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EIJA KOPONEN

# Increased BDNF signaling in adult brain

## An experimental study using transgenic mice overexpressing the functional trkB receptor

Doctoral dissertation

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## ABSTRACT

The neurotrophin brain-derived neurotrophic factor (BDNF) is a member of a protein family essential for nervous system development, neuronal survival, differentiation, and maintenance. In addition, the neurotrophins are suggested to regulate neuronal morphology by modulating dendritic, axonal and synapse structure and growth, and regulate the synaptic excitability and plasticity. Both molecular and behavioral evidence supports the role for BDNF in the development of memory traces. Furthermore, mutations in the BDNF gene are connected with pathologies such as epilepsy and Alzheimer's disease, and with psychiatric disorders like anorexia nervosa, depression and bipolar disorder. The plasticity-related functions of BDNF are mediated via trkB tyrosine kinase receptor. Ligand binding activates the receptor and initiates the intracellular signaling cascades emanating from trkB: Shc-MAP kinase, AKT/PI3-K and PLC$\gamma$. The activation of individual pathways is carefully regulated by the cellular environment and type of the activating stimulus, consequently leading to appropriate cellular responses.

In this study, we have addressed the role of increased BDNF signaling in adult nervous system. We have generated transgenic mice overexpressing the functional trkB receptor (trkB.TK+) under a neuronal, postnatally expressed Thy1 promoter. In these mice, transgenic trkB.TK+ protein was dramatically elevated in hippocampus and cerebral cortex, and more importantly, the receptor phosphorylation was equally increased. Therefore, the mice could be used as a model for augmented BDNF-trkB signaling in the mature central nervous system. Both transgenic and wild type mice were analyzed for the induction of downstream signaling molecules, for behavioral responses, for learning and memory and for development of epilepsy.

Increased trkB receptor activation selectively regulated the separate downstream pathways. The activation of phospholipase$C\gamma$-1 pathway was increased and the Akt pathway was downregulated whereas the signaling molecules Shc and MAP kinase were unaltered. The transcription of genes *c-fos*, *fra-2* and *junB*, GAP-43 and $\alpha$-CaMKII was regulated in transgenic mice, whereas the BDNF mRNA and protein levels were similar in both genotypes. These results suggest that the homeostasis in trkB downstream signaling is strictly regulated even if the receptor is continuously active. Our data supports the previous observations describing the refined regulation of BDNF-trkB signaling in different experimental systems.

Behaviorally, the increased BDNF signaling results in reduced anxiety and depression-like behavior. Additionally, by three independent testing paradigms, the trkB.TK+ mice exhibited improved learning and memory. In contrast, LTP, a molecular level measure for learning was attenuated in transgenic mice. Altogether, the behavioral data suggest that BDNF signaling via trkB is modulating some aspects of emotional and cognitive performance in rodents. Finally, augmented BDNF signaling in transgenic mice exacerbated the severity of *status epilepticus* and promoted the acute cell loss but the rate of epileptogenesis was not altered. These results suggest a damaging role for increased BDNF signaling during the acute phases of epilepsy.

In summary, this series of studies introduces a novel animal model with increased BDNF signaling in adult brain and provides new information on the relationship of BDNF-trkB system with neuronal plasticity.

National Library of Medicine Classification: WL 105, WL 104, WL 102, QY 60.R6
Medical Subject Headings: brain-derived neurotrophic factor; receptor; trkB; mice; transgenic; brain; behavior; learning; memory; depression; anxiety; long-term potentiation; epilepsy; hippocampus; neuronal plasticity; signal transduction

*"I don't see much sense in that," said Rabbit.*
*"No," said Pooh humbly, "there isn't.*
*But there was going to be when I began it.*
*It's just that something happened to it along the way."*

*From Winnie-the-Pooh by A.A.Milne*

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Kuopio, January 2005

Eija Koponen

## ABBREVIATIONS

| | |
|---|---|
| 5-HT | 5-hydroxytryptamine, serotonin |
| 5-HIAA | 5-hydroxyindoleacetic acid |
| AKT/PKB | serine/threonine protein kinase (protein kinase B) |
| BAD | proapoptotic Bcl-$_{XL}$-associated protein |
| BDNF | brain-derived neurotrophic factor |
| CA1/CA3 | hippocampal subfields 1-3 of the ammons horn |
| CaMKII | $Ca^{2+}$/calmodulin dependent kinase II |
| cAMP | cyclic adenosine monophosphate |
| CaRF | Ca2+ response factor |
| Cre | site-specific DNA recombinase |
| CREB | cAMP response element binding protein |
| DAG | diacylglycerol |
| DG | dentate gyrus |
| DRG | dorsal root ganglion |
| Erk | extracellular signal regulated kinase |
| EPSP | excitatory postsynaptic potential |
| Emx-BDNF$^{KO}$ | conditional BDNF knockout mice |
| Frs-2 | fibroblast receptor substrate-2 |
| GABA | $\gamma$-aminobutyric acid |
| GPRC | G-protein coupled receptor |
| GSK-3 | glycogen synthase kinase-3 |
| HPLC | high-performance liquid chromatography |
| i.p. | intraperitoneal |
| IP3 | inositol 1,4,5 triphosphate |
| K252a | tyrosine kinase inhibitor |
| LTP | long-term potentiation |
| L-LTP | late-LTP |
| MAPK | mitogen-activated protein kinase |
| mIPSC | miniature inhibitory postsynaptic current |
| MMP | matrix metalloproteinase |
| NA | norepinephrine |
| NF$\kappa$B | nuclear factor $\kappa$B |
| NGF | nerve growth factor |
| NPG | nodose-petrosal ganglion complex |
| NRIF | neurotrophin receptor interacting protein |
| NRAGE | neurotrophin receptor-interacting MAGE homologue |
| NMDA | *N*-methyl-D-aspartate |
| NR1 | NMDA receptor subtype 1 |
| NR2a/2b | NMDA receptor subtype 2a/2b |
| NT | neurotrophin |
| p75NTR | pan-neurotrophin receptor |
| PI3K | phosphatidylinositol-3-kinase |
| PIP2 | phosphatidylinositide |
| PKC | protein kinase C |
| PTB | phosphotyrosine binding |
| PLC$\gamma$ | phospholipase C, $\gamma$ subunit |
| Ras | GTP binding protein |
| RGC | retinal ganglion cell |
| SE | status epilepticus |
| SH2 | Src homology domain 2 |
| Shc | adaptor protein containing SH2 domain |
| SNP | single nucleotide polymorphism |
| TBS | theta burst stimulation |
| TNF | tumor necrosis family |
| trkA/B/C | tropomyosin-related kinase A/B/C |
| trkB-CRE | forebrain-specific trkB conditional knockout |
| trkB-PLC | PLC$\gamma$ site targeted mutant mice |
| trkB-Shc | Shc site targeted mutant mice |
| trkB.TK+ | mice overexpressing full-length trkB isoform |
| trkB.T1 | mice overexpressing truncated trkB isoform |

## LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications referred by their corresponding Roman numerals:

*I* Koponen E., Võikar V., Riekki R., Saarelainen T., Rauramaa T., Rauvala H., Taira T., Castrén E. (2004) Transgenic mice overexpressing the full-length neurotrophin receptor trkB exhibit increased activation of trkB/PLC$\gamma$ pathway, reduced anxiety, and facilitated learning. *Molecular and Cellular Neuroscience, 26, 166-181*.

*II* Koponen E., Lakso M., Castrén E. (2004) Overexpression of the full-length neurotrophin receptor trkB regulates the expression of plasticity related genes in mouse brain. *Molecular Brain Research 130, 81- 94.*

*III* Lähteinen S., Pitkänen A., Koponen E., Saarelainen T., Castrén E. (2003) Exacerbated status epilepticus and acute cell loss, but no changes in epileptogenesis, in mice with increased brain-derived neurotrophin factor signaling. *Neuroscience 122, 1081-1092.*

*IV* Koponen E., Saarelainen T., Võikar V., MacDonald E., Castrén E. Enhanced BDNF signaling regulates brain monoamines and produces an antidepressant-like behavioral response. *Submitted.*

In addition, some unpublished data are presented.

## TABLE OF CONTENTS

# 1 INTRODUCTION

The family of neurotrophic factors, the neurotrophins (NT), is appreciated for their versatile activities in both developing and mature nervous systems (reviewed by Huang and Reichardt, 2001; Lewin and Barde, 1996). The four mammalian neurotrophins are nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3) and neurotrophin-4 (NT4) that mediate their functions via the trk-family of tyrosine kinase receptors. Each neurotrophin has their specific corresponding receptor: NGF binds trkA, BDNF and NT4 bind trkB, and NT3 interacts mainly with trkC. However, a common p75 neurotrophin receptor (p75NTR) exists, that binds all NTs with similar affinity. P75NTR mediates some neurotrophic actions, especially apoptotic signals evoked by immature pro-NTs (review by Hempstead, 2002). The role for these two separate receptor systems is currently understood as follows: the trk receptors mediate survival signals emanating from the mature NTs whereas p75NTR mediates mainly apoptotic signals in response to pro-neurotrophins. Indeed, the initial observation of NTs as pro-survival factors has been broadened to a complex pattern of functions such as regulation of neurite outgrowth and sprouting, synapse function, cell differentiation, migration and proliferation as well as on functional plasticity in both peripheral and central nerve cells (McAllister et al., 1999; Poo, 2001; Schinder and Poo, 2000).

Emerging knowledge casts BDNF as a diverse modulator of the central nervous system (Bibel and Barde, 2000; Chao, 2003). Besides providing the target-derived survival signals during neuronal development, BDNF regulates neuronal structure, function and connectivity. BDNF and its corresponding receptor, trkB are widely distributed throughout brain with especially high expression in hippocampus and cerebral cortex. The trkB receptors are located on the cell membrane, dimerize and activate upon ligand binding, and initiate the downstream signaling cascades that ultimately lead to alterations in cellular functions (Patapoutian and Reichardt, 2001). Neuronal activity robustly regulates the levels and localization of both BDNF and trkB (Lu, 2003a; Schinder and Poo, 2000). Increased excitatory activity induced by pharmacological agents such as kainate, physical activity, seizures or learning increase BDNF in cortex and hippocampus whereas exposure to stress or activation of GABAergic inhibitory activity depresses BDNF expression.

The appropriate performance of the BDNF-trkB signaling system is required for neuronal activity that precedes learning (Poo, 2001; Tyler et al., 2002). Long-term potentiation (LTP), an experimental learning model at the cellular level, is affected if either BDNF or trkB are not present. In addition, behavioral evidence on genetically modified mice indicates that BDNF signaling is required for learning. To study more specifically the aspects of BDNF-trkB signaling, a variety of genetic mutants have been generated. Although null mutants of both BDNF and trkB are lethal, heterozygous and conditional mutants have been extensively studied. BDNF has been

shown to play a critical role in many forms of neuronal excitability, cellular morphology and behavioral responses ((Gorski et al., 2003a; Gorski et al., 2003b; Minichiello et al., 2002; Minichiello et al., 1998; Minichiello et al., 1999; Xu et al., 2000b).

The purpose of this study was to address the role of increased BDNF signaling in the adult nervous system. More specifically, we generated transgenic mice that overexpress the full-length trkB receptor isoform in postnatal neurons. Transgenic mice and their wild type littermates were examined with a battery of biochemical, histochemical, behavioral and electrophysiological analyzes. Although a variety of genetically modified animal models have addressed the role of BDNF and trkB by generating mice lacking either partner, the paradigm of increasing BDNF signaling has not been used before. Therefore, this study introduces a novel experimental model to elucidate the consequences of increased neurotrophin activity in adult brain.

# 2 REVIEW OF THE LITERATURE

## *2.1 Neurotrophins and their receptors*

The initial observation that led to the discovery of a protein family of neurotrophins (NT) was made more than 50 years ago, when researchers Levi-Montalcini and Hamburger found the survival promoting nerve growth factor, NGF (Johnson et al., 1986; Levi-Montalcini, 1987; Radeke et al., 1987). Since the discovery of NGF, three other mammalian neurotrophins - brain-derived neurotrophic factor BDNF (Barde et al., 1982; Leibrock et al., 1989), neurotrophin-3 (Ernfors et al., 1990a; Hohn et al., 1990; Jones and Reichardt, 1990; Maisonpierre et al., 1990b), neurotrophin-4 (Berkemeier et al., 1991; Hallbook et al., 1991) - and two members present only in fish- neurotrophin-6 (Gotz et al., 1994) and neurotrophin-7 (Nilsson et al., 1998)- have been isolated.

