors are the predominant forms of BDNF and NGF in the brain, and have been shown to accumulate in neurodegenerative diseases such as Alzheimer's disease (Fahnestock *et al.*, 2001; Lee *et al.*, 2001; reviewed in Chao, 2003). Indeed, proNeurotrophins are secreted as $p75^{NTR}$ ligand *in vivo* where they induce $p75^{NTR}$ -mediated death in damaged neurons (Harrington *et al.*, 2004). Whether proNeurotrophins play a role during development is unknown.

The expression of NTs in the mammalian brain is regulated during development and by neuronal activity. NT-3 shows the highest expression levels of all NTs during the perinatal period (Zhou and Rush, 1994) with most prominent expression in the hippocampus, neocortex and cerebellum.

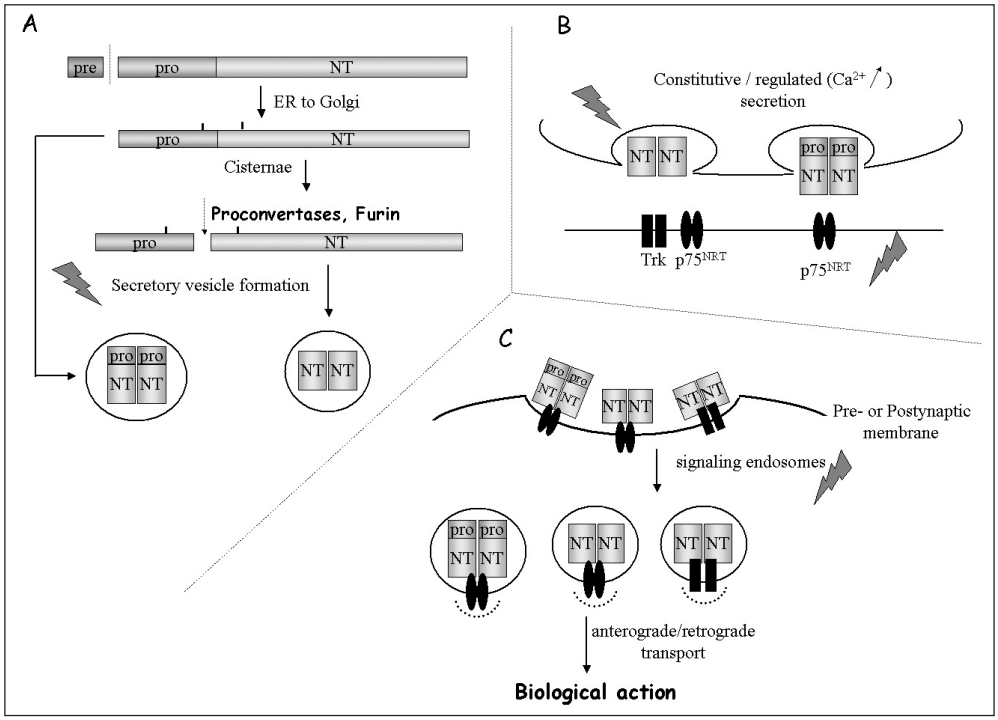

Fig. 1. Neurotrophin synthesis, transport and secretion. A) Neurotrophins are synthesized as prepro-proteins. Their mature forms (depicted as NT) result from cleavage by specific proteases before they are packed into vesicles in order to be secreted. In addition, unprocessed forms (proNT) are also secreted. B) Regulated secretion is linked to an increase in $[Ca^{2+}]i$. After release NT bind Trk and $p75^{NRT}$, whereas proNT only bind $p75^{NRT}$. C) The complex formed by the NTs and proNTs and their receptors are internalized in the pre- or postsynaptic membrane (depending on their location and autocrine or paracrine actions) and the signaling endosomes are transported anterogradely or retrogradely resulting in biological effects. Synaptic activity may regulate the synthesis, packaging and transport of NTs and their receptors (showed by a lightning arrow).

BDNF expression levels are low at birth but increase dramatically during the first weeks of postnatal development becoming abundant in hippocampus, neocortex, amygdala and cerebellum. In the adult, expression levels of NT-3 and BDNF are comparable in most areas of the brain (Hofer *et al.*, 1990; Maisonpierre *et al.*, 1990; Ernfors *et al.*, 1992). The expression of BDNF in hippocampal neurons is strongly increased by physiological and pathophysiological levels of neuronal activity (Zafra *et al.*, 1990; Ernfors *et al.*, 1991; Isackson *et al.*, 1991) providing a positive feedback on synaptic function and protecting neurons from excitotoxicity (Lessmann *et al.*, 2003). NGF expression shows very small changes during postnatal development (Ernfors *et al.*, 1990), except in the hippocampus. Expression of NT-4/5 is prominent postnatally in hippocampus, neocortex, cerebellum and thalamic nuclei, and continues to be expressed until adulthood (Friedman *et al.*, 1998).

Alterations in neurotrophin levels have profound effects on a wide variety of phenomena, including myelination, regeneration, pain, aggression, depression and substance abuse (reviewed in Chao, 2003). The biological properties of the neurotrophins have been analyzed in animals with deletions in their neurotrophin genes. Since complete lack of neurotrophins results in death early after birth, with the only exception of NT-4/5 (Conover *et al.*, 1995), the alterations in brain function and behaviour have been analyzed in heterozygous mice. NGF+/- mice show decreased cholinergic innervation of the hippocampus and deficiencies in memory (Chen *et al.*, 1997), together with a profound reduction of neurons of the peripheral nervous system (Crowley *et al.*, 1994). Behavioural tests on the BDNF+/- mice show that these mice are aggressive, hyperphagic and obese (Lyons *et al.*, 1999; Kernie *et al.*, 2000; Rios *et al.*, 2001) and present impairment in spatial memory consistent with defects in hippocampal long-term potentiation (Korte *et al.*, 1995; Patterson *et al.*, 1996; Bartoletti *et al.*, 2002). They also display elevated striatal dopamine levels (Dluzen *et al.*, 2001), and reduction of neurons and mechanoreceptors in the peripheral nervous system (Ernfors *et al.*, 1994a; Carroll *et al.*, 1998). The latter is also observed in the NT3+/- mice (Airaksinen *et al.*, 1996, Ernfors *et al.*, 1994b). Additionally, BDNF+/- mice are deficient in amygdala kindling activity (Elmer *et al.*, 1997) and present abnormal cardiovascular development (Donovan *et al.*, 1996). NT-4/5- deficient mice are long-lived and show no obvious neurological defects (Conover *et al.*, 1995) Overall, these phenotypes show that neurotrophins have essential functions for the developing and mature vertebrate nervous system as well as for some non-neuronal functions.

1.1.1.2 Neurotrophin receptors

To exert their biological actions, neurotrophins interact dually but with a different binding affinity with two types of receptors: the Trk receptors, and a tumor necrosis factor receptor like molecule, $p75^{NTR}$ (reviewed in Huang and Reichardt, 2003). While all neurotrophins display low affinity binding with p75, their interaction with Trk receptors is more specific with NGF activating TrkA, BDNF and NT-4/5 activating TrkB, and

NT3 activating TrkC (see Trk receptors section). As described above, the mature form of a neurotrophin displays high affinity interaction with its Trk receptor and normally a low affinity interaction with p75 ^{NTR}. On the contrary, the pro-form of a neurotrophin exerts high affinity binding to p75 ^{NTR} while it binds poorly to the Trk receptor. This dual binding system enables mature and immature neurotrophins to activate opposing pathways mediating opposing cellular functions, e.g. survival and death signals (Dechant, 2001; Miller and Kaplan, 2001; Roux and Barker, 2002). Nevertheless, different cellular responses to neurotrophins are not exclusively generated by differential ligand-receptor interaction with different signaling cascades coupled to the receptors involved. Modification by dynamic cellular processes such as changes in the lipid environment, internalisation into intracellular compartments, retrograde transport, and differential processing of the signaling endosomes are also involved, and currently under extensive study (reviewed in Dechant, 2001; Heerssen and Segal, 2002).

Trk receptors

A chimeric oncogene found in human colon carcinoma consisting of the fusion of a truncated tropomyosin and a protein tyrosine kinase sequences, and the

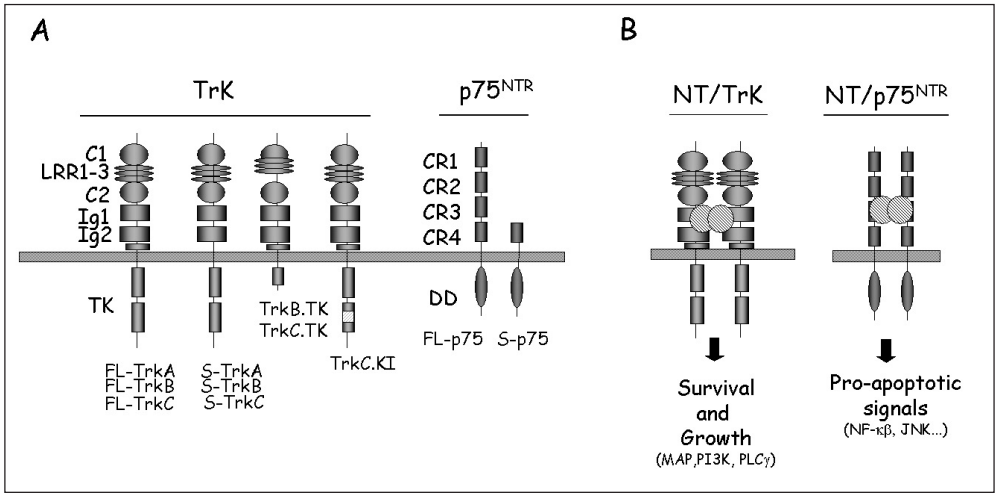

Fig. 2. Schematic structure of mammalian neurotrophin receptors and consequences of receptor-ligand binding. Full-length Trk receptors (FL) contain an extracellular domain consisting of three leucine-rich repeat (LRR) motifs separated by two cystein rich clusters (C1-2), and two inmunoglobulin-like domains (Ig1-2). The intracellular domain contains tyrosine kinase domains (TK). Different isoforms show deletions/insertions in their extracellular or intracellular structures (S, short-length receptors; TK, truncated receptors; KI, receptors with insertions). The p75^{NTR} contains four extracellular cystein-rich repeats (CR1-4) and an intracellular "death" domain (DD). S-p75 lacks CR1-CR3. B) NT binding to Trk receptors activates signal transduction pathways that induce neuronal survival and growth, whereas NT binding to p75^{NTR} induces cell death signals. Some intracellular signaling pathways are indicated. MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PLCγ, phospholipase-Cγ; JNK, Jun N-terminal kinase.

respective proto-oncogene, were cloned by Martin-Zanca and collaborators (Martin-Zanca *et al.*, 1989). Consequently the proto-oncogene was named tropomyosin-related kinase (Trk) and is now commonly referred to as TrkA. The *TrkB* and *TrkC* genes were identified by the virtue of their high homology to *TrkA* (Huang and Reichardt, 2003). There are three neurotrophin Trk receptors, TrkA, TrkB and TrkC, which bind neurotrophins with specific selectivity. NGF binds to TrkA (Kaplan *et al.*, 1991; Klein *et al.*, 1991), BDNF and NT-4/5 bind to TrkB (Klein *et al.*, 1989; Klein *et al.*, 1990), and NT-3 to TrkC (Lamballe *et al.*, 1991;). NT-3 is, although less efficiently, also able to activate each of the other Trk receptors (reviewed in Huang and Reichardt, 2001). In addition, all neurotrophins bind to the pan-neurotrophin receptor $p75^{NTR}$ (see explanations below and Fig. 2).

Structurally, all Trk receptors are members of the transmembrane receptor tyrosine kinase superfamily. They have an extracellular domain that contains leucine-rich motifs flanked by cystein clusters and IgG-like domains at the juxtamembrane region that are responsible for neurotrophin binding. The intracellular tyrosine kinase domain becomes activated upon NT binding. As a first step of this activation, ligand binding to the extracellular domain causes two Trk monomers to dimerize, thereby bringing the two intracellular tyrosine kinase domains into close contact to each other.

Binding of neurotrophins to Trk receptors causes dimerisation and autophosphorylation of intracellular tyrosine residues that act as docking sites for adaptor proteins, such as the src homology 2 domain containing transforming protein/FGF receptor substrate 2 (Shc/FRS-2), and to phospholipase-Cγ (PLCγ) (reviewed in Patapoutian and Reichardt, 2001; Huang and Reichardt, 2003. See Fig. 3 and 4). Subsequently to ligand binding, the activated receptors initiate several signal transduction cascades. Binding to Shc or FRS-2 can lead to activation of the Ras-ERK and phosphatidylinositol-3-OH kinase (PI3K)/Akt pathways, whereas binding of PLCγ leads to mobilization of Ca^{2+} from internal stores and other downstream effects. To mention some examples, the phospholipase activity of PLC-γ1 plays an essential role in nerve growth factor (NGF)-triggered Raf/MEK/MAPK pathway activation in PC12 cells (Rong *et al.*, 2003), and *in vivo* experiments in mutated mice have shown that a consequence of BDNF-triggered TrkB signaling in neurons is the activation of the transcription factor cAMP response element-binding protein (CREB; Finkbeiner *et al.*, 1997; Minichiello *et al.*, 2002; Huang and Reichardt, 2003).

Alternative splicing has been described for all Trk receptors. Trk A and TrkB transcripts are alternatively spliced at a small exon encoding their extracellular domain (Garner *et al.*, 1996; Strohmaier *et al.*, 1996). Receptor isoforms for TrkB (TrkB.T1-2) and TrkC (TrkC.TK1-5) may have truncations or insertions in the intracellular tyrosine kinase domains (Klein *et al.*, 1990; Lamballe *et al.*, 1993; Middlemas *et al.*, 1991). A summary of different isoforms of NT receptors is presented in Figure 2.

The functions of the isoforms are not completely known but evidence supports their importance in affecting the specificity of the responsiveness of Trk to

neurotrophins. In this context, truncated receptors may modify local effective NT concentration by capturing and presenting NT to neurons expressing full-length Trk receptors, inhibit activation of Trk kinases by forming non-productive heterodimers, regulate the surface expression of full-length receptors, and modify the substrate specificity altering cellular responses to their activation (for review see Roux and Barker, 2002; Huang and Reichardt, 2003). Activation of these pathways will culminate in the activation of transcription factors that affect gene expression and consequently modulate cell growth and survival of many neuronal populations (Patapoutian and Reichardt, 2001; Huang and Reichardt, 2003).

p75^{NTR}

Each neurotrophin binds to the transmembrane pan-neurotrophin receptor p75^{NTR}, a related member of the tumor necrosis factor receptor superfamily (reviewed in Hempstead, 2002). In the CNS, p75^{NTR} is expressed in a wide variety of cell populations during embryonic stages, whereas postnatally, the levels are reduced in most areas (reviewed in Roux and Barker, 2002).

A mature p75^{NTR} is a 399-aminoacid transmembrane receptor that contains four (1-4) cystein-rich repeats in the extracellular domain (ECD) (Fig.2). The third extracellular domain is responsible for the interaction with neurotrophins (reviewed in Roux and Barker, 2002). The intracellular juxtamembrane domain contains regions likely to mediate interactions with multiple p75^{NTR} adaptor proteins involved in biological activities comprising death and survival, cell cycle, axonal elongation and synaptic transmission (reviewed in Dechant and Barde, 2002). The C-terminal domain that contains a globular "death" domain (Liepinsh *et al.*, 1997) is implicated in the transduction of cell death signals (reviewed in Roux and Barker, 2002). Both domains present post-translational modifications with yet unknown functions, although roles in protein-protein interaction, intracellular receptor folding and cellular localization of the p75^{NTR} receptor have been suggested (reviewed in Roux and Barker, 2002).

Alternative splicing of the p75^{NTR} results in a short isoform (S-p75) that lacks the NT-binding domain 1-3 (reviewed in Dechant, 2001). The physiological role of this splice variant is currently unknown (Dechant, 2001). A recent report shows the existence of a homologous gene, NRH2, which encodes a protein structurally similar to the short form of p75^{NTR}, which regulates NGF binding to TrkA (Murray *et al.*, 2004). Further modifications of the full-length p75^{NTR} receptor have been reported to occur at the posttranslational level. They include cleavage of the receptor by metalloproteinases (DiStefano *et al.*, 1993) generating a soluble extracellular domain that is capable of binding neurotrophins, and a remaining truncated transmembrane domain. This modification is predominantly observed during development and following peripheral nerve injury, but its biological role, however, has remained elusive (reviewed in Roux and Barker, 2002).

Analysis of the ablation of *p75ntr* (von Schack *et al.*, 2001) has recently revealed that mice lacking p75^{NTR} phenotypically present severe defects in both nervous and vascular system (von Schack *et al.*, 2001).

By using a different set of adaptor proteins than Trk receptors, $p75^{NTR}$ is able to promote both survival and apoptosis in different cells as well as affect axonal growth (Huang and Reichardt, 2001) after neurotrophin binding. The death inducing capacity of $p75^{NTR}$ has been attributed to its ability to activate effectors such as proteases of the caspase family, p53, ceramides and Jun N-terminal kinase (JNK) signaling cascade (Aloyz *et al.*, 1998; Yoon *et al.*, 1998 Agerman *et al.*, 2000; Troy *et al.*, 2002; Huang and Reichardt, 2003). On the contrary, pro-survival pathways activated by $p75^{NTR}$ include NF-κB and Akt (Wooten *et al.*, 2001; Roux and Barker, 2002. See Fig. 3).

In addition to its ability to act as an independent receptor coupled to its own signaling apparatus, $p75^{NTR}$ has also been shown to modulate the affinity and signaling of Trk receptors. The association with $p75^{NTR}$ modulates affinity and ligand specificity of Trk receptors (Chao, 2003). Initial binding and transfection studies revealed that $p75^{NTR}$ bound NGF with low-affinity. The receptor was, therefore, termed 'low affinity NGF receptor' (LNGFR; Chao *et al.*, 1986; Johnson *et al.*, 1986; Radeke *et al.*, 1987), but later and coincident with the discovery of the other neurotrophin family members it was shown that $p75^{NTR}$ binds all neurotrophins with approximately equal affinity (reviewed in Huang and Reichardt, 2003). A recent report by Lee and co-workers (2001) demonstrated that the recombinant, cleavage resistant NGF precursor, proNGF, binds $p75^{NTR}$ with high-affinity and induces $p75^{NTR}$-dependent apoptosis of sympathetic neurons in culture, suggesting that neurotrophins may activate $p75^{NTR}$ in both pro-and mature forms (Lee *et al.*, 2001; Hempstead, 2002). In this scenario, the mature form of the neurotrophins preferentially binds to and activates the Trk receptor while the pro-form aims at the $p75^{NTR}$ receptor (Fig.3). Indeed, proNeurotrophins are secreted as $p75^{NTR}$ ligands *in vivo* inducing $p75^{NTR}$ -mediated death in damaged neurons (Harrington *et al.*, 2004). Whether proNeurotrophins play a role during development is unknown.

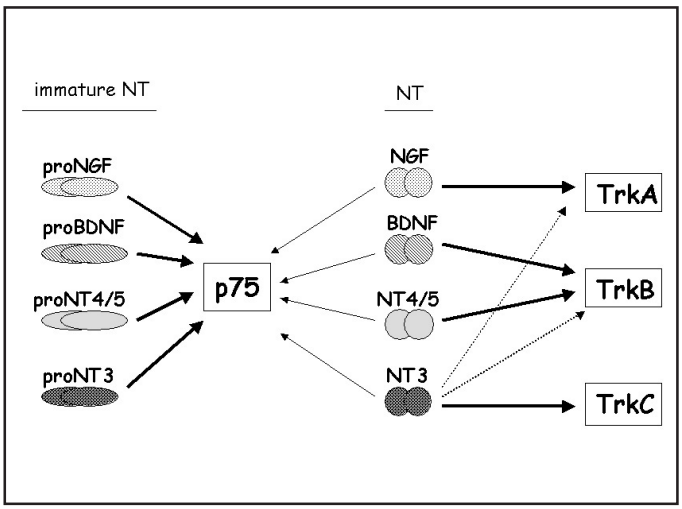

Fig. 3. Schematic representation of the binding of neurotrophins to their receptors. All immature (pro) neurotrophins bind to $p75^{NTR}$ with high affinity (bold arrows). Mature neurotrophins bind to both $p75^{NTR}$ and Trk receptors, but with higher affinity to Trk. NGF binds TrkA, BDNF and NT-4/5 bind to TrkB, and NT-3 preferentially binds to TrkC but can activate all the Trks (indicated with dashed arrows).

Recently, the work by Nykjaer and colleagues (Nykjaer *et al.*, 2004) indicated that proNGF induces cell death through binding $p75^{NTR}$ only when it is forming a complex with Sortilin, a protein that acts as a neurotensin receptor (Mazella, 2001). Further studies are needed to show whether other pro-neurotrophins use a similar, cleavage-dependent mechanism to target ligands derived from one precursor to different receptors. It will also be important to determine the role of these ligands, as independent effectors, or potentially as dominant negative modulators for mature neurotrophin functions.

Cross-talk with other membrane receptors and non-neurotrophin ligands

Beyond cell death and survival, Trk and $p75^{NTR}$ have been shown to promote other effects by binding to non-neurotrophin ligands or other membrane receptors. For instance, recent studies indicate that by modulating Rho activity (Yamashita *et al.*, 1999), $p75^{NTR}$ is able to associate with certain gangliosides (Yamashita *et al.*, 2002) and interact with membrane proteins such as the Nogo receptor (NgR1; Wang *et al.*, 2002), as well as with the recently identified transmembrane protein LINGO-1 (Mi *et al.*, 2004) in order to regulate axon elongation. Additionally, the $p75^{NTR}$ also binds non-neurotrophin ligands such as the neurotoxic prion protein fragment PrP (101-126) (Della-Bianca *et al.*, 2001), the Ab-peptide of the amyloid precursor protein (APP; Kuner *et al.*, 1998; Perini *et al.*, 2002), a rabies viral capsid glycoprotein (RV-Gs; Tuffereau *et al.*, 1998) and a mollusan cystein-rich neurotrophic factor (CRNF; Fainzilber *et al.*, 1996). In this context, it has been suggested that $p75^{NTR}$ may be a promising drug target for preventing cell death and activating axonal elongation (reviewed by Dechant and Barde, 2002).

Trk receptors also display cross talk with other membrane receptors. For instance, TrkB activation upon BDNF binding increases the ion influx through transient receptor potential (TRP) ion channel TRPC3 (Li *et al.*, 1999). This channel is highly expressed in brain regions where TrkB receptors are found, and their expression is especially abundant during neonatal development suggesting a role in neurotrophin-dependent plasticity of this type of receptors. Another example of interaction between Trk and TRP channels is the TRPV1 channel or capsaicin receptor (Bonnington and McNaughton, 2003). These channels are activated by heat or capsaicin, and NGF is known to potentiate the response of nociceptive sensory neurons to capsaicin. Mice lacking TRPV1 have a reduced nociceptive sensitization when injected with NGF. In addition, in NGF-responsive neurons, TrkA and TRPV1 are frequently co-expressed, and co-immunoprecipitation studies indicated that TRPV1, TrkA and PLCγ associate to form a complex that results in the activation of the channel after NGF application (Bonnington and McNaughton, 2003).

Furthermore, electrophysiological measurements in the hippocampus have shown that BDNF is also able to suppress the activity of AMPA receptor-mediated currents. This indicates that BDNF may regulate glutamatergic transmission at primary afferent synapses and implies a possible close association between TrkB

and AMPA receptors (Balkowiec *et al.*, 2000). Additionally, the recent work by Blum and collaborators (Blum *et al.*, 2002) showed that in both cell lines and hippocampal neurons, the voltage-gated sodium channel $Na_V1.9$, is activated by BDNF/TrkB binding, since an inward sodium current detected after BDNF application was prevented by activity blockers and Trk-specific inhibitors, and by blocking the expression of the channel. This work is providing molecular basis for the understanding of neurotrophin-evoked excitation in the CNS (i.e. see Kafitz *et al.*, 1999). Although the mechanisms for receptor-ion channel interactions are unknown, conformational changes in the structure of Trk are suspected to be the cause (reviewed in Chao, 2003). The biological consequences of the Trk interactions with ion channels are yet unknown. Further analysis on the molecular mechanisms underlying these interactions, i.e. in transgenic animals, may contribute to our understanding of the ability of neurotrophins to regulate synaptic plasticity in adult brain.

Another type of cross-talk observed between Trk receptors and other membrane proteins are the transactivation of Trk receptors by G-protein-coupled (GPC) ligands such as adenosine and the pituitary adenylate cyclase-activating polypeptide (PCAP) (Lee *et al.*, 2002). PACAP can exert trophic effects through a mechanism involving Trk receptors and utilization of tyrosine kinase signaling. These interactions result in neuroprotection, and suggests an explanation for the several neuroprotective actions of PACAP upon neuronal populations after injury, nerve lesion, or neurotrophin deprivation (Lee *et al.*, 2002).

The GDNF-family ligand (GFL) tyrosine kinase receptor, c-Ret (Airaksinen and Saarma, 2002) is another of the transmembrane receptor that has been shown to be activated by NTs. Surprisingly, binding of NGF to TrkA results in the activation of c-Ret without the requirement of GFL ligand binding, promoting survival in sympathetic neurons (Tsui-Pierchala *et al.*, 2002) and representing a new mechanism for transmitting survival signals within neurons.

Overall, these current data indicate that neurotrophin-mediated activation of neurotrophin receptors leads to a variety of biological responses, which includes proliferation, survival, and axonal and dendritic growth, but also assembly and remodeling of cytoskeleton, membrane trafficking, and modification of synaptic functions.

1.1.2 *Functional roles of neurotrophins*

1.1.2.1 Role of neurotrophins and their receptors in neuronal activity and synaptic plasticity

One of the most significant developments in the neurotrophin field has been the realization that these proteins and their receptors are modulated by neuronal activity and are involved in synaptic transmission and plasticity (Schinder and Poo, 2000). Experimental evidence so far strongly indicates that neuronal activity regulates synthesis, secretion and signaling of NTs, thus modifying synaptic transmission and connectivity as a consequence of specific changes in the

pre- and postsynaptic neurons (Schinder and Poo, 2000).

Studies on hippocampal slices and cortical neurons have shown that the levels of transcripts of NTs and their receptor genes, specially those encoding for NGF, BDNF and TrkB, are elevated in epileptogenic situations and after pharmacological modulation of glutamatergic receptor activation (Gall and Isackson, 1989; Zafra *et al.*, 1990; Ernfors *et al.*, 1991; Lauterborn *et al.*, 2000). Elevation of NT expression after electrical stimulation has been also reported in the visual cortex (Castrén *et al.*, 1992), and the peripheral nervous system (Wang and Poo, 1997). Not only the expression but the secretion and release of NT is regulated by neuronal activity. In hippocampal slices or dissociated cell cultures, robust depolarization (e.g. in response to high KCl, glutamate or patterned electrical stimulation) results in an elevated level of secreted and/or surface-bound NTs (Blöchl and Thoenen, 1995; Goodman *et al.*, 1996; Balkowiec and Katz, 2000). Moreover, NTs themselves can also function as a regulatory signal for NT secretion (BDNF: Canossa *et al.*, 1997; NGF: Kruttgen *et al.*, 1998). The secretion is mediated by an elevation of intracellular calcium in response to neurotrophin receptor signaling or by direct membrane depolarization induced by the NTs (reviewed in Lessmann *et al.*, 2003).