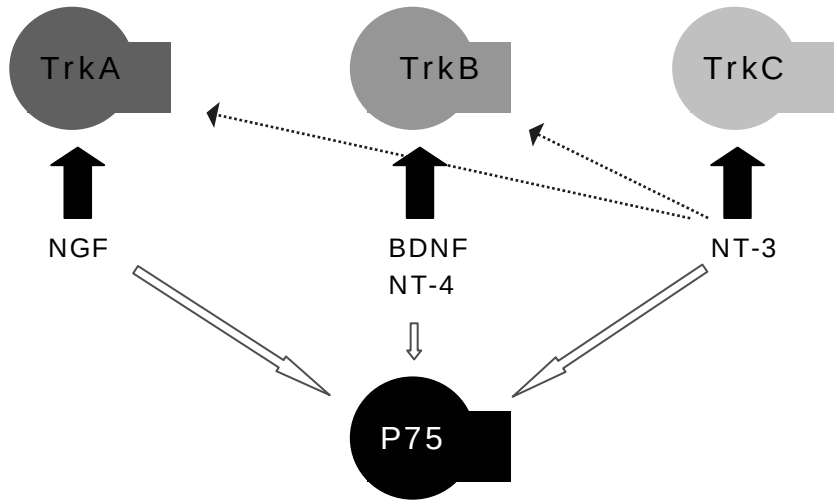


**Figure 1.** *Neurotrophins bind to their specific trk receptors and commonly to p75NTR.*

The functions of NTs are mediated through two distinct types of cell surface receptors. Each NT shows identical binding affinity for the common neurotrophin receptor, p75NTR that is a member of the tumor necrosis factor receptor family (Ernfors et al., 1990a; Rodriguez-Tebar et al., 1990; Rodriguez-Tebar et al., 1992; Bothwell, 1995). p75NTR is suggested not only to act as an accessory receptor for trk by modulating ligand binding and neurotrophin responses, but also to operate trk-independently in regulation of cell survival. Yet, most of the neurotrophin responses elicited in nerve cells are mediated by binding to transmembrane receptors of the trk tyrosine kinase receptor family. These interactions with trk receptors exhibit much higher ligand

specificity: trkA binds NGF (Kaplan et al., 1991a; Kaplan et al., 1991b; Klein et al., 1991a), trkB to BDNF and NT4 (Klein et al., 1992; Klein et al., 1991b; Soppet et al., 1991; Squinto et al., 1991) and trkC to NT3; however NT3 has some affinity also to trkA and trkB (Klein et al., 1991b; Lamballe et al., 1991; Soppet et al., 1991).

Upon ligand binding, trk receptors dimerize and the cytoplasmic tyrosine kinase residues phosphorylate thus activating the receptor. Subsequently, the active tyrosine residues act as initiation sites to several intracellular signal transduction cascades that ultimately result in cellular responses and altered physiological function (Patapoutian and Reichardt, 2001; Segal and Greenberg, 1996). The classic neurotrophin hypothesis states that neuronal survival is dependent on the limited target-derived secretion of NTs. The retrograde signal, from distal axon to the nucleus, initiated by NT binding to a receptor located in the axon terminals, is followed by internalization of the ligand-receptor complex and finally vesicular transport of complex to the nucleus where survival signals are provided. However, as trk receptors are found in both post- and pre-synaptic sites (Aloyz et al., 1999; Levine et al., 1995; Schinder and Poo, 2000; von Bartheld et al., 1996; Wu et al., 1996), an additional mode for anterograde signaling is acknowledged.

As pro-survival factors, NTs are suggested as potential therapeutic agents for many degenerative disorders, such as amylotrophic lateral sclerosis (ALS), Alzheimer's and Parkinson's diseases. Recently, mice lacking cortical BDNF were reported to show a phenotype similar to Huntigton's disease (Baquet et al., 2004; Gorski et al., 2003b). However many caveats are still encountered if NTs are applied as therapeutic agents (Dechant and Barde, 2002). Due to the complicated physiology NTs possess, the manner and location of NT administration as well as a proper knowledge of the mechanisms behind each degenerative disorder is necessary if rational therapeutic approaches are to be achieved (Thoenen and Sendtner, 2002).

### 2.1.1 p75 neurotrophin receptor

The 75-kDa neurotrophin receptor (p75NTR) is a member of the Fas/tumor necrosis factor receptor family (Johnson et al., 1986; Radeke et al., 1987) that commonly binds all neurotrophins. P75NTR is capable of both modifying the trk-mediated NT responses and signaling trk-independently (Hempstead, 2002). Structurally, p75NTR is a transmembrane glycoprotein that has an extracellular cysteine-rich domain and a cytoplasmic region lacking enzymatic kinase domain but instead it contains a death domain characteristic for the TNF family receptors (Roux and Barker, 2002). Besides the full-length p75NTR, a truncated splice variant exists that is lacking the extracellular region responsible for neurotrophin binding (von Schack et al., 2001).

The diverse functions of p75NTR as both pro-apoptotic and pro-survival factors are largely dependent on the cellular environment or to be precise, the presence of trk. p75NTR was identified as a co-receptor for trkA to create the high-affinity binding site for NGF (Benedetti et

al., 1993; Hempstead et al., 1991). In addition to modulating the binding affinity, p75NTR can alter ligand specificity of trk receptors. The interaction of p75NTR with trkA and trkB increases their specificity to NGF and BDNF, respectively, whereas the trkC specificity is actually broadened by p75NTR modulation (Hempstead, 2002; Vesa et al., 2000). Moreover, NT binding to p75NTR activates the nuclear factor κB (NFκB), an anti-apoptotic transcription factor, thus promoting cell survival (Miller and Kaplan, 2001a). Interestingly, another p75NTR-mediated survival pathway involves the activation of AKT/PI3K but in a trk-independent manner (Roux et al., 2001). Mechanistically, the concurrent interaction of p75NTR, trk receptor and the neurotrophin ligand is suggested to result in a conformational alteration either in trk (Zaccaro et al., 2001) or in the bound ligand partner such as NGF (He and Garcia, 2004).

Under conditions where trk signaling is impaired, 75NTR is an important mediator of cellular apoptosis. Numerous studies have reported a p75-dependent cell death occurring both in culture conditions and in p75NTR mutant mice (Dechant and Barde, 2002; Kaplan and Miller, 2000). Indeed, mice overexpressing the cytoplasmic p75NTR region, the death domain, show enhanced loss of peripheral sensory neurons (Majdan et al., 1997), whereas mice lacking p75NTR have reduced cell death of the cholinergic, sympathetic and sensory neurons (Agerman et al., 2000; Bamji et al., 1998; Naumann et al., 2002). Interestingly, the pro-neurotrophins have been recently characterized as potent pro-apoptotic molecules interacting selectively via p75NTR (Lee et al., 2001). Other identified p75NTR ligands are β-amyloid and prion peptide that both promote apoptosis via caspase activation (Hempstead, 2002; Yaar et al., 2002).

Besides trk receptors, several p75NTR –interactors with diverse signaling pathways have been identified (Huang and Reichardt, 2003; Roux and Barker, 2002). Recently a novel interacting protein, NRAGE was identified as a mediator in the p75NTR cell death pathway via activation of caspases and JNK (Salehi et al., 2000; Salehi et al., 2002). Another interacting protein, sortilin acts as a p75NTR co-receptor to produce a high-affinity binding site for pro-NGF and thus promote apoptosis (Nykjaer et al., 2004). Further p75NTR-associated cell death effectors include p53 and the neurotrophin receptor interacting factor (NRIF) (Hempstead, 2002). Besides regulating cell survival, p75NTR signaling regulates axonal outgrowth via modulation of RhoA activity (Gehler et al., 2004; Yamashita et al., 1999). Binding of NGF or BDNF to p75NTR reduces RhoA activity, subsequently promoting filopodial elongation of the growth cones (Gehler et al., 2004). Recently, p75NTR was identified as a co-receptor for the Nogo receptor that mediates inhibitory growth signals in myelin (Kaplan and Miller, 2003; Wang et al., 2002; Wong et al., 2002; Yamashita and Tohyama, 2003). Although the basal expression of p75NTR is rather low in many cell types, robust induction of expression is observed in many pathological conditions (Chao, 2003; Dechant and Barde, 2002). For example, p75NTR is induced following seizures (Roux et al., 1999), ischemia (Kokaia et al., 1998; Park et al., 2000), spinal cord injury (Beattie et al., 2002), and cortical axotomy (Harrington et al., 2004).

### 2.1.2 Proneurotrophins

NTs have biochemical characteristics of secretory proteins generated as immature precursors of about 260 amino acids, processed enzymatically and released as mature protein dimers. Intracellularly, pro-NTs are processed by pro-protein convertases and furin. Interestingly, both mature NTs and pro-NTs are secreted successfully and possess distinct biological activities. In fact, the unprocessed NTs are often more abundantly secreted than mature forms (Chao and Bothwell, 2002; Hempstead, 2002; Lessmann et al., 2003; Lu, 2003b).

Proteolytic cleavage is one crucial mechanism to regulate the location and tempo of neurotrophic actions. Lee and coworkers (2001) recently reported an intriguing observation of the physiologically important role for the neurotrophin precursor, pro-NGF (reviewed by Chao and Bothwell, 2002; Ibanez, 2002). The cleavage-resistant form of proNGF was identified to interact with p75NTR receptor with high affinity whereas trkA was not activated by pro-NGF (Lee et al., 2001). In contrast to the survival-promoting effect of trkA-NGF interaction, the newly identified association of proNGF with p75NTR robustly promoted apoptosis. Although this dual action is reported so far only for pro-NGF, one could expect similar actions by BDNF and NT3 pro-proteins. Possibly supporting this, both pro-NGF and pro-BDNF are cleaved by extracellular proteases plasmin and matrix metalloproteinases (MMPs; (Lee et al., 2001). According to literature, one vital phenomenon regulated by neurotrophins is synaptic transmission (Lu, 2003a). Indeed, BDNF is released at the synapse in response to neuronal activity and elicits effects on both pre- and post-synaptic sites to regulate synaptic transmission and plasticity (Kohara et al., 2001; Kojima et al., 2001; Tyler et al., 2002). If, and when, both plasmin and pro-BDNF would co-exist at synapses, the proneurotrophins could modulate also synaptic plasticity.

Intriguingly, a polymorphism in the pro-BDNF region has been connected with several pathologies. A single nucleotide polymorphism (SNP) at valine66 to methionine in the BDNF pro-domain increases the susceptibility towards disorders such as depression (Sen et al., 2003) bipolar disorder (Neves-Pereira et al., 2002; Sklar et al., 2002), eating disorder (Ribases et al., 2003) and memory impairments (Egan et al., 2003). While the exact mechanisms behind these abnormalities are unknown, the val66met polymorphism results in abnormal BDNF trafficking, distribution and activity-dependent release exclusively in neuronal cells (Chen et al., 2004). Therefore, the BDNF pro-region is likely to contain regulatory machinery for the subcellular sorting of BDNF and thus its biological activity. Taken together, mature neurotrophins signal survival via trk that is expressed either alone or combined with p75NTR. Instead, the proneurotrophins bind selectively to p75NTR thus inducing p75NTR-dependent signaling but no trk-mediated survival. However, the contribution of proneurotrophins on the currently identified functions of BDNF still needs further clarification.

## 2.2 TrkB receptor

### 2.2.1 TrkB gene

The expression of trkB gene begins early during embryonic development and persists during adulthood. The transcription pattern of the rodent trkB gene is complex with several mRNA transcripts ranging from 0.7 to 9 kb (Klein et al., 1990a; Klein et al., 1990b; Klein et al., 1989; Middlemas et al., 1991). The trkB locus codes for two major types of trkB mRNAs expressed in brain: the full-length receptor trkB.TK+ and truncated splice variants. The full-length trkB transcript encodes a typical tyrosine kinase receptor with extracellular, transmembrane and cytoplasmic domains. The two C-terminally truncated receptors have complete extracellular and transmembrane regions but only a short cytoplasmic tail (23 aa in trkB.T1 and 21 aa in trkB.T2) (Klein et al., 1989; Middlemas et al., 1991). In humans, the trkB gene is uncommonly large spanning at least 590 kb and contains 24 exons (Stoilov et al., 2002). Besides the large size, the trkB gene has remarkable structural complexity with alternative promoters, splicing sites and polyadenylation signals, and indeed 10 different trkB proteins in total can be produced from the human gene locus. However, similar to rodents, the full-length trkB.TK+ and truncated trkB.T1 isoforms are the major products. Additionally, a novel truncated isoform, TrkB.T-Shc, is expressed in human brain. T-Shc localizes to membrane but is not phosphorylated by trkB.TK+, therefore it may act as a negative regulator of trkB.TK+ (Stoilov et al., 2002).

### 2.2.2 TrkB mRNA and protein

The mRNAs encoding the different trkB isoforms are abundantly expressed in rodent brain as early as embryonic day 9.5 (Klein et al., 1990b). The expression of trkB.TK+ and the truncated isoforms is differentially regulated both spatially and temporally. Temporal regulation occurs in the hippocampus, where the trkB.TK+ mRNA reaches the adult expression levels already at birth whereas the truncated receptor peaks around two weeks postnatally (Dugich-Djordjevic et al., 1993; Fryer et al., 1996; Masana et al., 1993). A similar timing difference in the expression of splice variants during development is observed in cortex, amygdala, spinal cord and DRG of rodents (Ernfors et al., 1993; Fryer et al., 1996) as well as in human brain (Muragaki et al., 1997) and rat retina (Hallbook et al., 1996; Ugolini et al., 1995). Furthermore, the spatial distribution of trkB slice variants is different. Indeed, in the adult brain, the strongest trkB.TK+ expression is evident in neurons throughout the cortical layers, thalamus and the hippocampus (Armanini et al., 1995; Beck et al., 1993; Klein et al., 1990a). On the contrary, the truncated trkB is expressed in choroid plexus, ependyma and non-neuronal cells (Beck et al., 1993; Biffo et al., 1995; Frisen et al., 1993). However, in the adult motor neurons and

developing trigeminal ganglion cells, receptor variants co-localize (Armanini et al., 1995; Ninkina et al., 1996). Additionally, the distribution of trkB mRNA in forebrain region largely co-localizes with BDNF, particularly in hippocampus (Kokaia et al., 1993). All in all, the full-length receptor mRNA is the major form during early development, and the truncated receptor mRNA during later development and adulthood.

Similar to mRNA expression, the trkB.TK+ protein is the dominant receptor form in early development whereas truncated receptor protein governs later on. By immunohistochemistry, the trkB protein is typically present in neurons, with particularly strong immunoreactivity in the hippocampal and cortical structures (Cabelli et al., 1996; Fryer et al., 1996). More specifically, in hippocampus the truncated trkB receptor protein is expressed mostly in the somato-dendritic compartment of the cell whereas the catalytic trkB.TK+ receptor is associated primarily with axon initial segments (Drake et al., 1999). Furthermore, trkB.TK+ immunoreactivity was present in both excitatory and inhibitory nerve terminals. Postsynaptically, trkB.TK+ was found on the plasma membrane of dendritic spines (Drake et al., 1999). Interestingly, the differential sorting of trkB receptor isoforms is apparently maintained also in cultured hippocampal neurons (Haapasalo et al., 2002).

### 2.2.3 Regulation of trkB mRNA and protein

Like BDNF, the expression of trkB is regulated by diverse neuronal activity. First, brain insults regulate the amount of trkB mRNA and protein (Binder et al., 2001; Lindvall et al., 1994). Fiber transections (Beck et al., 1993), forebrain ischemia (Arai et al., 1996), and seizure-inducing activity (Aloyz et al., 1999; Binder et al., 1999; Dugich-Djordjevic et al., 1995; Merlio et al., 1993) all increase trkB transcription and receptor phosphorylation. Second, a simple potassium-induced neuronal depolarization increases the trkB.TK+ transcription (Kingsbury et al., 2003) and dendritic localization (Tongiorgi et al., 1997). Third, long-term locomotor activity is upregulating the trkB.TK+ mRNA and protein expression in spinal cord (Gomez-Pinilla et al., 2002; Skup et al., 2002). Fourth, hippocampal trkB protein is regulated by circadian rhythm (Dolci et al., 2003). Finally, learning and memory formation induce trkB transcription and receptor activation (Broad et al., 2002; Gomez-Pinilla et al., 2001; Mizuno et al., 2003b).

Regulating the number of trkB receptors available on the cell surface can modulate the responsiveness to BDNF. Neuronal activity, induced by either depolarization or tetanic stimulation, and elevation in the second messengers such as cAMP, both increase the amount of trkB receptors on the cell surface (Du et al., 2000; Meyer-Franke et al., 1998). The increase in the surface trkB levels is observed along dendrites, axons and cell soma. BDNF treatment, in contrast, reduces the trkB.TK+ expression on the cell membrane (Du et al., 2000; Meyer-Franke et al., 1998) via a mechanism that was recently reported to depend on the duration of the applied BDNF stimulation (Haapasalo et al., 2002). Indeed, the prolonged BDNF treatment has

been shown to result in receptor desensitization (Carter et al., 1995; Frank et al., 1996). Further, electrical stimulation, such as LTP-inducing theta burst stimulation (TBS), enhances the trkB internalization in a Ca2+ dependent manner therefore depleting trkB from the cell surface (Du et al., 2003). Accordingly, the tyrosine kinase activation was suggested to directly regulate receptor internalization (Du et al., 2003). Altogether, the activity-dependent regulation of trkB receptors on the cell surface provides one mechanism how BDNF signaling could be restricted to active neurons.

### 2.2.4 Structure of trkB receptors

The full-length trkB (trkB.TK+) mRNA encodes a 145-kDa glycoprotein that is 821 amino acids long and located on the plasma membrane (Klein et al., 1989). The mature receptor protein is mostly expressed in brain tissue and has notable homology to trkA, especially in the intracellular kinase region (Johnson et al., 1986; Klein et al., 1989; Middlemas et al., 1991; Schneider and Schweiger, 1991). In the N-terminus, the three leucine-rich repeats are flanked by two cysteine clusters. Adjacent to these there are two C2-type immunoglobulin-like domains that are followed by a single transmembrane domain and the cytoplasmic tyrosine kinase region (Schneider and Schweiger, 1991). The major ligand-binding structure has been localized to the second IgG domain (O'Connell et al., 2000; Urfer et al., 1998; Urfer et al., 1995), however also other extracellular structures contribute to ligand binding either directly or indirectly (Ninkina et al., 1997; Windisch et al., 1995). Additionally, the IgG domains are regulating the spontaneous dimerization in the absence of ligand (Arevalo et al., 2001). It is therefore

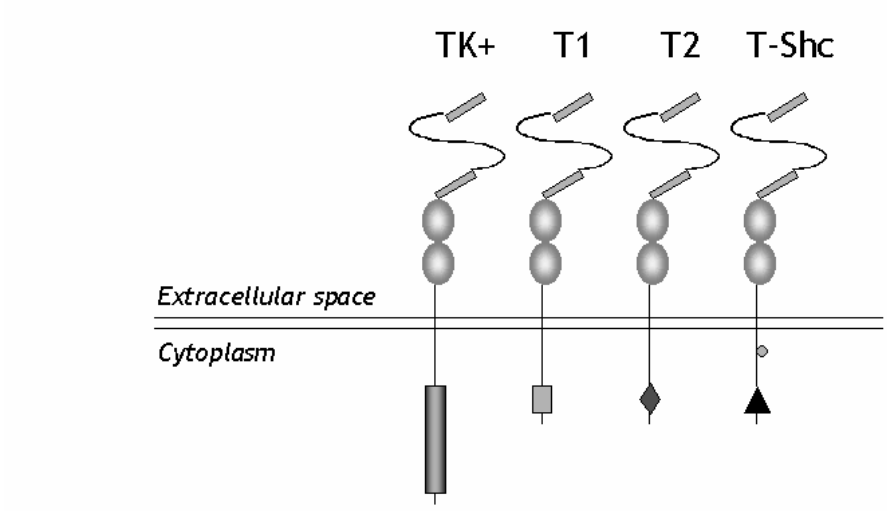


**Figure 2.** *A schematic representation of receptor structures encoded by the trkB gene.* The full-length receptor contains an intracellular kinase domain whereas the truncated splice variants have only short cytoplasmic tail region. Extracellularly, all receptor variants have identical structure. Abbreviations: TK+, full-length trkB.TK+; T1, truncated trkB.T1; T2, truncated trkB.T2; T-Shc, truncated trkB.T.Shc.

conceivable, that each of the separate extracellular regions somehow participate in the ligand binding and subsequent receptor dimerization. The N-terminus of the receptor is important in controlling the ligand specificity although in case of NT3 it is not known how it manages to activate three different receptors (see review by Huang and Reichardt, 2003 and references therein).

The intracellular domain is the most conserved region between trk family members (Klein et al., 1989; Middlemas et al., 1991). The intracellular region of the trkB.TK+ contains ten conserved tyrosine residues that activate in response to ligand binding and serve as docking sites for downstream adaptor molecules. Tyrosines 670, 674 and 675 (according to human trkA nomenclature) form the autophosphorylation loop that upon activation potentiates the phosphorylation of other tyrosines. The activity of Y670/674/675 loop is necessary for BDNF-inducible phosphorylation as well as for mediation of cell proliferation (McCarty and Feinstein, 1998). Additionally, these tyrosines may also directly bind downstream adaptor molecules (Huang and Reichardt, 2003). Tyrosine 490 in trkA (Y515 in human trkB) provides a docking site for Shc and Frs-2, and tyrosine 785 (Y816 in human trkB) binds phospholipase C$\gamma$ (Huang and Reichardt, 2003; Patapoutian and Reichardt, 2001). However, details on the contribution of the remaining five tyrosines on trk-signaling are still largely unknown (Inagaki et al., 1995). Nevertheless, the cytoplasmic adaptors near the phosphorylated tyrosines are numerous and most likely compete with each other for binding to active trk.

#### *2.2.4.1 Truncated trkB receptors*

The truncated 95-kDa trkB receptors (T1, T2 and T-Shc) have the analogous extracellular structure to trkB.TK+ thus suggesting that they are equally competent in ligand binding. However, truncated receptors lack the cytoplasmic kinase domain and they have short unique tails instead (Middlemas et al., 1991). Therefore, the suggested roles for the truncated receptors have been mostly modulatory. First, it was reported that truncated receptors expressed in non-neuronal cells could act as ligand scavengers by binding and releasing BDNF, and thus modify BDNF signaling (Beck et al., 1993; Biffo et al., 1995; Rubio, 1997). A second identified function for the truncated receptors is to act as a dominant-negative regulator for trkB.TK+ when co-expressed in neurons. Formation of the TK+/T1 heterodimer abolishes ligand-induced signaling thus resulting in altered cellular functions e.g. reduced survival (Eide et al., 1996; Haapasalo et al., 2001; Ninkina et al., 1996). In accordance, mice lacking all trkB receptors show increased cell survival in comparison to mutants where only the truncated trkB is expressed thus confirming the anti-survival role of truncated trkB receptors (Luikart et al., 2003). Furthermore, overexpression of truncated receptors increases the susceptibility to damage after stroke (Saarelainen et al., 2000a) but reduces epileptogenesis (Lahteinen et al., 2002). Finally, truncated trkB receptors might have signaling potential of their own (Baxter et al., 1997).

Supporting this, Rose and colleagues (2003) recently demonstrated that trkB.T1 mediates BDNF-dependent signaling in cultured astrocytes. Altogether, truncated trkB receptors mainly modulate BDNF signaling with both beneficial and adverse consequences, but they might also have regulatory actions independent of the full-length receptor.

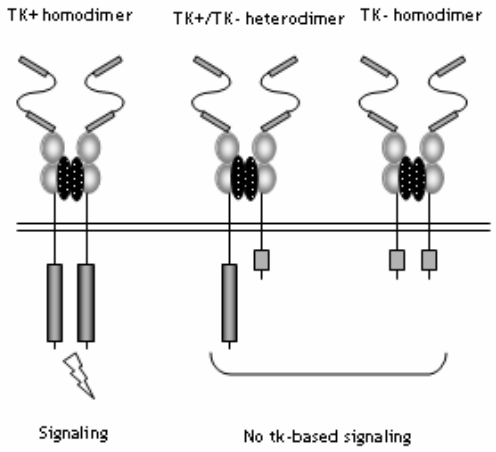


**Figure 3.** *Schematic representation of the possible trkB dimerizations occurring on the cell membrane upon neurotrophin binding.* Abbreviations used: TK+, full-length trkB receptor; TK-, truncated trkB receptor.