Neuronal activity also modifies synaptic strength (i.e. synaptic plasticity). It is thought that this phenomenon is the basis of the formation of memory traces in the brain, and that neurotrophins are involved in these processes. Indeed, NTs have profound effects on several signaling pathways in CNS neurons, which are necessary for the induction and maintenance of long-term changes in synaptic strength such as long-term potentiation (LTP) (Thoenen, 1995; McAllister *et al.*, 1999; Poo, 2001). LTP experiments (few seconds of high-frequency stimulation which enhances synaptic transmission in the hippocampus for days or even weeks) have demonstrated that synaptic plasticity results from the activation of cell surface receptors or ion channels that activate signaling systems coupled to the transcriptional machinery in the nucleus (reviewed in Poo, 2001). In this context, the role of BDNF and TrkB in LTP has been established (Korte *et al.*, 1995; Patterson *et al.*, 1996; Minichiello *et al.*, 1999), and recently, the creation of mice strains carrying a mutation in the intracellular domain of TrkB (Minichiello *et al.*, 2002) have been the key for demonstrating that phospholipase-Cγ (PLCγ) is the intracellular cascade involved in plasticity-related effects of BDNF in the hippocampus (Minichiello *et al.*, 2002; Ernfors and Bramham, 2003; Fig. 4).

1.1.2.2 Role of neurotrophins in pathophysiological conditions

The concept that neurotrophin expression in the brain is modulated in response to neuronal activity was first suggested by the demonstration of increased NGF mRNA levels following recurrent limbic seizures (Gall and Isackson, 1989). Subsequent studies demonstrated that the expression of NGF, BDNF and NT-3 mRNAs is drastically altered following seizures in response to electrolytic lesions (Isackson *et al.*, 1991; Rocamora *et al.*,

1992; Rocamora *et al.*, 1994), kindling (Ernfors *et al.*, 1992) or kainic acid injection (Dugich-Djordjevic *et al.*, 1992; Wetmore *et al.*, 1994). The up-regulation of BDNF and the activation of Trk receptors in the context of seizures appear to play a key role in the development of hyperexcitability *in vitro* and *in vivo* (Binder *et al.*, 2001).

In addition to seizure induction, other pathological conditions that clearly result in neuronal damage, such as mechanical injury, ischemia and hypoglycemia, also result in altered neurotrophin expression, and their low (Harrington *et al.*, 2004) or high-affinity receptors, TrkB and TrkC (Lindvall *et al.*, 1994). These damage-induced changes are thought to protect neurons undergoing acute metabolic stress after an insult and to stimulate sprouting and synaptic reorganization in the period of recovery.

Animal-models for neurodegenerative diseases such as Alzheimer's and Parkinson's diseases and amyotrophic lateral sclerosis (ALS), also demonstrate alterations in neurotrophin expression (reviewed in Dechant and Barde, 2002). For instance, in the adult brain, $p75^{NRT}$ is re-expressed under various pathological conditions, including epilepsy, axotomy and neurodegeneration. The levels of re-expression are comparable to those seen during early development. This suggests that an imbalance of neurotrophin receptor signaling may be involved in diseases of the nervous system, or that the disease may induce a switch to a developmental expression of this receptor (Rende *et al.*, 1993; Kokaia *et al.*, 1998;

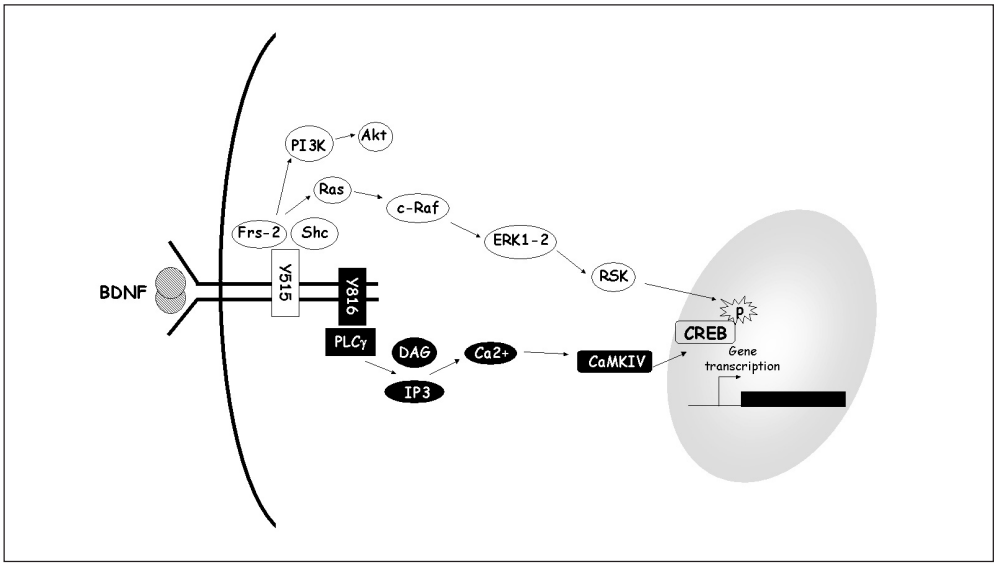

Fig. 4. Schematic representation of the signaling pathways associated with the Shc- and phospholipase-Cγ (PLCγ)-adaptor binding sites of TrkB [Tyr 515 (Y515) and Tyr 816 (Y816), respectively]. ERK, extracellular-signal regulated kinase; CREB, cAMP-response-element-binding protein; CaMKIV, Ca^{2+}-calmodulin-dependent kinase IV; PLCγ, phospholipase-Cγ; DAG, Diacylglicerol; IP3, Inositol 1,4,5-phosphate; PI3K, phosphatidylinositol 3-kinase (adapted from Ernfors and Bramham, 2003).

Mufson and Kordower, 1992; Giehl, 2001; Lowry *et al.*, 2001; Dechant and Barde, 2002). Furthermore, it has recently been discovered that a single amino-acid (Val 66 Met) polymorphism in the pro-domain of BDNF accounts for the ability of BDNF to undergo proper secretion and results in memory deficits (Egan *et al.*, 2003). These findings associate functional NT directly with psychiatric disorders.

1.1.2.3 Role of neurotrophins in corticospinal survival mechanisms

Corticospinal neurons (CSN) develop from subplate cortical precursors after embryonic day 13 (Armand *et al.*, 1994). Already during migration to their final destination, neocortical layer V, CSN extend axonal processes to subcortical targets. As CSN maturate, they maintain the collateral to their primary target, the spinal cord, and eliminate collaterals to the intermediate targets, e.g. the optic tectum (Armand *et al.*, 1994). During embryonic and early postnatal development, all target areas of CSN express the neurotrophins BDNF, NT-4/5 and NT-3, together with other neurotrophic factors such as CNTF and GDNF (reviewed in Giehl, 2001). During embryonic stages of development, however, the fate of cortical neurons is mainly determined by local signals. In this context, endogenous NT-3 acts as a differentiation signal for cortical precursors to develop into a neuronal phenotype (Ghosh and Greenberg, 1995) that depends on autocrine/paracrine BDNF supply for survival (reviewed in Giehl, 2001). The role of those neurotrophic factors that are expressed in the primary and intermediate target areas of developing CSN is unknown.

Mature CSN are located in layer V of the cortex, in areas associated with motor function (Miller, 1987; Nudo and Masterton, 1990). Virtually all adult CSN express TrkB and TrkC, and most of them also the GDNF receptor GFRα1 (Giehl *et al.*, 1998) and the CNTF receptor CNTFRα1 (Lee *et al.*, 1997). As suggested by *in vitro* studies (Giehl *et al.*, 2001), CSN appear to shift trophic dependencies during postnatal development from being dependent on BDNF (Ghosh and Greenberg, 1995) to other survival factors, CNTF, GDNF and NT-4/5 (Junger and Varon, 1997; Junger and Junger, 1998).

To study trophic dependencies in adult CSN, Giehl and collaborators (Giehl and Tetzlaff, 1996; Bonatz *et al.*, 2000) developed an *in vivo* model in which cell death of CSNs is induced by axotomy at internal capsule level (ICL). CSNs are specifically labeled by injecting retrograde tracers to the corticospinal tract of the spinal cord (Bonatz *et al.*, 2000). Once traced, axotomy of CSNs is stereotactically induced at the level of the internal capsule and analysis of survival can be assessed in CSNs of the sensorimotor cortex (Bonatz *et al.*, 2000). Intracortical infusions to the injured neurons serve to identify growth factors that might be relevant for the trophic support of CSN (Fig.5)

Accordingly, it has been shown that after injury BDNF is essential for the survival of CSN since application of function-blocking BDNF antibodies results in severe death of axotomized CSN, whereas exogenous application of BDNF results in total rescue of CSNs

(Giehl *et al.*, 1998). Axotomized CSNs are supported via a paracrine BDNF-mechanism that can be stimulated by BDNF- and NT-3 (Schutte *et al.*, 2000; reviewed in Giehl, 2001). GDNF is also a survival factor for lesioned CSN (Giehl *et al.*, 1997), and its survival effect depends on the presence of endogenous BDNF (Giehl *et al.*, 1998). In contrast to NT-3, GDNF does not exert its effect via stimulation of cortical BDNF expression (Schutte *et al.*, 2000). Inhibition of endogenous NT-3 and its TrkC or $p75^{NTR}$ receptor has been shown to promote CSNs survival, suggesting that endogenous NT-3 is responsible for the demise of a significant portion of injured CSNs. Even more, $p75^{NTR}$ is strongly induced in lesioned CSNs (Giehl *et al.*, 2001; Harrington *et al.*, 2004) while it is barely detectable in the unlesioned neurons. Consistently, blockade of $p75^{NTR}$ or neutralization of a $p75^{NTR}$ binding ligand, proNGF, rescues CSNs from axotomy- induced death. This indicates that in addition to TrkC/NT-3, also the $p75^{NTR}$/proNGF pathway promotes the death of lesioned CSNs. A schematic representation of the trophic dependences of CSNs is depicted in Fig. 6.

Recently, cortical neurons from the layer V have been shown to express the chloride cotransporter KCC2 (Kanaka *et al.*, 2001). As CSNs are a major subpopulation of this layer, this finding suggests that CSNs are a suitable model for studying possible relationships between NTs and chloride homeostasis.

1.2 Chloride homeostasis in brain development and pathophysiology

1.2.1 *GABA and neuronal maturation*

In the late 50's David Curtis and John Watkins were the first to show that γ-aminobutyric acid (GABA) inhibits the ability of mammalian neurons to fire

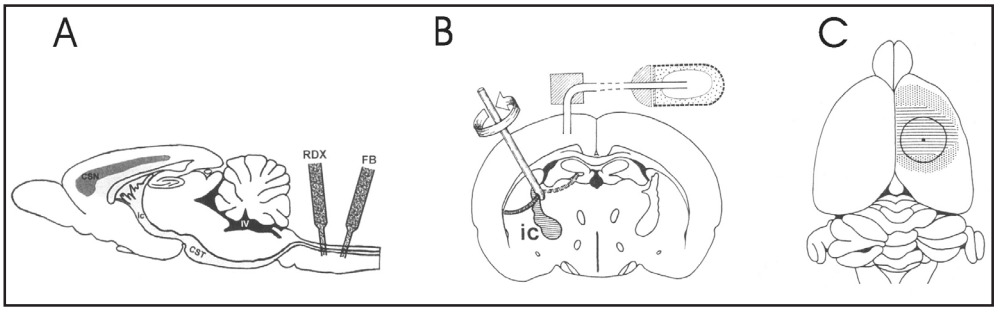

Fig. 5. Tracing and lesion of CSNs. A) To distinguish CSNs from other cortical layer V CSNs, neurons are retrogradely labeled by fluorescence tracer injections to the corticospinal tracts at cervical spinal cord levels C4/5. Fast blue (FB) is applied before lesion. B) After tracing, CSNs of one side of the brain are axotomized by unilateral internal capsule lesion (ICL) and the extension of the damage is controlled by the injection of rhodamine (RDX) immediately after lesion. This lesion results in axotomy of all corticospinal neurons of the sensory motor cortex of the lesion side. Osmotic minipumps are implanted intraparenchymally and connected to a steel cannula in the same operation session for intracortical delivery of solutions. C) Extension of the lesion (modified from Giehl and Tetzlaff, 1996 and Bonatz *et al.*, 2000; with permission)

action potentials (Curtis *et al.*, 1959). To date, it is of a general knowledge that most inhibitory neurons in the brain and spinal cord use either GABA or glycine as inhibitory transmitters, compensating the effects of excitatory transmitters (i.e. glutamate) and therefore generating inputs essential for the proper electric activity in the brain.

GABA, which is produced mainly through enzymatic decarboxylation of glutamic acid by glutamic acid decarboxylase (GAD), binds to two types of receptors, named $GABA_A$ and $GABA_B$. $GABA_A$ is a ligand-gated ion channel permeable to anions, and $GABA_B$ is a metabotropic receptor that by G protein transduction will open channels permeable to K^+ or inhibit channels permeable to Ca^{2+} (for review see Kaila, 1994).

$GABA_A$ receptors are highly permeable to chloride (Cl^-). When $GABA_A$ receptors are open, outward/inward currents of Cl^- may enter or leave the cell shifting the membrane potential

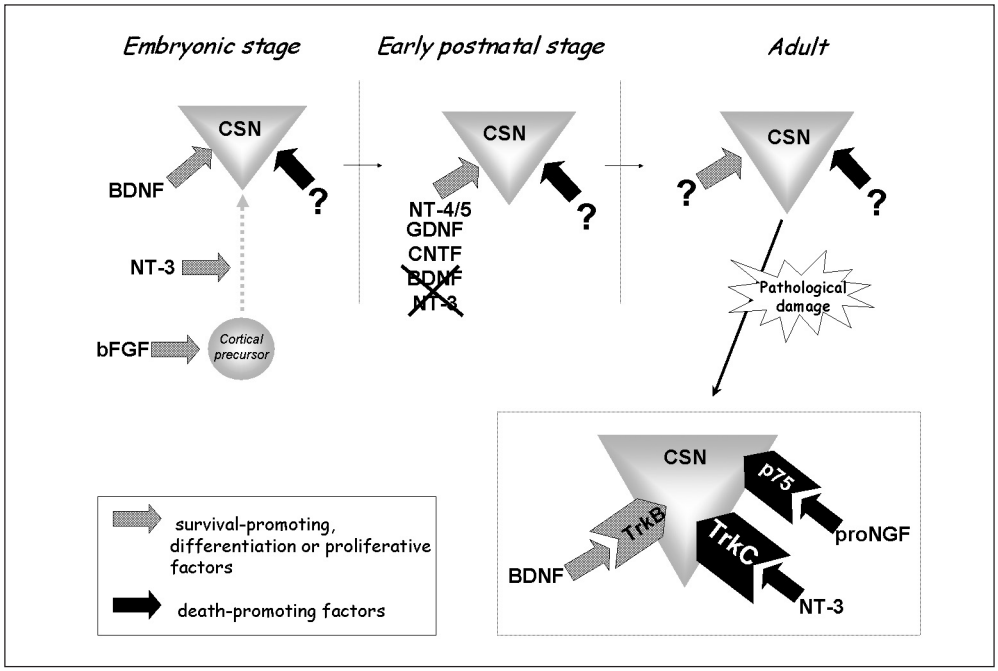

Fig. 6. Trophic dependencies of corticospinal neurons (CSNs). Cortical precursors require the presence of basic Fibroblast Growth Factor (bFGF) to proliferate and NT-3 to differentiate into a corticospinal phenotype. Survival of CSNs at embryonic stage is dependent on BDNF. Early postnatal CSNs lose BDNF-dependency to other survival factors such as GDNF, CNTF and NT-4/5. Death-promoting factors of CSNs at embryonic and early postnatal stages are unknown. Adult CSNs only require BDNF for survival when lesioned. The factors that promote survival/death of intact adult neurons are not known. The survival of adult CSNs depends on BDNF-mediated signaling through TrkB only under pathological conditions, and the death of injured neurons is promoted by the proNGF and NT-3, when binding to $p75^{NRT}$ and TrkC respectively. The factors that promote survival/death of intact adult neurons are not known.

close to the reversal potential for Cl^- (E_{Cl}) (Kaila 1994). Additionally, $GABA_A$ receptors are permeable to bicarbonate (HCO_3^-), as demonstrated by Kaila and collaborators (Kaila, 1994) when studying the effect of $GABA_A$-mediated responses on pH regulation in dissociated and cultured hippocampal neurons (Pasternack *et al.*, 1993) and in crayfish muscle fibers (Kaila and Voipio, 1987; Kaila *et al.*, 1992). Thus, the reversal potential of $GABA_A$ receptors (E_{GABA-A}) is set by E_{Cl} and $E_{HCO3.}$ Therefore, not only changes in intracellular chloride concentrations $[Cl^-]_i$ but also in intracellular bicarbonate concentrations $[HCO3^-]_i$ determine the polarity (hyper- or depolarizing) of the $GABA_A$-mediated response.

$GABA_A$ receptor-mediated responses vary during development. The well-known inhibitory synaptic actions of GABA occur in postnatal and mature neurons. At this stage and under normal conditions, $GABA_A$ receptor-mediated responses are hyperpolarizing (inhibitory). On the contrary, in immature neurons $GABA_A$ receptor-mediated responses are depolarizing (excitatory) and facilitate the generation of Na^+ and Ca^{2+} currents which will induce the activation of voltage-dependent Ca^{2+} channels (VDCCs) consequently generating $[Ca^{2+}]i$ transients that have a central role in neuronal differentiation, growth and maturation (Yuste and Katz, 1991; Barker *et al.*, 1998; Maric *et al.*, 2001; Owens and Kriegstein, 2002). This shift in GABAergic responses involves developmental and maturation processes tightly related to the expression and function of cation-chloride cotransporters (CCC).

1.2.2 *Cation Chloride Cotransporters*

CCCs are a group of proteins that participate in ion homeostasis and have lately been discovered to be intrinsically involved in mechanisms that control neuronal growth and maturation, synaptic development and plasticity, neuroendocrine functions and the generation of network rhythms (reviewed in Lauf and Adragna, 2000; Payne *et al.*, 2003).

Their nature is electroneutral and as such, they do not generate any current. Instead, they contribute to the inwardly or outwardly directed net flux of ions generated by gradients that are set by active transporters such as the Na^+/K^+-ATPase (see Fig.7). The hydrolysis of ATP by the Na^+/K^+-ATPase generates an inward gradient of K^+ and an outward gradient of Na^+ that by the action of CCCs will influence the net movement of Cl^-. Consequently, under physiological conditions, the gradient of K^+ is employed by the K^+/Cl^- cotransporters (KCC) to extrude Cl^- out of the cell, whereas the $Na^+/K^+/2Cl^-$ cotransporters (NKCC) will use the gradient of Na^+ to induce an increase of the intracellular Cl^- concentration. Together with the action of other secondarily active transport proteins, the Na^+-dependent and independent anion exchangers (NDAE and AE, respectively), which exchange Cl^- for $HCO3^-$, changes in the intracellular concentration of Cl^- will influence the overall electrochemical gradient of Cl^- and hence the resulting hyperpolarizing or depolarizing current across anion-permeable channels (i.e. GABA- and glycine-gated ion channels)

(reviewed in Kaila, 1994; Payne *et al.*, 2003) (Fig.7).

To date, seven electroneutral cation-Cl^- cotransporters have been described in mammals: one Na^+/Cl^- cotransporter (NCC), two $Na^+/K^+/2Cl^-$ cotransporters (NKCC1 and NKCC2) and four K^+/Cl^- cotransporters (KCC1-4). Structurally, they are 120-200 kDa glycoproteins with a relatively small intracellular N terminus followed by 12 putative transmembrane segments, and a large intracellular C terminus, which in the case of KCC1 and KCC2 presents a tyrosine kinase consensus site (Payne *et al.*, 1996).

The KCC1 isoform was the first CCCs discovered (Gillen *et al.*, 1996). It is activated by cell swelling and considered to be the house-keeping transporter that maintains volume homeostasis in all cells (Gillen *et al.*, 1996; Holtzman *et al.*, 1998; Gillen and Forbush, 1999). The two isoforms NKCC1 and KCC1 are ubiquitously expressed in all tissues (Gillen *et al.*, 1996; Plotkin *et al.*, 1997). NCC and NKCC2 are essentially restricted to the kidney (Russell, 2000), KCC3 and KCC4 are found in both CNS and non-neuronal tissues (Hiki *et al.*, 1999; Mount *et al.*, 1999; Race *et al.*, 1999) and KCC2 is only expressed in neurons (Payne *et al.*, 1996; Rivera *et al.*, 1999; for review see Lauf and Adragna, 2000; Payne *et al.*, 2003).

There is a tight correlation between the expression of some CCCs and changing levels of intracellular Cl^- during development (Li *et al.*, 2002). In that context, the cotransporters NKCC1 and KCC2 have a central role in the regulation of chloride homeostasis in neurons. For instance, the expression of NKCC1 is high during early postnatal development (Plotkin *et al.*, 1997; Lu *et al.*, 1999; Li *et al.*, 2002). Therefore,

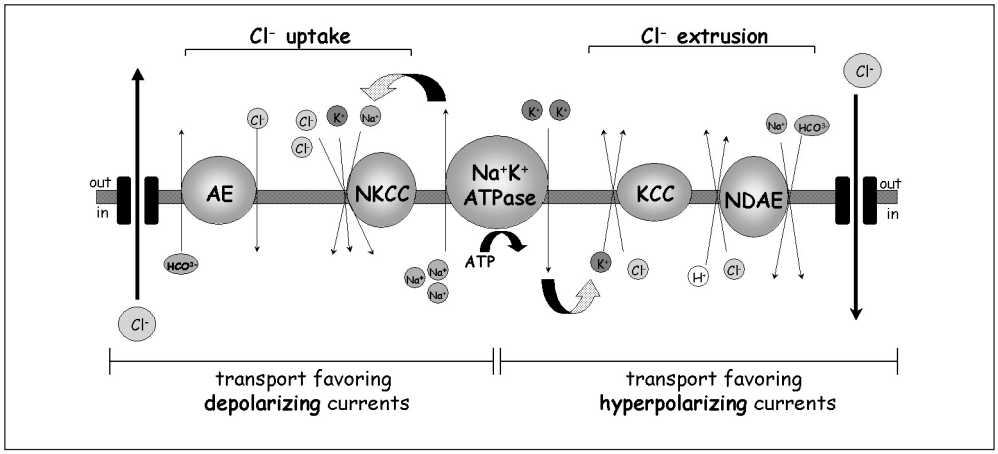

Fig. 7. Cathion Chloride transporters and their basic modes of operation. Under physiological conditions, NKCCs and AEs co-transporters mediate Cl^- uptake, whereas KCCs and NDAEs extrude Cl^-. The Na^+/K^+-ATPase generates a K^+ gradient that is employed by KCCs to extrude Cl^-.This results in an inwardly directed electrochemical gradient for Cl^- that generates hyperpolarizing currents across anion-permeable channels. Uptake of Cl^- by NKCCs is driven mainly by the energy taken from the Na^+ gradient, and the resulting outwardly directed Cl^- electrochemical gradient permits depolarizing Cl^- currents (modified from Payne *et al.*, 2003).

during developmental processes in immature neurons and in adult sensory neurons, the depolarizing GABAergic activity is regulated by NKCC1, raising the internal Cl^-. On the contrary, KCC2 expression increases during development (Lu *et al.*, 1999), thus, the inhibitory GABA responses in mature CNS neurons are regulated through KCC2 by lowering the $[Cl^-]i$ (Lu *et al.*, 1999; Rivera *et al.*, 1999; Hubner *et al.*, 2001; Li *et al.*, 2002; reviewed in Payne *et al.*, 2003)

The wide variety of physiological roles performed by the CCCs have been analyzed by gene disruption of the different transporters in mouse, and the phenotypes observed have been in some cases linked to human diseases. Summarizing, NCC, NKCC1 and NKCC2 may have an important role in regulating hypertension, KCC2 in epilepsy, KCC1 and KCC3 in hemoglobinopathies, and KCC4 in hearing disorders (Boettger *et al.*, 2002; reviewed in Delpire and Mount, 2002). However, the precise role of the individual pathways involved in the regulation of these phenotypes are yet unknown.

1.2.3 *KCC2*

1.2.3.1 Neuronal expression of KCC2

Identified using a combination of database searching, Polymerase Chain Reaction (PCR) techniques and cDNA library screening, KCC2 was characterized as a ~145 kDa protein with an amino acid sequence 67% identical to KCC1 (Payne *et al.*, 1996; Williams *et al.*, 1999). When comparing the primary structures of KCC1 and KCC2, the predicted structure for KCC2 is a glycosylated 12 transmembrane (TM) domains protein where TM2 is the most divergent segment, sharing only 60% amino acid identity (Payne *et al.*, 1996; Payne, 1997), and suggesting TM2 the responsible for ion binding.

In situ hybridization studies and PCR techniques demonstrated that KCC2 mRNA is located in the CNS (Payne, 1997, Rivera *et al.*, 1999), although it is also found in PNS as demonstrated by immunohistochemistry (IHC) and PCR in dorsal root ganglion (Lu *et al.*, 1999).

During development, KCC2 mRNA exhibits low levels of expression in rat hippocampal, cortical and retinal neurons, which increase with maturation (Clayton *et al.*, 1998; Lu *et al.*, 1999; Rivera *et al.*, 1999; Williams *et al.*, 1999; Vu *et al.*, 2000). In adulthood, the KCC2 protein is abundant in most neurons throughout the nervous system (Kanaka *et al.*, 2001), preferentially located in dendritic areas of the hippocampus (Rivera *et al.*, 1999; Williams *et al.*, 1999; Gulyas *et al.*, 2001) and the neocortex (Fukuda *et al.*, 1998a; DeFazio *et al.*, 2000).

1.2.3.2 Neuronal function of KCC2

The nature of GABAergic transmission, excitatory versus inhibitory, is determined primarily by the electrochemical gradient for Cl^-, which depends on the intra- and extracellular concentrations of Cl^-. This electrochemical gradient is mediated by K^+-Cl^- cotransport and sets the reversal potential for GABAergic mediated responses (E_{GABA-A}; the membrane voltage at which $GABA_A$ currents change their direction). The first indications about KCC2 being the cotransporter

responsible for the proper establishment of Cl^- currents in mature neurons, and hence the proper GABAergic transmission, were acknowledged by Payne and collaborators (Payne *et al.*, 1996; Payne, 1997). The fact that KCC2 was only expressed in neurons indicated that this isoform displayed unique functional characteristics. In addition, IHC studies colocalized KCC2 with $GABA_A$ receptors, suggesting a putative role of KCC2 in neuronal Cl^- extrusion and post-synaptic inhibition. This role, was finally confirmed by Rivera and collaborators (1999). Using antisense oligonucleotides exposed to rat hippocampal slices, they were able to demonstrate that the shift from depolarizing to hyperpolarizing actions of GABA that occurs during neuronal maturation, is correlated with an up-regulation of KCC2 mRNA expression (Rivera *et al.*, 1999).

The importance of KCC2 in the regulation of CNS excitability has been established by the characterization of mice with targeted deletion of the KCC2 gene. Disruption of *Kcc2* results in a failure of hyperpolarizing inhibition that leads to early postnatal death due to an abnormal muscle tonus, defects in motor control, and to an inability to breathe (Hubner *et al.*, 2001). Additionally, hypomorphic KCC2 gene-targeted mice show phenotypical variability according to their expression levels of KCC2 (Woo *et al.*, 2002;Vilen *et al.*, 2001). Consequently, hypomorphic animals with 20-30% of the normal KCC2 levels are viable and have no obvious behavioural abnormalities (Woo *et al.*, 2002), whereas mice with only 5-10% of KCC2 display spontaneous, generalized seizures and die shortly after birth (Vilen *et al.*, 2001). Thus, reduction in KCC2 expression results in an increased susceptibility to the development of seizures. Therefore the early function of KCC2 might be critical during brain development and the formation of brain circuitry.