### 2.2.5 TrkB.TK+ activation mechanisms

The identification of NGF as a trkA ligand established the functional connection between these two protein families (Kaplan et al., 1991b) and paved the way to recognition of trkB ligands BDNF, NT3 and NT4 (Klein et al., 1992; Klein et al., 1991b; Soppet et al., 1991; Squinto et al., 1991). The primary activating step for the full-length trkB receptor is naturally the ligand engagement by the extracellular domain that results in TK+/TK+ homodimerization and phosphorylation of tyrosines in the kinase activation loop (Ibanez et al., 1993; Jing et al., 1992). The subsequently activated tyrosines provide the docking sites for cytoplasmic downstream effectors. The adaptor proteins Shc and phospholipaseC-$\gamma$ were first named trkB substrates that bind to trkB tyrosines 515 and 816, respectively (Middlemas et al., 1991; Stephens et al., 1994; Vetter et al., 1991). However, the formation of TK+/T1 heterodimers or T1/T1 homodimers quenches the ligand-induced signaling (discussed above). NT signaling via trkB generally mediates actions such as survival and plasticity whereas the p75NTR-mediated actions often stimulate pro-apoptotic pathways (Huang and Reichardt, 2003; Kaplan and Miller, 2000; Patapoutian and Reichardt, 2001). Finally, the p75NTR can modify ligand specificity to trk

receptors (Benedetti et al., 1993; Bibel et al., 1999; Hempstead et al., 1991), binding kinetics (Mahadeo et al., 1994), and receptor activation (Vesa et al., 2000).

In the absence of NT ligands, trk receptors can also be activated in response to G-protein coupled receptor (GPCR) activation. This transactivation of trk receptors is reported to occur via GPCR-ligands adenosine and neuropeptide PACAP (Chao, 2003; Lee and Chao, 2001; Lee et al., 2002a; Lee et al., 2002b). Two main differences separate NT-induced trk activation from GPCR transactivation. First, trk phosphorylation via transactivation occurs much slower as NT-induced activation (Lee and Chao, 2001). Second, GPCR-mediated trk activation selectively promotes signaling via the PI3K/AKT pathway, therefore promoting survival (Lee and Chao, 2001; Lee et al., 2002b). Recently, the trk transactivation was reported to take place in the intracellular membranes instead of the cell surface (Rajagopal et al., 2004). Altogether, transactivation through GPCRs provides an alternative route for trk signaling in the absence of neurotrophin ligand.

## *2.3 TrkB.TK+ signaling pathways*

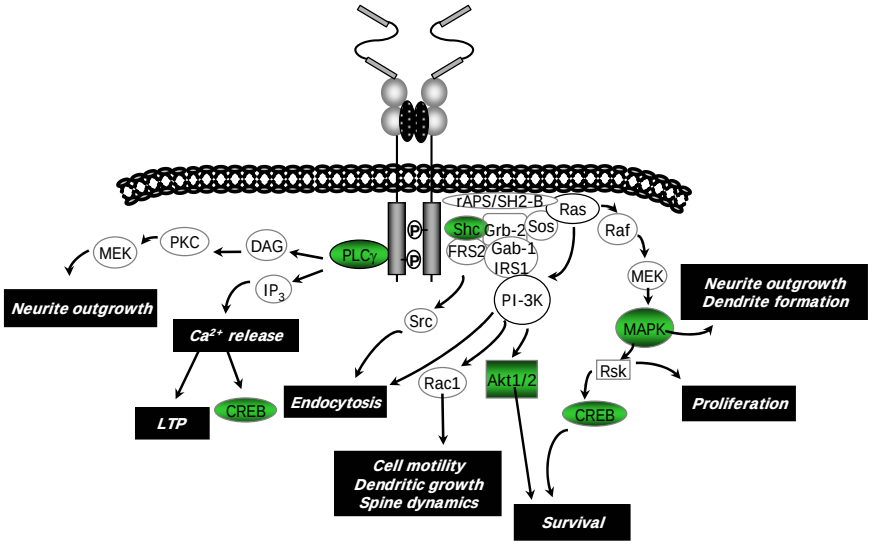


**Figure 4.** *Neurotrophin signaling pathways via trkB.TK+ receptor.* Abbreviations used:AKT,serine/threonine kinase; CREB, cAMP response element binding protein; DAG, diacylglycerol; FRS_2, fibroblast receptor substrate-2; MAPK, mitogen activated protein kinase; MEK, MAPK kinase; PI-3K, phosphatidylinositol-3-kinase; IP3, inositol-1,4,5-triphosphate; PLCγ, phospholipase Cγ. Modified from Kaplan and Miller, 2000.

### 2.3.1 PhospholipaseC-γ (PLCγ)

When the Y816 in trkB is activated upon ligand engagement, it recruits the cytoplasmic enzyme protein phospholipaseC (PLC) that directly binds to trkB through internal SH2 domain and is in turn itself activated by phosphorylation of the trk kinase (Patapoutian and Reichardt, 2001; Segal and Greenberg, 1996). Several mammalian PLC isoforms exists, however only PLCγ-1 has been shown to bind and be activated downstream of trk (Middlemas et al., 1994; Obermeier et al., 1994; Obermeier et al., 1993; Vetter et al., 1991). The activated PLCγ-1 binds to phosphatidylinositides (PIP2) and enzymatic activity hydrolyzes it to generate diacylglycerol (DAG) and IP3. IP3 induces the release of $Ca^{2+}$ from internal stores thus increasing the intracellular $Ca^{2+}$ levels. As a consequence, enzymatic pathways controlled by intracellular $Ca^{2+}$ concentrations, such as synaptic $Ca^{2+}$-calmodulin (CaM) kinases, are activated (Ouyang et al., 1997). On the other hand, DAG stimulates protein kinase C isoforms, such as PKCδ (Bibel and Barde, 2000; Huang and Reichardt, 2003). Moreover, increase in the intracellular Ca2+ may enhance neurotransmitter release (Lessmann, 1998). The PLCγ-1 pathway appears to be critically involved in synaptic plasticity. Indeed, targeted mutant mice where the PLCγ binding site has been disrupted by changing the tyrosine residue to phenylalanine (Y816F), demonstrate the importance of proper PLCγ signaling in hippocampal plasticity (Minichiello et al., 2002). Similar to trkB and BDNF null mice, the PLCγ targeted mutants show impaired hippocampal LTP. Additionally, phosphorylation of CaM kinases II and IV, and the expression of transcription factor Egr-1, were impaired. In agreement, *in vitro* studies have shown that PLC inhibitors block BDNF-dependent synaptic potentiation (Kleiman et al., 2000; Yang et al., 2001). Altogether, the PLCγ pathway is critical for the neurotrophin-mediated effects on synaptic plasticity.

### 2.3.2 Ras-MAPK

The trkB pathways leading to activation of Ras are rather complex (Huang and Reichardt, 2003; Kaplan and Miller, 1997; Segal, 2003). Upon initial phosphorylation at Y515, at least two possible adaptor molecules compete for the direct binding to phosphorylated tyrosine residue: Shc and Frs-2 (Huang and Reichardt, 2001; Huang and Reichardt, 2003; Meakin et al., 1999; Stephens et al., 1994). Signaling through the Shc pathway mediates the transient activation of ERK signaling (Grewal et al., 1999). Shc exists in three isoforms that differ in their expression and functions; ShcB and ShcC, but not ShcA, are highly expressed in nervous system (Segal, 2003). NTs can induce recruitment of each of these proteins; however in mature neurons ShcC binding is preferred (Conti et al., 2001). Upon ligand binding, Y515-site provides a recruitment site for the Shc PTB (phosphotyrosine binding) domain. Binding of Shc is followed by phosphorylation and recruitment of a protein complex of the adaptor Grb2 and the Ras exchange factor SOS. In the next step, SOS activates Ras and the activated Ras stimulates several

downstream pathways; PI3K, c-Raf/Erk and p38MAPK/MAPK-activating protein kinase 2 (Segal, 2003). The Ras activation is a critical event for NT-induced differentiation in PC12 cells (Segal et al., 1996).

Prolonged ERK activation is dependent on a separate signaling pathway initiated by the recruitment of fibroblast receptor substrate-2 (Frs-2). Activated Frs-2 provides several binding sites to downstream elements such as adaptors Grb-2 and Crk, the protein phosphatase Shp2, and Src-kinase (Huang and Reichardt, 2001; Huang and Reichardt, 2003; Meakin et al., 1999). Crk binds and activates the exchange factor C3G that in turn activates a small G protein Rap-1 that stimulates B-raf, which initiates the ERK cascade (Meakin et al., 1999; Segal, 2003). Therefore, the Frs-2 provides an alternative, Shc-independent mechanism to activation of Grb2/SOS/Ras pathway. Overexpression of the members of Frs-2 pathway in PC12 cells promotes differentiation (Hempstead et al., 1994; Meakin et al., 1999).

The standard Ras/MAPK-pathway model consists of a G-protein (such as Ras) initiated cascade where the three kinases activate one another in a cascade-like manner eventually leading to activation of MAP kinase such as Erk1/2 (Segal and Greenberg, 1996). Of the various MAP kinases (mitogen activated protein kinases) activated through Ras/Raf/MEK pathways, four are known to respond to NT/trk signaling: Erk 1,2,4 and 5 (Segal, 2003). The major role for neuronal Erks is the regulation of gene expression. For example, Erk 1,2 and 5 can activate the members of the RSK protein kinases that further activate the transcription factor CREB. Furthermore, Erks may act directly on the CREB-binding protein (CBP), however for this to happen, Erks have to be translocated to the nucleus. In the nucleus, Erks regulate transcription factors such as Elk-1 or Egr-1 (Grewal et al., 1999). Besides the nuclear actions, MAP kinase activity can regulate axonal elongation (Atwal et al., 2000). Taken together, the multiple Ras-MAPK signaling pathways of trkB provide a wide variability of signals, both divergent and convergent, in response to ligand stimulation.

### 2.3.3 PI3 kinase

Phosphatidylinositol-3-kinases (PI3Ks) are critical in mediating NT-induced survival and in regulating vesicular trafficking (Brunet et al., 2001; Datta et al., 1999). The heterodimeric PI3 kinase enzyme that is activated by neurotrophins consists of regulatory (p85) and catalytic (p110) subunits of which both have several splicing variants (Bartlett et al., 1999; Bartlett et al., 1997; Fruman et al., 1998). The catalytic and regulatory subunits are constitutively associated. Activated trks can stimulate PI3 kinase through at least two distinct pathways and the choice between pathways depends on the cell type (Vaillant et al., 1999). First, PI3 kinase is stimulated when the catalytic subunit p110 directly binds to active Ras (Kaplan and Miller, 2000; Rodriguez-Viciana et al., 1994). This Ras-dependent pathway is utilized by many survival-promoting signals in neurons (Huang and Reichardt, 2001; Vaillant et al., 1999). Alternatively, PI3K is activated

through Shc/Grb-2/Gab-1 pathway in a Ras-independent manner (Holgado-Madruga et al., 1997; Kaplan and Miller, 2000).

Lipid products generated by the activated PI3K, the phosphatidylinositides, bind and activate directly their target proteins such as protein kinase Akt (also known as protein kinase B, PKB) (Huang and Reichardt, 2001; Kaplan and Miller, 2000; Segal, 2003). Again, an alternative pathway to Akt activation exists. Phosphatidylinositides can also activate PDK-1 kinase that in turn activates Akt (Alessi et al., 1997). Akt substrates include several important survival-regulating proteins: BAD, transcription factors of forkhead family (FKH), IκB and glycogen synthase kinase 3 (GSK-3). The Bcl-2 family member BAD, promotes apoptosis via Bcl-XL/Bax-dependent mechanisms when dephosphorylated. However, Akt-dependent phosphorylation inactivates BAD and subsequently suppresses the BAD-induced cell death (Bonni et al., 1999; Datta et al., 1999). Interestingly, neurons from the BAD knockout mice show no alterations in apoptosis therefore suggesting a non-essential role for BAD in cell survival (Shindler et al., 1998). Another target, cytoplasmic IκB functions as a trapper for the transcription factor NF-κB (Datta et al., 1999). Upon Akt-induced phosphorylation, IκB is degraded and the NF-κB is translocated to nucleus where it promotes survival. Furthermore, Akt kinase phosphorylates members of the Forkhead family of transcription factors (FKHR; (Biggs et al., 1999; Brunet et al., 1999) and promotes cell survival through regulation of cell death genes. In the presence of Akt, the phosphorylated Forkhead stays in the cytoplasm whereas in the absence of Akt activation Forkhead is translocated into nucleus where it promotes the transcription of cell death genes such as Fas ligand (Biggs et al., 1999; Brunet et al., 1999). Finally, Akt kinase phosphorylates and inactivates the proapoptotic GSK-3 thus enhancing cell survival (Pap and Cooper, 1998). Altogether, the PI3K/Akt pathway is the major regulator of cell survival in neurons (Aloyz et al., 1998; Datta et al., 1999; Mazzoni et al., 1999). The Akt protein is at the center of several distinct regulatory pathways, probably mediating survival at a number of levels depending on the cellular surroundings. Besides survival, PI3K-Akt pathway may regulate also vesicular transport and mRNA translation (see references in reviews by Huang and Reichardt, 2001; Segal, 2003).