In this context, a potential role for KCC2 in diseases that involve seizure-induced phenotype such as the human epilepsy has been suggested. The human KCC2 is located on chromosome 20q (Song *et al.*, 2002). Although this arm of chromosome 20 contains two epilepsy genes, there is no linkage with the segment containing the KCC2 gene. This, however, does not exclude the possibility that KCC2 constitutes a modulating factor for epilepsy. In this context, it is important to note the work by Cohen and collaborators (Cohen *et al.*, 2002) in finding the grounds of epileptic activity in the human hippocampus. Performing electrophysiological recordings in hippocampal tissue surgically removed from epileptic patients, they demonstrated that a subpopulation of pyramidal neurons from the sclerotic CA1 hippocampal region are depolarizing in response to the GABA released by the interneurons. These abnormal responses from the pyramidal neurons supported by the activity of the interneurons result in a pathological synchronized excitation and initiates epileptic activity. Whether these depolarizing effects of GABA are linked to a low expression of the KCC2 in pyramidal neurons, or how this decrease is related to seizure activity, is open up for investigation.

1.2.3.3 KCC2 regulation

The molecular mechanisms behind the regulation of KCC2 are currently under

study. Nevertheless, several reports have already provided insights for the understanding of the regulatory mechanisms controlling KCC2 during development and mature situations.

Localization of a canonical tyrosine phosphorylation site in the carboxy terminal region of KCC2 suggests that KCC2 regulation may occur by protein phosphorylation. In that context, biochemical experiments have shown that cultured hippocampal neurons initially contain an inactive form of the KCC2 protein, which becomes activated during subsequent maturation. That process can be accelerated by insulin-like growth factor-1 (IGF-1) and can be reduced by the application of membrane-permeable protein tyrosine kinase inhibitors (Kelsch *et al.*, 2001). These results suggested that the activation of KCC2 during development might be dependent upon growth factor-mediated tyrosine kinase phosphorylation. However, Stein and collaborators (2004) have recently demonstrated that phosphorylated KCC2 was detectable early in the spinal cord development when the functional GABA switch had not yet occurred, indicating that tyrosine phosphorylation seems to be less important for the functional switch than the transcriptional up-regulation of KCC2.

Other molecules that might contribute to the intracellular regulation of KCC2 have been described by using a yeast two-hybrid screening (Inoue *et al.*, 2004). The experiments indicate that the C-terminal region of KCC2 interacts with an ATP-generating enzyme, the brain-type creatine kinase (CKB). This interaction was also detected in cultured cells and brain extracts. The authors suggest that CKB might provide ATP to modulate the activity of KCC2, but the functional role of this interaction is not yet evident.

In an attempt to investigate whether synaptic activity can regulate the switch of GABAergic transmission, Ganguly and collaborators (Ganguly *et al.*, 2001), found that in mouse hippocampal cultures KCC2 expression is under the control of $GABA_A$ receptor-mediated transmission. The level of KCC2 mRNA was reduced by chronic blockade of $GABA_A$ receptors and increased by chronic depolarization with KCl. Their results suggested that GABA itself acts as a critical maturation factor for the switch of the physiological and biochemical properties of GABA signaling. However, this statement is currently under debate. Recently published reports have shown that hyperpolarizing inhibition may develop without trophic support of GABA. Titz and collaborators (Titz *et al.*, 2003) found the existence of a switch in GABAergic responses from depolarizing to hyperpolarizing in cultured midbrain neurons even when $GABA_A$ receptors had been blocked throughout development, providing conflicting evidence that GABA itself is not required for the neuronal developmental switch. On the other hand, using both dissociated as well as organotypic hippocampal cultures (a more physiological approach), Ludwig and collaborators, showed that the developmental up-regulation of KCC2 mRNA expression as well as the protein levels, were not altered by chronically GABAergic blockage (Ludwig *et al.*, 2003).

The up-regulation of the KCC2 mRNA at early developmental stages has also recently been shown by Aguado and collaborators (Aguado *et al.*, 2003). The authors showed an increase in KCC2 expression paralleled with an increase in

spontaneous network activity in the hippocampus of mice overexpressing BDNF that exhibit neither an increase in the expression of GABA nor glutamate ionotropic receptors, thus suggesting a role of BDNF in the regulation of KCC2 expression. Whether the effect of BDNF on KCC2 expression during development involves a direct or indirect molecular mechanism is not known.

Overall, the molecular mechanisms behind the regulation of KCC2 still need to be elucidated. An open question is whether this essential protein is self-regulatory or might be regulated in concerted action with other proteins (i.e. maybe with CCC members or even unrelated proteins).

1.2.4 *Chloride homeostasis and trauma*

Traumatic insults to the brain (contusion, epileptic seizures or hypoxia–ischemia) cause excessive release of glutamate that leads to elevated $[Ca^{2+}]_i$ and to excitotoxic cellular damage and death (Lipton, 1999). Additionally, $GABA_A$ receptor-mediated responses after trauma have been shown to be depolarizing as a result of an increase in $[Cl^-]_i$ (Katchman *et al.*, 1994; van den Pol *et al.*, 1996; Fukuda *et al.*, 1998b; Nabekura *et al.*, 2002) and, consequently, the excitatory actions of GABA after trauma contribute to neuronal damage by increasing even more cytosolic Ca^{2+} levels in injured cells.

In rat hippocampal pyramidal neurons $GABA_A$ receptor-mediated currents are depolarizing at birth, but the resting potential of GABA (E_{GABA}) becomes progressively more negative during early postnatal maturation, indicating a decrease in $[Cl^-]_i$ during development (reviewed in Ben-Ari, 2002). Nevertheless, in mature neurons, the same embryonic GABA and glycine receptor-mediated depolarizing responses have been shown under some pathophysiological conditions such as trauma (van den Pol *et al.*, 1996; Nabekura *et al.*, 2002; Toyoda *et al.*, 2003), and epilepsy (Köhling *et al.*, 1998; Cohen *et al.*, 2002).

Interestingly, the elevation in neuronal $[Cl^-]_i$ and depolarizing action of $GABA_A$ receptors that occur after trauma appear to be mainly caused by alterations in KCC2 expression. In this context, down-regulation of KCC2 mRNA expression has been observed after focal cerebral ischemia-induced excitotoxicity (Galeffi *et al.*, 2004), and it seems to be a general early response involved in various kinds of neuronal trauma (Nabekura *et al.*, 2002; Coull *et al.*, 2003; Malek *et al.*, 2003; Toyoda *et al.*, 2003). In addition, a dramatic loss of KCC2 protein has been observed after sustained interictal-like activity induced in hippocampal slices in the absence of Mg^{2+} (ref. Thomas-Crusells, *et al.* 2000).

The functional significance of the depolarizing action of GABA after injury has been suggested to be related to an attempt from the neurons to recapitulate ontogenic programs in which they have greater developmental plasticity for recovery (van den Pol *et al.*, 1996; Cramer and Chopp, 2000; Payne *et al.*, 2003). However, whether these programs imply neuronal survival remains unknown.

2. AIMS OF THE STUDY

The present work has been conducted under the general aim of elucidating the significance of the interplay between neurotrophins and chloride homeostasis under normal and pathological conditions.

The specific aims of the study were:

- To analyze the effect of neurotrophins on the expression and function of KCC2 in intact adult rodent hippocampus and under pathophysiological conditions *in vivo* and *in vitro.*
- To develop a simple and reliable method for detecting surface membrane protein trafficking changes in hippocampal tissue, in order to study the regulation of the KCC2 trafficking.
- To characterize the molecular mechanisms involved in the activity-dependent regulation of KCC2 by using both *in vivo* and *in vitro* models of altered patterns of neuronal activity, in order to elucidate the intracellular signaling pathways involved.
- To study the effect and potential role of neurotrophins on KCC2 in neuronal survival after *in vivo* injury.

3. MATERIALS AND METHODS

The methods used are described in detail in the "Materials and Methods" section of the respective publications. All experimental procedures using laboratory animals were performed according to ethical guidelines approved by local authorities.

3.1 Animals and tissue preparation

Experimental animals are described in I-IV. Young and adult NMRI (II) and C57BL/6 (I) mice, adult mice from a BDNF- mutant strain (IV) (Korte *et al.*, 1995), NT-3- deficient mice (Airaksinen *et al.*, 1996), adult mice from $TrkB^{PLC/PLC}$-, $TrkB^{SHC/SHC}$- and $TrkB^{W/W}$ (III)-mutant strain (Minichiello *et al.*, 2002), and Sprague-Dawley (I, IV) and Wistar (III) rats were housed under conditions of 12-14h light and 10-12h darkness cycles, receiving food and water *ad libitum*. On the day of experimentation, animals were anesthetized in a CO_2 chamber and sacrificed by cervical dislocation followed by decapitation (I-III), or anaesthetized with a combination of chloral hydrate/sodium pentobarbital for surgical procedures, and sacrificed by an overdose of sodium pentobarbital before transcardial perfusion (IV).

3.2 Tissue processing

Acute and organotypic hippocampal slices were processed and maintained as described in I-III and elsewhere (Stoppini *et al.*, 1991; Lahtinen *et al.*, 2001). For experiments that required fixed tissue, brains were either quickly removed and immediately frozen on powdered dry ice for later fixation (I-III), or brains from animals previously transcardially perfused with PBS followed by 4% PFA, were post-fixed in PFA, cryoprotected in 20% sucrose in PBS, frozen in dry ice cooled isopentane, and cryosectioned (IV).

3.3 Biotinylation of cell surface proteins

Biotinylation procedure of acute hippocampal slices, homogenate preparation of surface biotynilated slices, and measurements of protein turnover rates experiments are detailed in II.

3.4 *In situ* hybridization and quantification of mRNA expression

The protocols for radioactive *in situ* hybridization performed on brain cryostat sections are described in I, III and IV. Non-radioactive *in situ* hybridization of free-floating sections from acute hippocampal slices is described in II and III and in Nieto *et al.*, 1996. The cRNA probes used are described in Table 2. Quantification of hybridization signals for KCC2, BDNF and TrkB mRNAs in the hippocampus is described in I and III. The method of quantification of KCC2, GAP-43 and Tα1-tubulin mRNAs levels in CSNs is detailed in IV and in McCabe *et al.*, 1989. The controls were performed by competition with an excess of cold probe and with sense mRNAs probes.

3.5 Immunohistochemistry and western blotting

The procedures for both IHC and Western blotting detection are described in I, II and III. The antibodies used in both procedures are presented in Table 3. In

the IHC experiments samples in which the primary antibodies were omitted were used as controls. Non-specific labeling was not detected in any case. For Western blots, and according to the experiment, antibodies detecting proteins with known turnover rates, neuronal specific structural proteins and intracellular proteins, were used as a controls.

3.6 RT-PCR

The protocol for total extraction of mRNA from rat hippocampal slices and the PCR amplification are described in II and III and previously in Rivera *et al.*, 1999. Total RNA from the slices was extracted with Trizol (Sigma) using a modified version of the protocol by Chomczynski and Sacchi, 1987. Total RNA was reverse transcribed (RT) using random hexamer primers and Superscript II reverse Transcriptase (Life Technologies Inc.).

3.7 ELECTROPHYSIOLOGICAL RECORDINGS

The protocols for electrophysiological recordings are described in I, II and III. Evoked field potentials were measured from rat CA1 pyramidal cells from acute and/or organotypic hippocampal slices using glass microelectrodes filled with 150 mM NaCl and 50mM of the spiking blocker QX-314, and were recorded with MED64 multichannel recording system (I) or with an Axoclamp 2B amplifier in bridge or discontinuous mode (II, III).

3.8 *IN VIVO* KINDLING AND AXOTOMY PROCEDURES

Fast kindling protocol in which a direct stimulation of the ipsilateral hippocampus is applied, was used in our *in vivo* kindling experiments. This experimental model of epilepsy differs from the hypothalamic kindling protocol in which increased susceptibility appears after a long period of daily stimulation. The description of the kindling protocol is detailed in I and in Kokaia *et al.*, 1999. Control animals were operated but did not receive stimulation.

The axotomy procedure performed in the study IV of this thesis is based on a unilateral lesion at the level of the internal capsule (IC) region where all axons of the corticospinal neurons of the sensory motor cortex converge. To distinguish CSNs from other cortical layer 5 neurons, they were retrogradely labeled by fluorescent trace injections to the corticospinal tracts at cervical spinal cord levels C4/5 before axotomy. Fast Blue (FB; 2% in 0.2% DMSO) and/or rhodamine tracer mixture (RDX; 15% rhodamine dextran 10,000, 10% rhodamine dextran 3000, and 10% rhodamine-β-isothiovyanate in 0.2% DMSO) were used as tracers. The unlesioned contralateral side of the brain is the lesion control. The coordinates for stereotaxic lesion and intracortical neurotrophic factor delivery are described in IV and elsewhere (Giehl and Tetzlaff, 1996; Bonatz *et al.*, 2000). The neurotrophic factors and intracortically infused antibodies are listed in Table 4.

3.9 NEURONAL SURVIVAL ANALYSIS

The analysis of survival of rat and mouse CSN is described in IV and in Bonatz *et al.*, 2000. The number of surviving CSN was assessed by blinded cell counts of every second section collected for cell counts (i.e. every fourth section of the mice and every 10th section of the rat

brains). The criterion for a CSN was a tracer-filled pyramidal-shaped profile of >4 mm (rats) and >3 mm (mice) in diameter. For the quantitative survival data, only the data from the cell-death areas were used (the cell death area spans from the frontal level of the anterior commissure 2.8 and 1.6 mm in posterior direction in rats and mice, respectively. See Bonantz *et al.*, 2000) Within the cell death area, percentage of survival is defined as number of fast blue-labeled CSN on the lesion side divided by the number of fast blue-labeled CSN in contralateral to the lesion side x 100%.

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

Probe	Specie	Lenth	Nucleotides	Reference	Genbank no.	Used in
TrkB	mouse	483	517-882	Klein et al., 1989; Hiltunen et al., 1996	X55573	I
Kcc2	mouse	1039	4605-5566	Payne et al., 1996	AA982489	I, III
Bdnf	rat	366	517-882	Hiltunen et al., 1996	M61178	I, III
Kcc2	rat	35	2981-3016	Kanaka et al., 2001	U55816	IV
Gap-43	rat	51	220-270	Basi et al., 1987	-	IV
Tα1-tubulin	rat	50	1548-1594	Ginzburg et al., 1986	-	IV

Table 3. List of antibodies used for immunohistochemistry (IHC), Western blotting (WB) and Biotinylation assay (BT).