### *2.4 Regulation of trk signaling*

The regulation of trk signaling is carefully controlled on several levels. Firstly, the ligand specifies the downstream responses elicited. Site-directed mutagenesis in trkB mice demonstrated the importance of the stimulating ligand (Minichiello et al., 1998). In these mice, the Shc docking site was disabled (Y490F mutation) and as a consequence, the NT4 dependent survival was dramatically reduced whereas the BDNF dependent cell populations were only modestly affected (Minichiello et al., 1998). These results suggest that trkB ligands use separate

downstream pathways when mediating survival. The distinct activities of trkB ligands were further confirmed by another mice model where the NT4 gene was inserted in the BDNF gene locus (Fan et al., 2000). This study showed differences in the survival promoting potential of trkB ligands and further corroborated that trkB-Shc signaling pathway is more crucial for NT4 actions (Fan et al., 2000). Secondly, the timing of the ligand binding regulates downstream responses. A rapid 2-minute pulse of NGF activates efficiently the PLC-γ signaling (Choi et al., 2001). Additionally, a brief pulse of BDNF triggers a postsynaptic action potential (Kafitz et al., 1999) and exerts the BDNF-derived effects on LTP in slices (Schuman, 1999).

Finally, the location of the ligand-receptor interaction determines the activated downstream pathways. Local signaling at the axon terminals regulates i.e. the axonal outgrowth. Axonal neurotrophin stimulation leads to phosphorylation of axonal trks and activation of the Ras/MAPK pathway (Atwal et al., 2000; Riccio et al., 1997; Senger and Campenot, 1997; Watson et al., 2001). Conflicting evidence has suggested that the trk signaling pathways via Shc or PLCγ account for the growth cone guidance (Atwal et al., 2000 vs. Ming et al., 1999). Recently, an interesting observation stated that semaforin-3F, a chemorepellat, antagonizes the actions of NGF-induced PI3K-MEK-ERK activation in growth cones (Atwal et al., 2003). Additionally, local neurotrophin signaling within axons contributes to axonal elongation and promotes endocytosis (Beattie et al., 2000; Kuruvilla et al., 2000).

In contrast, the long-term trk signaling in the cell body is essential for the survival and differentiation effects. If neurotrophins are applied to distal axons, trk activation rapidly occurs along axons and in the cell body in a complex with the stimulating neurotrophin (Bhattacharyya et al., 1997; Riccio et al., 1997; Tsui-Pierchala and Ginty, 1999; Watson et al., 1999). These complexes are found within vesicles designated as signaling endosomes together with downstream signaling factors PI3 kinase, PLCγ and Shc (Beattie et al., 1996; Grimes et al., 1997; Grimes et al., 1996; Howe et al., 2001). The signaling endosome is formed when the ligand-induced receptor activation leads to internalization of the ligand –receptor complex through clathrin-mediated endocytosis. The transport of the signaling endosome is most probably carried by the motor protein dynein along the microtubules (Heerssen and Segal, 2002). The receptors within the endosome remain catalytically active and continue signaling as they travel towards the cell body however it is unclear how trk activity is maintained in the vesicles. Presumably, the basal trk activity is enough to maintain the active state and the vesicular localization of the complex would protect the phosphorylated trk against the actions of phosphatases (Miller and Kaplan, 2001b). Interestingly, in the DRG cultures, neurotrophin stimulus applied on the cell body, activates two separate MAP kinase pathways within the cell body: Erk1/2 and Erk5 (Watson et al., 2001). However, if the stimulation is applied on distal axons, only the Erk5 activation occurs in the cell body. Similarly, within the retinal system BDNF has opposing effects on the dendritic growth depending on the location of stimulation (Lom et al., 2002). Together

these results suggest that the location of the neurotrophin stimulus is an important regulatory step for the responses elicited.

## *2.5 BDNF*

### 2.5.1 BDNF mRNA and protein

Brain-derived neurotrophic factor (BDNF) was identified as a second member of the neurotrophin growth factor family with close structural homology to NGF (Barde et al., 1982; Leibrock et al., 1989). The genomic structure and regulation of rat BDNF gene is rather complex (Metsis et al., 1993; Timmusk et al., 1993; West et al., 2001). There are four 5' exons, each with its own promoter that are combined onto one common 3' exon with one alternative splice site consequently producing eight different transcripts in total. Individual promoters direct the expression of rat BDNF tissue-specifically; transcrips containing exons I, II and III are preferably expressed in brain whereas exon IV transcripts are present in heart and lung (Metsis et al., 1993; Timmusk et al., 1993). The transcript from exon III responds strongly to neuronal stimulation and so far two different transcription factors, CREB and CaRF, have been identified to bind to BDNF promoter III for transcription regulation (Lu, 2003a; West et al., 2001). Altogether, the presence of multiple promoters highlights the fact that the BDNF gene must be under careful control in order to execute the variety of functions it has. As suggested by nomenclature, BDNF mRNA is abundantly expressed in brain. BDNF expression levels are low during fetal development, increase after birth and then reduce to adult levels (Maisonpierre et al., 1990a). In the adult brain, especially high expression is observed in hippocampus, cortex, cerebellum, amygdala, and in various hypothalamic nuclei (Castren et al., 1995; Dugich-Djordjevic et al., 1995; Ernfors et al., 1990b; Hofer et al., 1990). Inside hippocampus, the pronounced expression is located into dentate granule cells and pyramidal neurons of the CA1-CA3 regions. Only few brains areas, such as striatum, completely lack BDNF mRNA (Castren et al., 1995). Finally, BDNF mRNA is present also in glia (Murer et al., 2001).

The mature BDNF protein is a 13.5-kDa protein that is secreted as a dimer into extracellular space (Kolbeck et al., 1994). BDNF is generated as a precursor, pre-pro-BDNF protein, where the pre-sequence is cleaved off after sequestration to endoplasmic reticulum. The remaining pro-BDNF is further processed via Golgi apparatus into *trans*-Golgi network and packed there into secretory vesicles. The pro-BDNF is cleaved intracellularly by either enzymes furin or pro-convertases and secreted as a mature peptide. Alternatively, protein is secreted as a pro-BDNF and cleaved by extracellular proteases such as MMPs and plasmin (Lee et al., 2001; Lessmann et al., 2003). The mature BDNF protein expression resembles the mRNA distribution (Nawa et al., 1995). Further, immunohistochemical and overexpression studies have revealed that in hippocampal and cortical regions the BDNF protein is mainly somatodendritically

localized (Fawcett et al., 1997; Goodman et al., 1996; Hartmann et al., 2001; Kojima et al., 2001; Tongiorgi et al., 1997; Wetmore et al., 1991) whereas also axonal BDNF is detected (Kohara et al., 2001). Finally, BDNF protein has been co-localized with synaptic markers therefore suggesting a presence within a synapse (Fawcett et al., 1997; Goodman et al., 1996). However, the subcellular localization of BDNF protein still needs further clarification.

The first identified function for BDNF was the ability to promote survival of peripheral sensory neurons during programmed cell death and these observations have been confirmed on the system level in genetically modified mice (Conover et al., 1995; Ernfors et al., 1994; Huang and Reichardt, 2001; Jones et al., 1994; Sendtner et al., 1992). In the central neurons, BDNF and NT4 responsive neurons include cerebellar granule neurons, mesensephalic dopaminergic neurons, hippocampal neurons, cortical neurons and retinal ganglion cells (Lindholm et al., 1993; Okoye et al., 2003; Segal et al., 1992; Tong and Perez-Polo, 1998). Interestingly, as exogenous BDNF is reported to protect neurons against insults like energy deprivation, excitotoxins or free radical accumulation it may enhance the toxic effects, as well (Murer et al., 2001). Finally, as BDNF expression is reduced in brains of both Alzheimer's and Parkinson's disease patients it may well play a role in the pathogenesis of these degenerative disorders (Murer et al., 2001; Murray et al., 1994; Parain et al., 1999).

### 2.5.2 Activity-dependent regulation of BDNF

#### *2.5.2.1 Regulation of BDNF mRNA*

The BDNF transcript levels are easily regulated by diverse neuronal activity. An upregulation of the BDNF mRNA is observed in response to epileptiform activity induced by lesions, kindling or pharmacological agents such as kainate (Ballarin et al., 1991; Dugich-Djordjevic et al., 1992; Ernfors et al., 1991; Isackson et al., 1991; Zafra et al., 1990). The response to seizures is specifically pronounced in the hippocampus and occurs rapidly. Interestingly, if BDNF signaling is repressed as in trkB.T1 overexpressing mice, the kainate-induced increase in BDNF transcript is less pronounced (Saarelainen et al., 2001). Furthermore, neuronal depolarization by either glutamate receptor agonist MK-801 or high potassium (Zafra et al., 1991; Zafra et al., 1990), osmotic stimulus (Castren et al., 1995), or brain insults like ischemia (Kokaia et al., 1996; Lindvall et al., 1992), all strongly elevate BDNF mRNA expression in brain. Besides glutamate, the neurotransmitter GABA ($\gamma$-aminobutyric acid) regulates BDNF expression. Stimulation of the GABAergic system by GABA agonists reduces hippocampal BDNF mRNA whereas inhibiting the GABAergic system has the opposite effect (Lindholm et al., 1994).

In addition to these rather dramatic stimulations, also subtle more physiological stimuli evoke BDNF transcription. Light or visual deprivation rapidly regulates levels of BDNF mRNA and protein (Capsoni et al., 1999; Castren et al., 1992). Even such delicate stimuli as whisker stimulation can induce BDNF mRNA expression (Rocamora et al., 1996). Furthermore, voluntary

exercise increases hippocampal BDNF mRNA (Gomez-Pinilla et al., 2001; Neeper et al., 1996; Russo-Neustadt et al., 1999; Vaynman et al., 2003), whereas diet rich in fat and sugar has the opposite effect (Molteni et al., 2002; Molteni et al., 2004). Induction of hippocampal long-term potentiation (LTP) has been reported to upregulate BDNF mRNA both *in vivo* and *in vitro* conditions (Castren et al., 1993; Patterson et al., 1992). Equally, induction in the hippocampal BDNF expression is observed after hippocampus-dependent forms of learning (Gomez-Pinilla et al., 2001; Hall et al., 2000; Kesslak et al., 1998) whereas amygdala-dependent fear conditioning increases amygdaloid BDNF mRNA (Rattiner et al., 2004). Conversely, stress that is induced by repeated footshock delivery downregulates BDNF transcript (Rasmusson et al., 2002). Finally, the neurotrophin levels are regulated by NTs themselves (Lindholm et al., 1994; Patz and Wahle, 2004). Subcellularly, the nuclear transcript may be selectively transported to active dendrites and translated locally. Depolarization of hippocampal neurons leads to more dendritic distribution of BDNF and trkB transcripts and is suggested to induce local protein synthesis in dendrites (Righi et al., 2000; Tongiorgi et al., 1997). Altogether, these results indicate that BDNF expression is regulated by neural activity.