Antibody/Antigen	Host	Source/Reference	Dilution	Method	Used in
GluR1	gt	Santa Cruz	1:1000	WB	II
Transferin Receptor	mo	Zymed Laboratories	1:2000	WB	II, III
MAP2	rb	Chemicon	1:2000	WB	II
KCC2	rb	Payne et al.,1996	1:1000	WB	I-III
			1:600	IHC	II
	rb	B2C/Rivera et al., 1999	1:5000	WB	I-III
			1:200	IHC	III
αTubulin (DM1A)	mo	Sigma-Aldrich	1:5000-1:10000	WB	I, III
pCREB	rb	Upstate Biotech	1:1000	WB	III
HRP-IgG	mo	Sigma	1:4000	BT	II-III
HRP-IgG	rb	Sigma	1:4000	BT	II-III
goat-HRP	do	Amersham Pharmacia	1:3000	IHC	II
goat-Cy3	do	Jackson Lab	1:200	IHC	II
FITC-IgG	gt	Jackson Lab	1:200	IHC	III
TRITC-IgG	gt	Jackson Lab	1:200	IHC	III

gt, goat; mo, mouse; rb, rabbit; do, donkey

Table 4. List of intracortically infused substances

Infusion contents	Concentration	Source/Reference	Dilution
PBS	20mM	Sigma	
BDNF	500µg/ml in PBS	Amgen	
RAB*	1mg/ml in PBS	Amgen/ Yan et al., 1997b	
anti-TrKB	serum	Amgen/ Yan et al., 1997b; Kang et al., 1997	1:7
anti-TrKC	serum	Amgen/ Yan et al., 1997b; Giehl et al., 2001	1:7
preTrkC	serum	Amgen	
Mab#21/27**	1mg/ml in PBS	Amgen/Korsching and Thoenen, 1987	
mouse-IgG	1mg/ml in PBS	Sigma	
TrkB-Fc	60µg/ml in PBS	Amgen	
KCC2-AS"	9µg/ml	Institute of Biotechnology, Univ. Helsinki	
Random"	9µg/ml	Institute of Biotechnology, Univ. Helsinki	

All solutions contained penicillin/streptomycin in a final contentration of 50U/ml

* affinity purified BDNF-neutralizing rabbit polyclonal anti-BDNF antibody

**anti-NGF antibody "KCC2 anti-sense, and random oligonucleotides

"KCC2 anti-sense and random oligonucleotides

4. RESULTS AND DISCUSSION

4.1 PATHOLOGICAL ACTIVITY ALTERS THE EXPRESSION OF KCC2 (I-III)

Altered patterns of activity as well as different types of traumatic insults are known to induce long-term effects on neuronal Cl^- regulation that result in changes in the GABAergic transmission (Payne *et al.*, 2003). Since the neuron-specific K^+/Cl^- cotransporter KCC2 is a key regulator of chloride homeostasis (Rivera *et al.*, 1999; DeFazio *et al.*, 2000; Kakazu *et al.*, 2000; Hubner *et al.*, 2001), it is imaginable that these changes would affect both the expression and the function of KCC2. To address this possibility, we performed *in vitro* and *in vivo* experiments in mice and rat hippocampus.

4.1.1 *Effect of pathological spontaneous activity on KCC2 mRNA and protein expression*

In order to study the effects of neuronal activity on the KCC2 mRNA and protein expression *in vivo*, we examined the effects of kindling-induced seizures in the mouse hippocampus. Strikingly, after *in vivo* hippocampal kindling–induced seizures the KCC2 mRNA decreased in all hippocampal regions *(I, Fig. 4A)*. In the *dentate gyrus* (DG) granule layer, a large decrease in KCC2 mRNA expression was already observed 2 h after kindling (55 ± 2%), and the lowest level was reached after 6 h (30 ± 3%). After 24 h, KCC2 mRNA levels had partially recovered (77 ± 3%). The KCC2 mRNA levels in the CA1 and CA3 regions were significantly lower than in control animals, and did not recover within 24 h**.** At the protein level, a general decrease in KCC2 immunostaining was found 6 h after kindling *(I, Fig. 4B)*. A regional comparison of these data with *in situ* hybridization results showed that the *in vivo* kindling-induced changes in KCC2 mRNA were directly translated into corresponding alterations in protein expression. These results suggested that the expression of KCC2 could be down-regulated by pathophysiological activity.

We also examined the effects of spontaneous activity on KCC2 expression *in vitro* in acute hippocampal slices that had been induced to interictal-like activity by withdrawing Mg^{2+} from the extracellular solution (Anderson *et al.*, 1986; Mody *et al.*, 1987). By using rodent hippocampal slices we also had the possibility of detecting membrane trafficking changes in KCC2 protein expression (see explanations below) as well as perform electrophysiological recordings in order to detect possible functional alterations due to the trafficking changes in KCC2. Additionally, previewing experimental analysis of genetically mutated mice strains in order to study KCC2 in regards to its possible molecular mechanisms of activity, using the 0-Mg^{2+}*in vitro* model that emulates epileptogenetic conditions was thought to be the most adequate.

RT-PCR experiments and *in situ* hybridization of KCC2 mRNA expression in hippocampal slices under Mg^{2+}-free (0-Mg^{2+}) conditions clearly showed a down-regulation of the expression of KCC2 mRNA that was detectable already after 30min and continued up to 3h exposure to 0-Mg^{2+} solution *(III, Fig.1A)*. The expression was recovered to control levels when slices had been treated in normal medium

conditions containing Mg^{2+}, or by adding a combination of glutamate activity blockers (NBQX and AP5) into the medium prior to exposure to 0-Mg^{2+} conditions *(III, Fig. 1B)*. Western blot analysis of slices treated under the same conditions, also showed down-regulation of the KCC2 protein and the levels were rescued in slices that had been exposed to a combination of pharmacological activity blockers *(III, Fig. 1C-D)***.** Non-radioactive *in situ* hybridizations of free-floating sections that were treated with the convulsant drug 4-AP, showed a strong down-regulation of KCC2 *(III, Fig. 3A)***.** Western blot analysis of the same slices revealed a decreased level of the KCC2 protein *(III, Fig. 3B)***.** In addition, inmunofluorescent staining of acute hippocampal slices exposed to the 0-Mg^{2+} solution revealed an area-specific decrease of KCC2 showing a strong reduction in staining intensity in CA1 dendritic region *(III, Fig. 2)***.** This is an interesting finding also in the light of recent evidence that suppression of dendritic inhibition is strongly pro-convulsant (Cossart *et al.*, 2001).

Overall, these results indicate an activity-dependent down-regulatory effect on the expression of KCC2, and supported our hypothesis that pathophysiological activity would affect the main key regulator of chloride homeostasis.

4.1.2 *Analysis of the degradation rate of membrane KCC2 in hippocampal slices*

Most of what is known about the intracellular trafficking of a variety of physiologically important membrane bound and transmembrane proteins are derived form studies using *in vitro* cell surface biotinylation. However, these experiments are either performed on cell lines or dissociated primary neuron cultures. In order to study trafficking of KCC2 protein in the same experimental paradigm (hippocampal slices) used in our experiments (I and III), we developed a surface membrane biotinylation technique to study membrane protein trafficking in the adult rodent hippocampal slice preparation **(II).** This should allow us to address the role of trafficking on the regulation of KCC2 function in an organotypic, i.e. physiologically relevant, context.

In order to examine the efficiency of the biotinylation in a complex tissue slice spanning several cell layers, we used a peroxidase amplification assay to detect the presence of bound biotin. After 1h of incubation, complete penetration of the biotinylation reagent into the acute hippocampal slices was detected in fixed transversal sections from hippocampal slices *(II, Fig. 2)*. Biotinylation of intracellular proteins such as MAP2 was not detected by immunohistochemistry, ensuring that the labeling was exclusively at the cell surface.

To assure the viability of the tissue, electrophysiological recordings were performed directly on the biotinylated slices. Normal extracellular evoked field responses in the CA1 region upon stimulation of Schäffer collaterals were obtained in labelled slices, up to 1h after slice preparation. Furthermore, lowering the Mg^{2+} concentration in the bath solution increased the number of population spikes evoked by a single stimulation *(II, Fig. 3)* showing that the 0-Mg^{2+}-induced increase in the excitability was maintained in the preparation. These results indicate that the biotinylisation

procedure affects cell surface proteins throughout the entire slice, and that the slice remains viable during the preparation.

To further validate the method, we studied the degradation kinetics of the Transferrin receptor (TfR) and the Glutamate receptor subunit A (GluR A). The kinetics of both membrane proteins has already been well established by other approaches (Mammen *et al.*, 1997; Horikawa and Nawa, 1998; Lok and Loh, 1998). TfR was our control protein since it is known to escape degradation by recycling back to the cell membrane (for review see Lok and Loh, 1998), whereas GluR A is known to present a fast degradation rate in hippocampal cell cultures (Mammen *et al.*, 1997; Horikawa and Nawa, 1998). Homogenates of biotynilated slices were precipitated with streptavidin and analysed by Western blots *(II, Fig. 4)*. By using immunoprecipitation of biotynilated protein with streptavidin beads we reduced possible problems of erroneous detection of endogenous biotin. Our results showed a high rate of degradation of GluR A and no significant degradation of TfR within the time window used, corroborating previously studied membrane trafficking changes of these two receptors in dissociated cell cultures, and thus validating our new methodology.

Taken together, we were able to establish a new method that offers the advantage of assessing protein trafficking in an organotypic situation. Obviously, this setting is significantly closer to the physiological situation than dissociated cultures. A constraint of the method is that it is more difficult to follow organelle distribution in neurons that are maintained in their organotypic context, than in neurons kept under dissociated culture conditions. In addition, acute slices placed on a one-side perfused planar channel recording system cannot be maintained viable for more than 5 or 6 h (Oka *et al.*, 1999), therefore restricting the time window in which the method can be applied. Thus, our system represents a manoeuvre to quantitatively study acute membrane protein changes in a physiological condition.

As demonstrated above (see section 4.1.1), the activity-specific down-regulation of KCC2 took place at a transcriptional level resulting also in decreased levels of KCC2 protein. However, we could not rule out that changes of KCC2 mRNA stability and/or alterations of KCC2 protein trafficking contributed to this phenomenon. Having established a method to assess protein trafficking in acute organotypic slices, we now addressed the question whether activity also affected the trafficking of KCC2 between cell surface and intracellular compartments. Our biotinylation assays revealed that KCC2 protein displays a strikingly fast turnover rate under Mg^{2+}-free conditions (*III, Fig. 4)*. The rate of decay was significantly reduced when a combination of activity blockers was applied to 0-Mg^{2+} exposure conditions, indicating that the KCC2 membrane protein was regulated in an activity-dependent manner. Accordingly, the activity-dependent increase in the turnover of the plasmalemmal KCC2 transporters enhances their susceptibility to transcriptional down-regulation that was detected at the transcriptional level in the previous experiments (see section 4.1.1).

Altogether, these fast modifications suggest that activity-dependent KCC2 regulation plays a role in synaptic plasticity.

4.1.3 *Effect of spontaneous activity on the KCC2 function*

The generation and maintenance of the chloride gradient required for hyperpolarizing ionotropic responses in CNS neurons in general, and the CA1 region of the hippocampus in particular, is attributed to the neuron-specific K^+–Cl^- cotransporter, KCC2 (Thompson and Gahwiler, 1989; Kaila, 1994; Rivera *et al.*, 1999; DeFazio *et al.*, 2000; Kakazu *et al.*, 2000; Hubner *et al.*, 2001).

To examine the functional consequences of the decreased expression of KCC2 **(I, III)** on neuronal Cl^- extrusion, we measured the inhibitory postsynaptic potential ($IPSP_A$) reversal potential (E_{IPSP-A}) in CA1 pyramidal neurons from acute hippocampal slices exposed to 0-Mg^{2+} conditions *(III, Fig. 5)*. The neuronal E_{IPSP-A} was measured in hippocampal slices and dissociated neurons subjected to a tonic chloride load with a 0.5 M Cl^-containing sharp microelectrode in the presence of the activity blockers (NBQX plus AP-5). The results obtained showed that 0-Mg^{2+}-induced activity led to a decrease in the efficacy of Cl^- extrusion, indicating that the predicted fall in plasmalemmal KCC2 was paralleled by an activity-dependent impairment in the efficacy of Cl^- extrusion by pyramidal neurons. These results also shed light on previous findings from *in vivo* and *in vitro* models of epilepsy as well as for human epileptic tissue which have demonstrated a positive shift in the driving force of $GABA_A$-mediated inhibition, which is sometimes sufficient to change the postsynaptic response from hyperpolarizing to depolarizing (Tasker *et al.*, 1992; Kapur *et al.*, 1995; Köhling *et al.*, 1998; Cohen *et al.*, 2002).

4.2 KCC2: A LINK BETWEEN NEUROTROPHINS AND CHLORIDE HOMEOSTASIS (I, III)

Among their well-known effects on neuronal survival and differentiation (Huang and Reichardt, 2001), neurotrophins have been reported to be involved in synapse formation and functional maturation (Marty *et al.*, 1997; Huang and Reichardt, 2001). In addition, in the adult brain, experimental paradigms involving strong stimulation such as *in vivo* kindling are known to lead to a very strong up-regulation and release of BDNF and to an activation of its receptor TrkB. This is thought to predispose cortical areas to seizures due to, at least in part, on the effects of fast GABAergic inhibition. (Binder *et al.*, 2001; Huang and Reichardt, 2001). Keeping this in mind, the activity dependent effects on the expression of KCC2 **(I, III)** could involve neurotrophin and/or neurotrophin receptor modulation. In order to address this hypothesis, we analyzed the effect of BDNF and NT-4/5 and NGF on KCC2 expression in acute and long term cultured organotypic hippocampal slices, and in *in vivo* kindling paradigms. For these studies, we used material from wild type rats and from TrkB mutant mice.