#### *2.5.2.2 Regulation of BDNF release*

The efficiency of proteolytic cleavage controls BDNF functions. The generated precursor proteins are translocated to Golgi complex for further processing. The pro-neurotrophins are cleaved by furin or specific pro-convertase enzymes and packed into secretory vesicles (Lessmann et al., 2003; Mowla et al., 2001). Both mature and pro-NTs can be secreted and they have distinct biological actions upon release (Lee et al., 2001; Lu, 2003b). The secretion from the cell can be either constitutive or regulated depending on the cellular context and the efficiency of furin cleavage (Farhadi et al., 2000; Mowla et al., 1999). Genetic mutations that alter the balance between secretory pathways can cause physiological consequences. As an example, the valine66-to-methionine single nucleotide polymorphism (SNP) in the human pro-BDNF gene, cuts down the regulated secretion and results in deficits of episodic memory (Egan et al., 2003).

BDNF functions are controlled temporally by activity-dependent secretion. The regulated BDNF secretion from hippocampal neurons is induced by a variety of stimuli such as high potassium, glutamate or neurotrophins themselves (Blochl and Thoenen, 1995; Canossa et al., 1997). Both depolarization and neurotrophin-induced BDNF release depend on increase in the intracellular Ca2+ concentration (Canossa et al., 1997; Goodman et al., 1996). Moreover, electrical stimulation robustly induces BDNF secretion (reviewed by Poo, 2001). In cultured peripheral neurons, the LTP-inducing tetanic stimulation is the most effective in promoting endogenous BDNF secretion (Balkowiec and Katz, 2000). Using an elegant ELISA *in situ* assay, that study demonstrated first that actually the pattern of electrical stimulation is regulating

BDNF release response. Analogous results have been gained in the central neurons (Gartner and Staiger, 2002; Goodman et al., 1996). Additionally, these studies have demonstrated that BDNF can be released from both postsynaptic and presynaptic compartments (Balkowiec and Katz, 2002; Hartmann et al., 2001; Kohara et al., 2001). Finally, BDNF is more potent in modulating active synapses (Boulanger and Poo, 1999) and expectedly, secretion occurs in active synapses (Hartmann et al., 2001; Kojima et al., 2001). As the effects of BDNF are restricted within 60 μm distance from the release site (Zhang and Poo, 2002), the local synaptic release provides an additional way to enhance BDNF signaling specificity.

## *2.6 Physiological roles of BDNF*

### 2.6.1 BDNF and structural plasticity

Since the first neurotrophin was originally identified as a stimulator of neurite outgrowth in peripheral neurons, it may well be conceivable to suggest a similar role for BDNF in central neurons. Indeed, BDNF regulates the dendritic growth of cortical neurons (Horch and Katz, 2002; Horch et al., 1999; McAllister et al., 1996; McAllister et al., 1995) and dentate granule cells (Danzer et al., 2002). Exogenous BDNF application or transfection, results in increased dendritic length and complexity in a layer-specific manner. Furthermore, the effect could be blocked by either inhibiting neuronal activity or with tyrosine kinase inhibitor K252a (Danzer et al., 2002; McAllister et al., 1996). Accordingly, scavenging the endogenous BDNF causes dramatic dendritic retraction (McAllister et al., 1997). Interestingly, that study also demonstrated a spatial distinction in BDNF responses of cortical neurons: basal dendrites were more affected by BDNF shortage. Besides BDNF, trkB receptor isoforms differentially regulate dendritic morphology. As transfection of trkB.TK+ to ferret slices promoted the proximal dendritic branching and inhibited elongation, the transfected trkB.T1 had counteracting actions instead and the ratio of T1 to TK+ was suggested to serve as a switch between the distinct modes of dendritic growth (Yacoubian and Lo, 2000). If so, it may provide an important mechanism to regulate the dendritic structure, i.e. during development when T1/TK+ ratios are changing. Analogous to the above *in vitro* data, transgenic mice overexpressing BDNF display increased dendritic complexity in the dentate gyrus (Tolwani et al., 2002) whereas both BDNF and trkB knockout mice exhibit reduced dendritic structure (Gorski et al., 2003b; Xu et al., 2000b). In the latter case, BDNF or trkB is ablated from cortex and hippocampus, and as a result, substantial loss of dendrites and cell soma shrinkage is observed. Further information on the spatial restrictions of BDNF responses is provided by studies on *Xenopus* retinal ganglion cells (RGCs). Increase in the tectal target-derived BDNF supports the RGC dendritic formation whereas locally increased BDNF levels within retina lead to decreased dendritic branching (Lom et al., 2002; Lom and Cohen-Cory, 1999). Finally, neuronal activity is a key signal for dendrite formation in general (Lohmann et al., 2002; Miller and

Kaplan, 2003). Two main signaling pathways, the calcium/calmodulin kinases (CaMKs) and the MEK/MAPK pathway are suggested to regulate the activity-dependent dendrite formation, often in cohort (Miller and Kaplan, 2003; Redmond et al., 2002; Vaillant et al., 2002; Wu et al., 2001). Interestingly, neurotrophins activate both the CaMKII and CaMKIV via a mechanism suggested to engage trk-mediated activation of PLC$\gamma$, generation of IP3 and subsequent release of $Ca^{2+}$ (He et al., 2000; Kaplan and Miller, 2000; Minichiello et al., 2002). Therefore, neural activity and neurotrophins might act in convergence to promote dendrite formation.

In addition to dendrites, exogenous BDNF can potently enhance the axonal arborization of RGCs (Cohen-Cory, 1999; Cohen-Cory and Fraser, 1995; Inoue and Sanes, 1997) and dentate granule cells (Danzer et al., 2002) and these effects are abolished by antibodies to BDNF or by activity blockade. In cultured *Xenopus* spinal neurons, BDNF may act in a chemoattractive manner and trigger growth cone turning via a mechanism requiring cAMP/ protein kinase A signaling (Markus et al., 2002; Song et al., 1997). Knockout mice lacking trkB have reduced number of axonal collaterals and varicosities in hippocampus (Martinez et al., 1998). Likewise, axonal fragmentation is present in the amygdala of trkB/trkC double heterozygous mutants (von Bohlen und Halbach et al., 2003).

Since BDNF is known to contribute to synaptic transmission, it is likely to modulate morphology of synapses and spines. Indeed, BDNF does promote the formation and stabilization of both excitatory and inhibitory synapses (Alsina et al., 2001; Huang et al., 1999b; Martinez et al., 1998; Seil and Drake-Baumann, 2000; Vicario-Abejon et al., 1998; Vicario-Abejon et al., 2002). Treatment of hippocampal slices with BDNF enhances spine formation in apical dendrites, also in the absence of action potentials (Tyler and Pozzo-Miller, 2003; Tyler and Pozzo-Miller, 2001). Additionally, the activity-dependent synapse control in the adult cortex appears to require BDNF, as the whisker stimulation does not induce increased spine density in heterozygous BDNF mutant mice as in controls (Genoud et al., 2004). Furthermore, BDNF regulates synapse maturation. Specifically, the number of synaptic vesicles at the active zones is reduced in mice lacking BDNF or trkB (Martinez et al., 1998; Tyler and Pozzo-Miller, 2001). In conclusion, BDNF signaling via trkB regulates the formation and maintenance of dendrites, axons and synapses therefore promote the establishment of functional neuronal circuitry.

### 2.6.2 BDNF and synaptic transmission

Besides the traditional role as a survival factor during development, extensive evidence points to a perhaps even more important role for BDNF in the regulation of synaptic transmission (Lu, 2003a; Poo, 2001; Thoenen, 1995; Vicario-Abejon et al., 2002). BDNF potentiates excitatory synaptic transmission by promoting presynaptic transmitter release. Acute BDNF application to developing *Xenopus* neuromuscular synapses rapidly potentiates basal synaptic transmission by increasing neurotransmitter release (Lohof et al., 1993). In the central excitatory synapses,

similar enhancement is observed upon BDNF application into hippocampal and cortical cultures (Lessmann, 1998; Levine et al., 1995), slice preparations (Kang et al., 1996; Kang and Schuman, 1995; Kang et al., 1997) and intrahippocampal infusion (Messaoudi et al., 1998; Ying et al., 2002). Furthermore, addition of BDNF causes hyperexcitability in slices (Scharfman, 1997) that is similarly observed in BDNF transgenic mice (Croll et al., 1999). As expected, the BDNF-induced potentiation of synaptic transmission is enhanced by simultaneous presynaptic neuronal activity (Boulanger and Poo, 1999). The site of BDNF action is still a matter of controversy. Some studies have demonstrated that BDNF acts postsynaptically (Henneberger et al., 2002; Kovalchuk et al., 2002; Levine et al., 1995; Suen et al., 1997) whereas strong data supports actions via presynaptic transmitter release as well (Frerking et al., 1998; Gottschalk et al., 1998; Lohof et al., 1993; Olofsdotter et al., 2000; Vicario-Abejon et al., 1998). In line with presynaptic actions of BDNF, a reduced number of docked vesicles are observed at excitatory synapses on CA1 dendritic spines of BDNF knockout mice (Pozzo-Miller et al., 1999). Additionally, a reduced amount of vesicular proteins synaptobrevin and synaptophysin is observed in BDNF mutants. Furthermore, presynaptic but not postsynaptic, expression of dominant negative trkB.T1 receptor inhibites synaptic potentiation in cultured neurons (Li et al., 1998).

BDNF is also a modulator of the GABAergic transmission although the mechanisms are not clear. Evidence suggests that BDNF application reduces GABAergic inhibitory transmission and mIPSCs in hippocampal CA1 region (Brunig et al., 2001; Frerking et al., 1998; Tanaka et al., 1997) and depresses the excitatory synaptic transmission to GABAergic cortical neurons (Jiang et al., 2004) in a trkB-dependent manner. In contrast, in hippocampal slice preparations of mice lacking BDNF, synaptic inhibition was enhanced and granule cell excitability reduced (Henneberger et al., 2002; Olofsdotter et al., 2000). In support of this, BDNF regulates the development and maturation of GABAergic inhibitory interneurons (Huang et al., 1999a; Marty et al., 1996; Marty et al., 1997), and modulates the expression of $GABA_A$ receptors by recruiting trkB (Brunig et al., 2001; Elmariah et al., 2004; Jovanovic et al., 2004; Thompson et al., 1998). Chloride transport, which is maintained by $K^+$-$Cl^-$ transporter (KCC2), plays a critical role in the development and maintenance of inhibitory GABAergic transmission (Ben-Ari, 2002; Kaila, 1994; Rivera et al., 1999). Interestingly, the expression and activity of KCC2 is regulated among others by BDNF signaling via trkB (Rivera et al., 2002; Rivera et al., 2004; Wardle and Poo, 2003). Taken together, BDNF modulates inhibitory synaptic transmission through regulation of postsynaptic expression levels of $GABA_A$ receptors and chloride transporter KCC2.

### 2.6.3 BDNF in LTP

Long-term potentiation (LTP) is an experimental approach to study the early stages of memory formation and learning. Application of high-frequency stimulation of presynaptic excitatory pathway rapidly induces a long-lasting enhancement of synaptic strength that is

measured as increased amplitude of EPSPs in the postsynaptic neuron. Furthermore, LTP is restricted to the activated synapse and can be sustained for weeks or months. LTP occurs in many brain regions, however the classic experimental design in the hippocampal slices monitors the Schaffer collateral → CA1 (CA3 → CA1) synapse responses to stimulation. During measurement, test stimuli are first delivered repeatedly to induce stable EPSPs for baseline determination and eventually LTP is induced by high-frequency stimulation. If LTP is evoked, the subsequent test stimuli will produce enhanced EPSPs.

The contribution of BDNF-trkB signaling system to LTP is crucial. Induction of hippocampal LTP rapidly increases BDNF mRNA expression (Castren et al., 1993; Dragunow et al., 1993; Patterson et al., 1992). Furthermore, induction of LTP at the CA3 → CA1 synapse is impaired in two independent lines of BDNF knockout mice (Korte et al., 1995; Patterson et al., 1996; Pozzo-Miller et al., 1999) of which only one line shows defects in the basal synaptic transmission (Patterson et al., 1996; Pozzo-Miller et al., 1999). Interestingly, LTP is rescued by re-expression of BDNF either by virus-mediated gene transfer or exogenous application (Korte et al., 1996; Patterson et al., 1996) therefore suggesting that the impairment is not due to developmental deficits in these mice. Likewise, cortical LTP impairment is observed in heterozygous BDNF null mice of a third independently generated mutant (Bartoletti et al., 2002). Additional evidence to support the role of BDNF was provided by studies where LTP was attenuated if function-blocking BDNF antibodies or BDNF scavenging trkB-IgG proteins are applied on *in vitro* slices (Chen et al., 1999; Figurov et al., 1996; Kang et al., 1997). Besides the immediate actions in the hippocampal potentiation, BDNF is essential during the late-phase LTP (L-LTP) that requires new protein synthesis (Bradshaw et al., 2003; Kang et al., 1997; Korte et al., 1998). Interestingly, microinfusion of BDNF directly into dentate gyrus induces a long-lasting enhancement of transmission at the perforant path → granule cell (PP → GR) synapse (Messaoudi et al., 1998; Messaoudi et al., 2002; Ying et al., 2002). This BDNF-LTP shares properties analogous to high-frequency stimulation-induced late LTP, such as upregulation of the transcription factor *Arc* ((Ying et al., 2002) and refs therein).