4.2.1 *Fast down-regulation of KCC2 is associated with BDNF and its receptor TrkB*

In organotypic hippocampal cultures, application of the TrkB receptor ligands BDNF and NT-4/5 resulted in a clear down-regulation of KCC2 mRNA, as shown by RT-PCR *(I, Fig. 1A)*. Application of NGF, which acts via TrkA, or heat-inactivated BDNF and NT-4/5, had no effect on the KCC2 expression levels (unpublished data). Western blot analysis indicated that the effects of BDNF were dose-dependent, with maximum decrease of KCC2 expression at a dose of 100ng/ml BDNF *(I, Fig. 1B)*. Both RT-PCR and Western blot analysis showed that the effect after BDNF and NT-4/5 treatment was abolished when TrkB was inhibited by the tyrosine kinase inhibitor K252a, and by depletion of these ligands with soluble TrkB-Fc fusion receptor bodies (TrkB-Fc). When TrkB-Fc was added without any combination of NTs, the levels of KCC2 increased. That suggested that endogenous neurotrophin pathways mediate the effect, i.e. that the modulation is physiologically relevant (*I, Fig. 1C*). Nevertheless, the fact that the effect of TrkB-Fc was smaller than the effect after application of K252a may suggest that other endogenous trophic factors in addition to BDNF may have a small contribution to this process, or that TrkB maintains ligand independent baseline activity. Interestingly, inhibition of network activity by tetrodotoxin (TTX) or by glutamate blockers (CQNX and AP5), did not inhibit the down-regulation of KCC2 by BDNF *(I, Fig. 1D)*, indicating that the TrkB-mediated effects on KCC2 were not caused by a general increase in neuronal excitability and network activity (Scharfman *et al.*, 1997; Kafitz *et al.*, 1999; Scharfman *et al.*, 1999). The same type of experiments on acute hippocampal slices exposed to 0-Mg^{2+} showed similar results *(III, Fig. 6 A and B)*. In this regard, application of K252a to the slices did not affect the expression of neither KCC2 mRNA nor the protein, whereas the down-regulation of KCC2 was decreased by the presence of TkB-Fc. These results further supported our conclusion that BDNF/TrkB mediates the effect of activity dependent modulation of KCC2.

To examine possible functional consequences of BDNF-induced changes in KCC2 expression, we exposed acute hippocampal slices to 100–200 ng/ml BDNF for different periods of time. Free-floating *in situ* hybridization of acute slices showed that KCC2 mRNA levels were down-regulated in all hippocampal regions after 3 hours of BDNF treatment *(I, Fig. 2A)*. Western blots show that the reduction of KCC2 protein was already detectable after 2 h of incubation, and became even more pronounced after 4 h *(I, Fig. 2B)*. This brief time delay may reflect a number of steps, including the diffusion of BDNF into the slice and the activation of TrkB-mediated signaling cascades, and was in agreement with the consequent changes in KCC2 expression observed in all experiments done.

Electrophysiological recordings on acute hippocampal slices exposed to endogenous BDNF for up to 4h *(I, Fig. 3)* showed an impaired capacity of Cl^- extrusion in CA1 hippocampal neurons. These findings reveal that the BDNF-induced reduction of KCC2 expression has a functional consequence on neuronal Cl^- extrusion.

As described above (see section 4.1.1), the KCC2 mRNA decreased in all hippocampal regions, and most significantly in the *dentate gyrus* (DG), after *in vivo* hippocampal kindling–induced seizures *(I, Fig. 4A)*. BDNF and TrkB expression after kindling is most pronounced in the DG (Bengzon *et al.*, 1993; Binder *et al.*, 1999). In several epilepsy models, the time course of up-regulation of BDNF protein levels has been shown to follow its mRNA levels with a 4 hours delay starting in the DG, followed by the *hilus* and CA3 *stratum lucidum* at 12–24 h after stimulus (Binder *et al.*, 2001). In agreement with previous data (Bengzon *et al.*, 1993), the expression levels of TrkB and BDNF mRNAs showed a time-dependent increase upon kindling-induced seizures. In our experiments, TrkB mRNA levels increased bilaterally in all hippocampal regions with a prominent peak after 2 h in the granule cells of the DG (160 ± 5% of control), returning to control levels after 6 h. The increase of TrkB mRNA levels in CA1 and CA3 was not as steep as in the DG, but remained high 6 h after kindling (138 ± 2% in CA1, 139 ± 3% in CA3) and returned to control values after 24 h. BDNF mRNA showed a strong increase in all hippocampal regions with a 3–5-fold increase over control levels 2 h after kindling. The BDNF mRNA levels gradually returned back to control values after 24 h, except for the DG that still showed elevated levels (198 ± 5% of control).

Altogether our data reveals a novel mechanism whereby BDNF/TrkB signaling suppresses chloride-dependent fast GABAergic inhibition. By affecting the expression of KCC2, BDNF/trkB activation alters the efficiency of neuronal chloride extrusion and therefore alters the global inhibitory action of $GABA_A$-mediated responses. This mechanism most likely contributes to the well-known role of TrkB-activated signaling cascades in the induction and establishment of epileptic activity.

4.2.2 *Dissection of TrkB activated intracellular pathways involved in mechanisms of activity-induced reduction of KCC2 expression (I, III)*

BDNF binding to TrkB induces receptor tyrosine phosphorylation followed by the activation/inhibition of downstream proteins, including the activation of the transcription factor cAMP response element-binding protein (CREB) (Finkbeiner *et al.*, 1997; Bibel and Barde, 2000; Minichiello *et al.*, 2002).

Using an antibody that specifically recognizes phosphorylated CREB (anti phospho-CREB), we were able to detect an increase in phospho-CREB immunoreactivity in acute hippocampal slices exposed to 0-Mg^{2+} *(III, Fig. 7A)*. The signal was visible in the CA1 region and the DG after 1h exposure, becoming more robust after 3h. After 3h exposure, also the CA3 region showed activated CREB. Using Western blot analysis, we compared CREB and KCC2 expressions on 0-Mg^{2+} superfused (3h) hippocampal slices in the presence or absence of TrkB-Fc. These experiments showed that scavenging of endogenous BDNF blocks CREB phosphorylation *(III, Fig. 7A)*.

Overall these results indicated that interictal-like activity-induced CREB phosphorylation is mediated by TrkB, and its BDNF-induced spatio-temporal pattern of activation, previously reported by Finkbeiner and collaborators

(Finkbeiner *et al.*, 1997), correlates with the KCC2 down-regulation *(III, Fig. 2)*.

The recent generation of two strains of TrkB gene mutated animals (Minichiello *et al.*, 2002) have been the key for demonstrating that PLCγ and Shc/FRS-2 are the two major intracellular cascades involved in plasticity-related effects of BDNF in the hippocampus. While a single amino acid-targeted mutation of the TrkB gene on the Shc/FRS docking site shows that PLCγ-CREB signaling is necessary for LTP, a mutation in the PLCγ docking site indicates that Shc/FRS-2 is not required (Minichiello *et al.*, 2002; Ernfors and Bramham, 2003).

In order to identify the signaling mechanisms required for BDNF/TrkB-mediated KCC2 down-regulation, we used hippocampal slices from wild type ($TrkB^{W/W}$) and transgenic animals ($TrkB^{PLC/PLC}$ and $TrkB^{SHC/SHC}$). Different sets of acute hippocampal slices were exposed to exogenous BDNF or to 0-Mg^{2+}solution for 3-4h. As expected, Western blot analysis showed that, in both situations, KCC2 levels were down-regulated in the wild type animals. In the $TrkB^{SHC/SHC}$ mutants, the expression of KCC2 was not affected, indicating that the Shc/FRS-2 coupled pathway is important for KCC2 down-regulation. To our surprise, in both BDNF- and 0-Mg^{2+}-exposed slices from $TrkB^{PLC/PLC}$ mice, KCC2 expression was significantly up-regulated *(III, Fig. 8)*. Taken together, these results indicate that the BDNF-mediated activation of the Shc pathway is crucial for both the down-regulation and the up-regulation of KCC2. Down-regulation of KCC2 appears to take place if the Shc pathway is activated in conjunction with the PLCγ cascade. In turn, the Shc pathway acting in the absence of the PLCγ cascades triggers KCC2 mRNA up-regulation.

During maturation, an increase in the expression of KCC2 is required for a functional postsynaptic inhibition in the central nervous system (Rivera *et al.*, 1999). Additional studies will be required to elucidate whether this developmental up-regulation is controlled by the same mechanisms.

4.3 BDNF/ KCC2-DEPENDENT SURVIVAL OF LESIONED ADULT NEURONS (IV)

Trophic support embraces multiple parameters such as the regulation of neuronal plasticity, axonal and dendritic growth, and survival. Recently, it has been suggested that damaged neurons switch trophic support in favor to recapitulate ontogenic programs of plasticity (for see reviews of Cramer and Chopp, 2000; Payne *et al.*, 2003). As for the neurotrophin dependent regulation of KCC2 expression, there are apparently two different modes of regulation in adult versus immature neurons: while BDNF appears to up-regulate KCC2 mRNA expression in embryonic hippocampus (Aguado *et al.*, 2003), our own data show that BDNF mediated TrkB activation in adult hippocampal neurons results in a down-regulation of the expression of KCC2. As depicted, this down-regulation impairs Cl^- extrusion and, hence, alters GABAergic transmission.

In the light of these observations, we tested the hypothesis that lesion of adult neurons re-activates a developmental mode of neurotrophin mediated KCC2 regulation. We used the corticospinal system as an experimental model because corticospinal neurons (CSNs) express TrkB receptor and are rescued from

axotomy-induced death by BDNF (reviewed in Giehl, 2001). In addition, KCC2 is strongly expressed in the adult neocortex, with highest levels in cortical layer V, where the cell bodies of CSNs are located (Giehl *et al.*, 2001; Nabekura *et al.*, 2002). KCC2 expression was assessed using semiquantitative *in situ* hybridization techniques (Giehl *et al.*, 1998). The effect of neurotrophic factors on the expression of KCC2 was studied in intact and lesioned animals that received intracortical infusions of different neurotrophins, antibodies and carrier solutions using a mini-pump perfusion system. The procedure is depicted in detail in IV *(see IV, Fig. 1)*.

4.3.1 *Axotomy-induced switch of BDNF-mediated KCC2 regulation in corticospinal neurons.*

To examine the effects of BDNF on KCC2 expression in adult CSNs, we infused BDNF for 7 days in the frontal cortex of intact and lesioned animals in which CSNs were identified by retrograde tracing. Coronal sections of the brain were processed for *in situ* hybridization of KCC2 mRNA and the levels of expression quantified. BDNF decreased KCC2-expression 0.4-fold in intact CSNs, and increased KCC2-expression 1.8-fold in lesioned CSNs *(IV, Fig. 2)***.** Comparing the expression levels of the lesion with the respective control sides revealed that KCC2 expression is down-regulated in CSNs after axotomy *(IV, Fig. 3)*. Analysis of the time course of the BDNF effects on KCC2 expression shows that the BDNF induced up-regulation was already fully established at day 1 after the injury, the time point when all lesioned CSNs are still alive (Giehl and Tetzlaff, 1996). These data showed that axotomy switches BDNF-mediated KCC2 regulation from down-regulatory in unlesioned to up-regulatory in lesioned adult CSNs. The switch to an embryonic mode of regulation coincides with a shift of the KCC2 expression to low levels after axotomy and suggests a reactivation of a developmental program in adult CSNs after injury.

4.3.2 *Corticospinal neurons reactivate developmental programs of trophic support to survive after axotomy*

In order to address the functional context of the lesion-induced switch, we next examined whether the trophic requirements of CSNs change after axotomy and become reminiscent of the trophic regulation in the embryonic cortex.

All embryonic cortical neurons (E17/18) depend on endogenous BDNF for survival. Since adult CSNs require endogenous BDNF after axotomy (Giehl *et al.*, 1998), we examined whether they also require this factor while still intact. Quantification of CSNs in aged mice of a BDNF knockout strain (Korte *et al.*, 1995) revealed that the heterozygous mutants have virtually identical CSNs numbers as their wild-type counterparts *(IV, Fig. 4A)***.** As survival of axotomized CSNs is severely decreased in heterozygous BDNF mutants (Giehl *et al.*, 2001), neurotrophins appear to mediate their survival effects on CSNs in a dose-dependent manner. Thus, our data showed that BDNF is not required for the survival of intact adult CSNs. This finding is in line with the previous notion that acute neutralization of BDNF does not affect the survival of intact CSNs

(Giehl *et al.*, 1998). Thus, adult intact CSNs do not require BDNF for survival but switch to BDNF-dependency after axonal injury.

Since not only BDNF but also NT-4/5 acts via TrkB (Barbacid, 1995), and both factors are present in the adult cortex (Timmusk *et al.*, 1993; Kang *et al.*, 1997) it is conceivable that endogenous NT-4/5 compensates for the reduced BDNF levels in the BDNF knockouts, and sustains a TrkB activity that is sufficient to support survival of intact but not lesioned (Giehl *et al.*, 2001) CSNs. To address this issue, we intracortically infused a polyclonal TrkB antibody for 7 days to intact and lesioned CSNs. TrkB blockade dramatically reduced the survival of lesioned CSNs while the survival of intact CSNs was not affected *(VI, Fig. 5)*. These data indicated that CSNs switch from BDNF/TrkB-independency in the intact state to BDNF/TrkB-dependency after axotomy. Since also embryonic cortical neurons are supported by endogenous BDNF for survival (Ghosh *et al.*, 1994), this switch represents the reactivation of a developmental mechanism for the promotion of neuronal survival after a pathological damage.

The observation that lesioned neurons require the same survival factors as developing neurons is not sufficient to state that these cells reassume an embryonic mode of survival regulation after lesion. To this end, it is necessary that other aspects of survival-regulation such as the cell death promotion function similar as during development. For embryonic cortical neurons, NT-3, acts as a differentiation factor causing cortical precursors to adopt a neuronal phenotype (Ghosh and Greenberg, 1995) and to become dependent on BDNF (Ghosh *et al.*, 1994). To address the possibility that, after lesion, NT-3 may cause BDNF-dependence and thus lead injured adult CSNs back into a situation similar to their embryonic stage, we simultaneously eliminated the survival signal (BDNF) and the respective death signal (NT-3 or proNGF) by intracortically infusing the respective combinations of ligand neutralizing antibodies.

Axotomized CSNs survived BDNF withdrawal when NT-3 was simultaneously neutralized (Giehl *et al.*, 2001) (*VI, Fig. 5C)* indicating either (1) that the death inducing signal is dominant, or (2) that NT-3 is required for the BDNF-dependence. However, the death-promoting signal *per se* was not dominant since a significant proportion of BDNF dependent CSNs died when both BDNF and proNGF were simultaneously neutralized *(VI, Fig. 5A,C)*. The results indicated that NT-3 promotes death by causing the BDNF-dependency of CSNs after the lesion, while proNGF acts as the death-executing signal for those CSNs that are insufficiently supplied with BDNF. Thus, also NT-3 reassumes a role for adult lesioned CSNs, which is similar to its role during development, i.e. it cause the dependency of CSNs on BDNF for survival.

The question remained whether the lesion specifically activates this function of NT-3, or whether NT-3 physiologically maintains the dependence of intact mature CSNs on a survival-promoting factor. To address this question, we examined the role of endogenous NT-3 for intact CSNs in 1.5 year-old mice of a NT-3 knockout strain (Airaksinen *et al.*, 1996). CSNs numbers were virtually

identical in both heterozygous and wild type animals *(VI, Fig. 4B)* suggesting that NT-3 does not affect the survival of intact CSNs, but becomes important for the survival regulation after the lesion. Thus, adult CSNs reactivate a developmental program for survival regulation after axonal injury.

Lesion-induced reactivation of ontogenetic programs has been addressed in the context of regeneration or increased plasticity (Tetzlaff *et al.*, 1994; Dechant and Barde, 2002; Cramer and Chopp, 2000). In order to investigate whether the lesion-induced switch in KCC2 regulation was as well part of a general activation of developmental programs including regeneration, we analyzed the expression of two regeneration associated proteins, Tα1-tubulin and the growth associated protein 43 (GAP-43), which are both elevated during axonal growth and increased plasticity (Tetzlaff *et al.*, 1994; Plunet *et al.*, 2002), and the expression of $p75^{NTR}$, which is induced during neuronal death induction (Giehl *et al.*, 2001; Dechant and Barde, 2002). Tα1-tubulin and GAP-43 mRNA levels were decreased in lesioned CSNs *(VI, Fig. 6A)* showing that regeneration- or plasticity-associated gene expression was not induced. In contrast, lesioned CSNs strongly up-regulated p75 mRNA expression *(VI, Fig. 6A)*. Thus, axotomized CSNs reactivate developmental programs primarily associated with survival-regulation, but not regeneration or increased plasticity, hence associating KCC2-regulation with the regulation of neuronal survival. Nevertheless, this does not exclude the possibility of proximal sprouting and rewiring of axotomized neurons.

4.3.3 *KCC2 is associated with recovery from neuronal injury.*

A significant portion of lesioned CSNs recovers from the injury (Giehl *et al.*, 1997). To corroborate the association of KCC2 with survival-regulation after injury, we examined whether KCC2 levels are recovered in CSNs that survive the initial injury period, and whether this coincides with a loss of the injury specific survival-regulation. Indeed, KCC2 mRNA levels were recovered by day 42 after the lesion and even slightly exceed the KCC2-levels in CSNs of the intact control side *(VI, Fig. 6C)*. This recovery was coincident with a loss of the BDNF-dependency of lesioned CSNs since the application of BDNF-neutralizing antibody at such a late stage after the lesion did not result in additional CSNs-death *(VI, Fig. 6B)*. Thus, lesioned CSNs appear to re-establish mature KCC2 regulation and trophic support after an initial post injury period. By increasing KCC2 levels after axotomy, BDNF might have a second function in addition to survival promotion: It might also promote maturation that eventually leads to switching off the BDNF-dependency.

4.3.4 *Does KCC2 have a role in* CSN *survival mechanisms?*

Our data suggest that the lesion induced switch of KCC2 regulation is involved in the regulation of neuronal survival. To test the hypothesis that KCC2 is directly involved in the establishment of the trophic dependency of injured CSNs, we depleted KCC2 levels in intact CSNs by infusing KCC2 antisense

oligonucleotides (Phosphothionate dideoxy oligonucleotides, PODNS) into one cortical hemisphere. Our preliminary results using this procedure show that KCC2-PODN significantly reduced the survival of intact CSNs when it was applied in combination with BDNF-blocking antibodies (see Fig. 8, here presented as unpublished data). In contrast, application of KCC2 antisense alone did not reduce the survival of unlesioned CSNs indicating that endogenous levels of BDNF are able to sustain CSNs. Additionally, application of random antisense did not affect the survival of unlesioned adult CSNs therefore assuring that their survival was not altered by the infusions applied nor by the methodology used. In addition, CSNs were not affected when combined infusions of random antisense and TrkB fusion bodies were supplied, corroborating the correlation observed between KCC2 expression and survival of CSNs. To our surprise, combined application of KCC2 antisense and TrkB-Fc did not result in death of CSNs. This might be due to a problem in the concentration of TkB-Fc applied. The survival of adult CSNs was neither affected by the administration of BDNF nor by BDNF-blocking antibodies reassuring that unlesioned adult CSNs do not require BDNF for survival. Altogether, these finding shows that down modulation of KCC2 is required for the installation of BDNF dependency.

Additional experiments regarding the regulation of KCC2 will be needed to elucidate whether the implication of

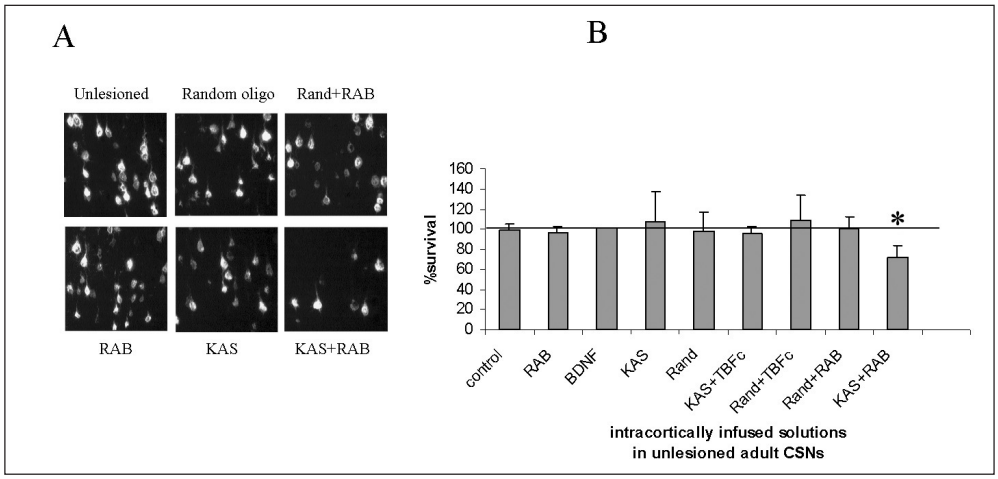

Fig. 8. KCC2 depletion affects survival of adult CSNs. (A) Photomicrographs of representative unlesioned (n=5) Fast Blue-labeled CSNs in cortical layer V after intracortically infused solutions of BDNF (n=1), BDNF-neutralizing antibodies (RAB, n=4), KCC2-antisense oligonucleotides (KAS, n=4), random oligonucleotides (Rand, n=4) or a combination of oligonucleotides and BDNF-neutralizing antibodies (Rand+RAB, n=2; KAS+RAB, n=3). (B) Plot of the survival analysis of the same treatments described in (A) in unlesioned CSNs as well as of infusions of TrkB-neutralizing antibodies (TBFc, n=2) and combination of oligonucleotides and TrkB-neutralizing antibodies (KAS+ TBFc, n=3). (means ± SEM; ANOVA: Rand+TBFc vs. TBFc $P<0.01$; Rand+TBFc vs. KAS+RAB ul $P<0.1$; KAS ul vs. TBFc $P<0.01$; KAS ul vs. KAS+RAB ul $P<0.1$).

KCC2 in the survival of CSNs is a result of KCC2 regulation on itself and/or is due to concerted actions with other proteins (i.e. CCC members or even proteins that do not belong to the CCC-family).

To summarize, the results indicate that *in vivo* axotomy induces corticospinal neurons to switch their neurotrophin- and KCC2-mediated survival regulation to a developmental mode. This suggests that, after injury, adult neurons simultaneously activate developmental patterns of GABA- and NT regulation, which probably interact in order to regulate neuronal survival and to promote recovery in a critical post-injury phase. The molecular mechanisms involved clearly indicate that BDNF/TrkB is a key survival pathway, that proNGF/p75^{NTR} represent the death inducing signal, and NT-3 would be "the" or "one" essential signal for the initiation of the lesion-specific functional antagonism of BDNF and proNGF on survival regulation. This embryonic, lesion-induced program is restricted to a few weeks after injury with both neurotrophin and KCC2 regulation reassuming their pre-injury state *(VI, Fig.7)*.

The findings might be important for the development of therapeutic strategies that aim at diseases involving neuronal death. This work further emphasizes that approaches to foster neuronal survival have to take into consideration whether the target neuron is in a "lesion mode" or an "intact mode".

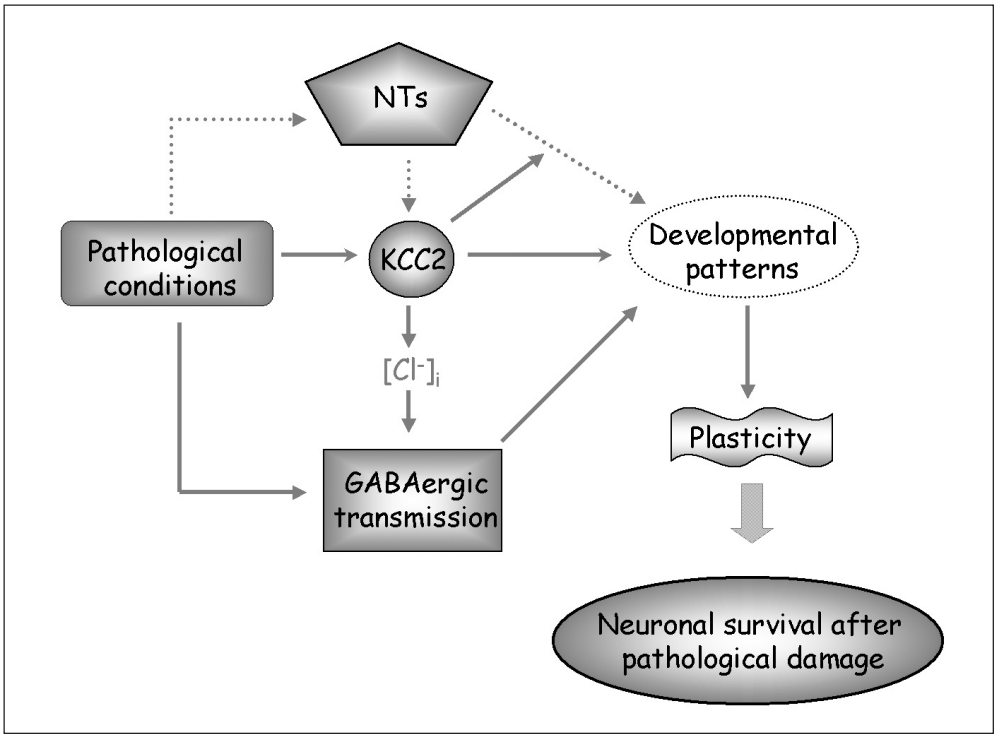

Fig. 9. Under pathological conditions adult neurons simultaneously activate developmental patterns of $[Cl^-]_i$ and neurotrophin regulation (dotted arrow), which are probably interacting to regulate neuronal survival and to promote plasticity and recovery after damage.

5. CONCLUDING REMARKS

1. The expression and function of KCC2 is affected by altered patterns of activity and axotomy and it is regulated by BDNF/TrkB activation. The BDNF-mediated TrkB activation induces a decrease in the expression of KCC2 in mature neurons that leads to an impairment of Cl^- extrusion that suggests a reestablishment of developmental patterns of chloride homeostasis.
2. The time-window of the KCC2 expression at transcriptional and translational level, together with the downstream molecular mechanisms involved in the BDNF-mediated regulation of KCC2, suggest a role of KCC2 in manifestations of neuronal plasticity.
3. The BDNF-mediated KCC2 regulation switches from down regulatory in intact to up regulatory in lesioned adult corticospinal neurons. This switch coincides with a shift of the KCC2 expression to low levels after axotomy. Both suggest that developmental programs are reactivated in adult corticospinal neurons after injury. In addition, the findings show that neurotrophins and KCC2 interact *in vivo*, and that this interaction is subjected to a lesion induced change.
4. Axotomy induces also a switch in the trophic dependency of adult neurons to a developmental mode of neuronal survival regulation, with endogenous NT-3 acting as a conditioning signal for BDNF-dependent survival. The ontogenetic reactivation is temporarily restricted which allows the neurons to acquire plasticity for functional adaptation in a critical post-injury period. Blockage of KCC2 expression in adult CSNs results in neuronal death, suggesting that KCC2 is a key regulator for the establishment of ontogenetic programs of survival regulation in lesioned CSNs. Overall, these findings suggest that lesion induced embryonic programs also apply to the regulation of neuronal survival.

Our findings may be important for the development of treatment strategies for neurological disorders involving neuronal death.

A summary of the concluding remarks of this thesis is depicted in Fig.9

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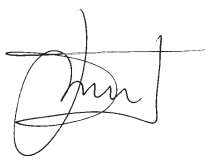

Helsinki, July 2004

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