LTP-inducing tetanic stimulation enhances regulated BDNF release and leads to increased CREB activation through a trkB-ERK pathway (Gooney and Lynch, 2001; Patterson et al., 2001). Interestingly, as demonstrated by Patterson et al (2001) during L-LTP, trkB signaling seems to regulate the redistribution of activated MAPK towards the nuclear compartment. Genetically modified mice have also corroborated the role of trkB signaling in synaptic potentiation. Conditional trkB mutant mice, in which the full-length receptor is eliminated forebrain-specifically (trkB-CRE), exhibit markedly reduced CA3 → CA1 potentiation in response to stimulation (Minichiello et al., 1999), a deficit comparable to that observed in BDNF null mice (Korte et al., 1995; Patterson et al., 1996). In accordance with the BDNF null mice, control

experiments indicated that basal synaptic transmission was normal in these trkB conditional mutants (Korte et al., 1995; Minichiello et al., 1999). Another conditional mice model lacking all trkB isoforms in the forebrain region (*trkB* CA1-KO) confirms the results (Xu et al., 2000a). In these mice, the absence of trkB in hippocampal CA1 region leads to reduction in CA3 $\rightarrow$ CA1 synapse potentiation and provides evidence for presynaptic BDNF action in the modulation of LTP. Recently, mice with targeted mutations in the trkB binding sites for Shc and PLC$\gamma$ have demonstrated some functional differences among trkB downstream pathways (Korte et al., 2000; Minichiello et al., 2002). These studies reported that successful induction of TBS-induced LTP in the CA3 $\rightarrow$ CA1 synapse requires PLC$\gamma$ signaling (Minichiello et al., 2002). Interestingly, trkB-Shc mutants display intact LTP (Korte et al., 2000) therefore suggesting that signaling via Shc pathways is not required for hippocampal LTP. These results are surprising since previous data connects Ras/MAPK pathway to synaptic potentiation (English and Sweatt, 1996; English and Sweatt, 1997; Patterson et al., 2001; Ying et al., 2002). However it is possible that the cross-talk between trkB downstream signaling pathways might rescue the Shc-deficit in terms of LTP induction. Altogether, it is likely that complexity of factors modulating presynaptic as well as postsynaptic actions will contribute to generation and maintenance of LTP; however, the evidence in favor of BDNF-trkB is undeniably strongly.

### 2.6.4 BDNF and learning

The BDNF/trkB signaling system is a likely player in memory acquisition and learning, especially in hippocampus-dependent paradigms such as spatial learning. Indeed, the BDNF mRNA expression is upregulated in response to spatial learning (Gomez-Pinilla et al., 2001; Kesslak et al., 1998; Mizuno et al., 2000). Further, enriched-environment housing upregulates BDNF mRNA and protein, and is associated with improved spatial learning (Falkenberg et al., 1992; Ickes et al., 2000). Surprisingly, artificially increased BDNF levels have not revealed improved cognitive function (Pelleymounter et al., 1996). On the contrary, mice overexpressing BDNF develop a passive avoidance deficit that is dependent on BDNF overexpression levels (Croll et al., 1999). Further evidence for role of BDNF in learning is pointed out by function blocking studies, in which BDNF withdrawal by antisense oligonucleotides or anti-BDNF antibodies severely impairs spatial learning (Ma et al., 1998; Mizuno et al., 2000; Mu et al., 1999). In accordance, heterozygous BDNF null mutants display a spatial learning deficit (Linnarsson et al., 1997 but see Montkowski and Holsboer, 1997). Forebrain-resticted Emx-BDNF$^{KO}$ mice further corroborate that BDNF is required for spatial learning (Gorski et al., 2003a). Selective removal of BDNF from the dorsal cortex and hippocampus profoundly impaired spatial learning and complex discrimination whereas the hippocampus-dependent fear conditioning was not affected (Gorski et al., 2003a). Besides spatial learning, BDNF signaling appears to have a role in fear-motivated

learning (Alonso et al., 2002; Hall et al., 2000), and specifically during initial consolidation of long-term fear memory (Lee et al., 2004).

Spatial learning stimulates the activation of trkB receptor and downstream signaling partners (Gooney et al., 2002; Mizuno et al., 2003a). Accordingly, alterations in the trkB receptor function lead to learning deficits. Indeed, trkB-CRE targeted mutants show a dramatic deficit in complex and stressful learning paradigms such as Morris water maze or eight-arm radial maze whereas the less demanding learning tests are performed normally (Minichiello et al., 1999). Additional studies in a more naturalistic environment suggested that the trkB-CRE mice could actually learn a spatial task; however, they were unable to rapidly switch to another behavioral strategy (Vyssotski et al., 2002). Similar lack of behavioral flexibility is known to be associated with hippocampal lesions (Terry et al., 1989; Vyssotski et al., 2002). Furthermore, mice overexpressing the dominant negative form of trkB receptor, trkB.T1, display a mild learning deficit in the Morris water maze task (Saarelainen et al., 2000b). Interestingly, as these transgenic mice learned to find the hidden platform like their wild-type littermates there was a significant impairment in the long-term memory retention in a retest after two months. In conclusion, convincing behavioral evidence implicates BDNF signaling in hippocampus-dependent learning, although the exact mechanisms need yet to be examined in more detail.

### 2.6.5 BDNF in epilepsy

Epilepsy, the second most common neurological disease, is characterized by recurrent abnormal electrical activity in brain that eventually leads to appearance of spontaneous seizures and possible decline in cognitive skills. The initial insult, such as head trauma, inflammation or status epilepticus (SE), induces a variety of both acute and delayed responses that count for the progress of the disease. During epileptogenesis, factors such as neuronal loss, hippocampal neurogenesis, gliosis and neuronal sprouting cause alterations in the cellular homeostasis and lead to reorganization of the neuronal network (Lukasiuk and Pitkanen, 2004; Pitkanen et al., 2002; Scharfman et al., 2002). Additionally, various gene expression changes occur during SE and epileptogenesis (reviewed by Zagulska-Szymczak et al., 2001). During the early stages of epileptogenesis, genes mediating responses to stress and injury are regulated (Becker et al., 2002; Becker et al., 2003; Lukasiuk and Pitkanen, 2004) whereas studies focusing to later phases of epileptogenesis have reported expression changes in genes regulating synaptic plasticity, axonal growth or cellular signaling (Becker et al., 2003; Lahteinen et al., 2004; Lukasiuk et al., 2003; Lukasiuk and Pitkanen, 2004). Extensive evidence suggests a role for BDNF-trkB system as an important underlying contributor to epilepsy (Binder et al., 2001; Lindvall et al., 1994; Scharfman et al., 2002). First, both BDNF and trkB mRNAs are upregulated by seizures, showing the most dramatic increase in hippocampal pyramidal neurons, in dentate granule cells and in hilar neurons (Ernfors et al., 1991; Jankowsky and Patterson, 2001; Merlio et al., 1993). All trkB

isoforms are upregulated at 4 hours after seizure onset in hippocampal CA1-CA3 regions, however in the dentate gyrus, the truncated trkB mRNA is rapidly elevated already at 1-hour timepoint (Dugich-Djordjevic et al., 1995). Further, the hippocampal BDNF mRNA elevation is rapid and transient in response to seizures (Dugich-Djordjevic et al., 1992; Zafra et al., 1990). Second, BDNF and trkB protein expression is also regulated by epileptiform activity; however, the upregulation in the protein occurs slightly delayed after seizure onset and persists longer (Binder et al., 2001; Goutan et al., 1998; Nawa et al., 1995). Third, trkB receptor protein is activated by seizure activity. Both kainate-induced seizures and partial kindling increase trkB phosphorylation, especially in the hippocampal mossy fibers that sprout massively in response to epileptic activity (Aloyz et al., 1999; Binder et al., 1999). *In vivo* studies with targeted mutations have suggested that trkB activation during limbic epileptogenesis is independent of the TrkB-Shc site (He et al., 2002) whereas the role of PLC$\gamma$ site is still unresolved. Fourth, exposure to increased BDNF levels results in hyperexcitability that may promote the occurrence of spontaneous seizures. Exogenous BDNF added on *in vitro* slices enhances synaptic transmission (Scharfman, 1997) and transgenic mice overexpressing BDNF display increased seizure severity (Croll et al., 1999). Finally, decreased BDNF signaling reduces epileptogenesis. Mice overexpressing the dominant-negative trkB receptor display also less frequent and behaviorally milder seizures after kainate treatment (Lahteinen et al., 2002). Moreover, both heterozygous BDNF mice ($BDNF^{+/-}$) and mice treated with trkB-IgG show delayed development of the kindling response (Binder et al., 1999; Kokaia et al., 1995). Curiously, also chronic BDNF infusion into hippocampus is reported to inhibit development of kindling (Larmet et al., 1995). The latter observation, however, may be confounded by outside factors. Indeed, long exposure to BDNF is known to desensitize the receptor and results in receptor translocation from the membrane (Knusel et al., 1997; Haapasalo et al., 2002). Furthermore, the effect of BDNF on seizure development is dependent on the administration protocol used (Xu et al., 2004). In conclusion, strong evidence suggests the BDNF-trkB system as one pathway through which the enhanced neuronal activity is promoting the epileptogenesis and the structural reorganization of neuronal network.

## *2.7 Genetically modified mice in the BDNF/trkB system*

### 2.7.1 Knockout models

Several genetically modified mice models either lacking or overexpressing BDNF and trkB are described in the literature. The first knockout mice for trkB receptor ($trkB^{K}$-/-) were generated by Klein and coworkers (1993). In their approach, a targeted disruption in the trkB intracellular kinase domain completely abolished the full-length receptor expression and BDNF downstream signaling whereas the truncated receptor expression was reduced only about 50 %. The homozygous mice developed to birth but died shortly after, possibly due to abnormal

feeding behavior (Klein et al., 1993). These mice showed markedly reduced neuronal number in the sensory ganglia and a significant loss of facial motor neuron population in the spinal cord (Klein et al., 1993). However, the few trkB$^{K}$-/-mice that were able to survive up to three weeks displayed increased number of apoptotic central neurons, especially in the dentate gyrus (Alcantara et al., 1997). Interestingly, another trkB mutant mouse line totally lacking all trkB isoforms, suggests distinct roles for the truncated trkB receptor (Luikart et al., 2003). Here the survival of sensory neurons in the total knockouts was actually enhanced in comparison to trkB$^{K}$-/- mice, thus suggesting a pro-apoptotic effect for the truncated trkB. In contrast, the survival rate for trkB$^{K}$-/- mice was better than for total trkB knockouts suggesting yet an additional role for the truncated trkB in promoting long life through still unidentified mechanism (Luikart et al., 2003).

TrkB and trkC receptors act in concert to promote survival. Although double knockout mice lacking both trkB and trkC die by birth, the heterozygous mice expressing one allele of either gene (trkB-/-;trkC+/- or trkB+/-;trkC-/- ) survive for a few weeks (Minichiello and Klein, 1996). The dentate granule cells undergo enhanced cell death in the heterozygous double mutants compared to the single mutants already at P12 (Minichiello and Klein, 1996). Similarly, by old age, the double heterozygous trkB/trkC mutants (trkB+/-;trkC+/-) show neuronal loss and axonal degeneration in the hippocampus and amygdala (von Bohlen und Halbach et al., 2003). Altogether, these results suggest co-operative actions of the trkB and trkC signaling pathways when regulating survival *in vivo*.

Interestingly, the disruption of the BDNF gene had slightly less severe outcome than the trkB knockout, although the homozygous mutation was lethal as well (Ernfors et al., 1994; Jones et al., 1994). BDNF-/- mice show dramatic deficits in the sensory ganglia; especially the vestibular system is severely affected, whereas the motoneuron population appears normal. In contrast to trkB-/- mice, the BDNF knockouts show marked behavioral abnormalities: defects in the coordination and balance, ataxia, hyperactivity, and breathing irregularities ((Ernfors et al., 1994; Jones et al., 1994). Additionally, no gross structural deficit was present in neocortex, hippocampus and cerebellum although reduction in the calcium-binding proteins calbindin and parvalbumin was observed in both cortex and hippocampus (Jones et al., 1994). However, when the mechanism behind the ataxic gait in BDNF null mutants was later examined in detail, it revealed decreased cerebellar trkB activation and impaired cerebellar layering and foliation with reduced dendritic arborization of the Purkinje cells (Schwartz et al., 1997). In contrast to lethality of trkB and BDNF null mutations, the mice lacking NT4 are viable with no obvious phenotype (Conover et al., 1995). Additionally, double mutant mice lacking both BDNF and NT4 are not more affected than BDNF null mice. Interestingly, the motoneuron population that is severely affected in the trkB null mutants was intact in the double BDNF/NT4 null mice

therefore suggesting other mechanisms regulating the motoneuron survival (Conover et al., 1995; Liu et al., 1995).

In contrast to cooperative actions observed for the central neurons, a thorough analysis on the sensory neuron survival in null mutants revealed distinct effects for the trkB ligands. The vestibular and trigeminal ganglion neurons were BDNF-dependent whereas cells in the nodose-petrosal complex were dependent on BDNF, NT4, or both (Conover et al., 1995; Erickson et al., 1996). Loss of a specific subpopulation of the nodose-petrosal neurons participating in the control of ventilation is a likely cause for the irregular breathing phenotype of the BDNF-deficient mice (Erickson et al., 1996). Interestingly, if the BDNF gene is replaced by either NT3 or NT4, many severe neuronal deficits observed in BDNF null mutants are rescued to some extent (Agerman et al., 2003; Fan et al., 2000). By expressing NT3 in place of BDNF in the sensory system, the severe defects of the cochlea innervation and hearing observed in BDNF null mice are rescued, whereas the vestibular innervation is not (Agerman et al., 2003). Further contrast to BDNF null mice, heterozygous mice expressing NT4 under BDNF promoter are viable, fertile and show no obvious behavioral phenotype (Fan et al., 2000). However, the homozygous NT4 knock-in mice where levels of NT4 are multiplied are smaller, infertile and show cutaneous sensory abnormality. Further, NT4 expression is able to rescue the defects observed in BDNF -/- mice sensory ganglion neurons (Fan et al., 2000).

### 2.7.2 Conditional mutants

Since the publication of total trkB/BDNF knockouts with lethal symptoms, several approaches with different genetic combinations have addressed the role of BDNF and its receptor in nervous system. Studies on heterozygous null mice have demonstrated that BDNF is involved in the regulation of locomotor activity and food intake (Dluzen et al., 2001; Kernie et al., 2000; Lyons et al., 1999; Rios et al., 2001). BDNF mutants display symptoms associated with human obesity; mutants consume more food, their adipocytes are enlaged and they have high plasma levels of leptin and insulin. Although the caloric homeostasis is regulated by various satiety and adiposity signals, the results suggest BDNF/trkB system as one possible regulator of the food intake. In addition, lack of one BDNF allele results in hyperactivity and increased anxiety-like behavior, exaggerated aggressiveness, and a progressive loss of forebrain serotoninergic fibers (Kernie et al., 2000; Lyons et al., 1999; Rios et al., 2001). Interestingly, exogenous BDNF infusion reverses the obese phenotype whereas a treatment with selective serotonin reuptake inhibitor fluoxetine significantly alleviates the aggressive phenotype (Kernie et al., 2000; Lyons et al., 1999).

Conditional BDNF mutant mice (Emx-BDNF$^{KO}$) lack BDNF specifically from the early embryonic development onwards in the forebrain regions (Gorski et al., 2003b). These mice exhibit shortened lifespan, mild obesity, increased intermale aggressiveness and infertility

(Gorski et al., 2003b) combined with substantial deficits in spatial and nonspatial learning but not in anxiety (Gorski et al., 2003a). Despite these behavioral deficits, the forebrain cytoarchitecture was grossly normal. However by closer examination, at 5 weeks of age the null mice showed reduced cortical thickness, suggested to result from reductions in the neuronal soma size and dendritic morphology. Indeed, around 3 weeks of age when the endogenous BDNF expression would occur, a neuronal shrinkage and dendritic retraction were observed in the Emx-BDNF$^{KO}$ mice (Gorski et al., 2003b). Similarly, in striatum that receives most its BDNF via cortical anterograde transport, neurons had shrunken somas, thinner dendrites and neuronal loss at old age (Baquet et al., 2004). The opposite, however, is observed in transgenic mice overexpressing BDNF that display increased dendritic length and complexity in the dentate granule cells (Tolwani et al., 2002).

Interestingly, a similar requirement for BDNF in the maintenance of neuronal structure was seen in late-onset forebrain-restricted trkB mutant mice (Xu et al., 2000b). In these mice, the cortical pyramidal neurons are subjected to 50 % loss of trkB protein and as a result, the cortical structure is compressed due to reduced dendritic tree and shrunken cell soma (Xu et al., 2000b). Whereas the Emx-BDNF$^{KO}$ mice have no obvious loss of cortical neurons, the trkB conditional knockouts show marked loss of trkB-dependent cortical neurons (Gorski et al., 2003b; Xu et al., 2000b). However, the hippocampus of these same trkB-KO mice was morphologically intact (Xu et al., 2000a). In accordance, an independently created floxed trkB mutant lacking the kinase specific form also reports normal brain morphology besides a mild reduction in cortical thickness (Minichiello et al., 1999). However, the most remarkable finding in these two separate trkB conditional knockout mice is the reduced hippocampal LTP and severe impairment in complex learning paradigms (Minichiello et al., 1999; Xu et al., 2000a).

Targeted mutations in the trkB docking sites have revealed both similarities and differences in trkB signaling. Mice with targeted mutation in the trkB Shc-site (Y515F) show reduced and transient activation of MAPK in response to ligand stimulation. Interestingly, the NT4-dependent activation was effected more than BDNF induced (Minichiello et al., 1998). Accordingly, in trkB-Shc mice almost total loss of NT4 dependent sensory neurons is observed whereas the BDNF-dependent population is virtually normal. Together, these results suggest that signaling via the Shc-site is differentially activated by the two trkB ligands (Minichiello et al., 1998). Moreover, a mutation in trkB PLC$\gamma$ docking site (Y816F) abolishes the PLC$\gamma$ binding and activation of CREB, CaMKII and CaMKIV whereas the Sch and MAPK activation was not affected (Minichiello et al., 2002). Both trkB-Shc and trkB-PLC mutants have no major deficits in the central nervous system, therefore suggesting that mechanisms for BDNF/trkB- mediated neuronal differentiation are independent of both Shc and PLC$\gamma$ signaling pathways (Minichiello et al., 2002; Minichiello et al., 1998). Interestingly, the trkB-PLC mutation causes a prominent

impairment on the hippocampal LTP whereas trkB-Shc site is not required for hippocampal plasticity (Minichiello et al., 2002).

### 2.7.3 Overexpressing models

As the exogenous administration of BDNF to the brain is often difficult due to poor diffusion, genomic approaches to increase BDNF tissue levels have been used. Homozygous mice overexpressing BDNF under the β-actin promoter exhibit relatively mild phenotype with 30-40 % increased level of BDNF protein in brain (Croll et al., 1999). These mice could not be distinguished from the wild type littermates by appearance, behavior or hippocampal morphology (Croll et al., 1999; Qiao et al., 2001). Behaviorally, they exhibited significant passive avoidance deficit that was dependent on the BDNF expression levels. Additionally, the transgenic mice showed more severe seizures in response to systemic kainate administration. In response to repetitive stimuli, BDNF overexpressing mice showed increased excitability and appearance of spreading depression but increased resistance to LTP induction (Croll et al., 1999). In another BDNF transgenic mice, postnatally increased levels of BDNF lead to accelerated maturation of the GABAergic innervation and inhibition in visual cortex (Huang et al., 1999b). Additionally, in these mice, the effects of dark rearing are rescued, most propably due to increased BDNF expression (Gianfranceschi et al., 2003). In contrast to recognized pro-survival role, BDNF overexpression in skin promotes neurite innervation but not the survival of cutaneous sensory neurons (LeMaster et al., 1999).

Although several mice models lacking the trkB receptor are reported, only some mutants show increased receptor expression. Transgenic mice overexpressing the dominant-negative trkB receptor, trkB.T1, in postnatal neurons exhibit impaired learning and increased susceptibility to damage after ischemic insult (Saarelainen et al., 2000a; Saarelainen et al., 2000b). In these mice, the truncated T1 receptor variant mRNA is about 20-fold overexpressed, thus resulting in reduced activation of the full-length trkB (Saarelainen et al., 2003) possibly via sequestering the available BDNF. Interestingly, these mice together with heterozygous BDNF null mice were resistant to antidepressant induced behavioral and biochemical changes thus suggesting that BDNF signaling is required for the acute effects of antidepressive drugs (Saarelainen et al., 2003). Finally, the transgenic mice overexpressing the full-length trkB receptor are introduced comprehensively in the experimental part of this thesis (Koponen et al., 2004; Lahteinen et al., 2003), unpublished results). Altogether, the results from genetically modified animal studies suggest that deficiency in the BDNF/trkB system is more detrimental for the function of central neurons than the increased expression, which produces milder phenotypes.

## 2.8 Neurotrophin-4 (NT4)

The neurotrophin-4 was initially isolated from *Xenopus* and viper (Hallbook et al., 1991), and soon after the mammalian counterparts were reported (NT5, Berkemeier et al., 1991; NT4, Ip et al., 1992). The early reports showed a high structural homology to the other NGF family members, and identified trkB as the main signaling receptor. The NT4 is ubiquitously expressed and is a survival factor for the cultured DRG neurons (Berkemeier et al., 1991; Hallbook et al., 1991; Ip et al., 1992). Since these primary observations, NT4 is reported to promote the survival of the preganglionic nerves innervating adrenal medulla (Schober et al., 1998), the nigral dopaminergic neurons both *in vitro* (Hynes et al., 1994; Meyer et al., 2001), and *in vivo* (Alexi and Hefti, 1996; Lingor et al., 2000), the retinal ganglion cells (Cohen et al., 1994; Cui and Harvey, 1994) as well as the developing superior colliculus neurons (Spalding et al., 2002). In addition, NT4 promotes dendritic outgrowth of the mesensephalic dopamine neurons (DeFazio et al., 2000; Hagg, 1998) and retinal ganglion cells (Bosco and Linden, 1999) and activity-dependent inhibitory synaptogenesis (Seil, 1999; Seil and Drake-Baumann, 2000). In the developing neocortex, increased levels of NT4 disturbs normal cortical cytoarchitecture by forming heterotopias (Brunstrom et al., 1997). NT4 is strongly expressed in the muscle and is activity-dependent trophic factor for adult motor neurons (Funakoshi et al., 1995). Similar to BDNF, NT4 has a role in the maintenance of synaptic functions of the developing *Xenopus* motoneurons (Liou et al., 1997). Further, the overexpression of NT4 in *Xenopus* myocytes results in increased spontaneous activity in neuromuscular synapses that is tightly localized into activated synapse (Wang et al., 1998; Wang and Poo, 1997). Also, long-lasting NT4 treatment on hippocampal slices increases spontaneous excitatory activity in CA3 cells (Schwyzer et al., 2002).

Although BDNF and NT4 mediate their effects via the same trkB receptor and share a lot of similar effects, distinctions in their actions have been reported as well. Indeed, both NT4 and BDNF protect cerebellar granule cells against apoptosis (Kubo et al., 1995) and support hippocampal neuron survival (Lindholm et al., 1996). However, infusion of NT4 but not BDNF is able to improve spatial memory in aged rats (Fischer et al., 1994). Unlike BDNF knockout mice, mice lacking NT4 have normal lifespan, growth, fertility and no obvious neurological defects (Conover et al., 1995; Ernfors et al., 1994; Jones et al., 1994). In contrast to the trkB knockouts, the NT4 knockouts have normal motoneuron populations and the majority of the sensory neurons are unaltered (Conover et al., 1995; Klein et al., 1993). However, the nodose-petrosal ganglion complex (NPG) was reduced in both NT4 and BDNF single mutants and even greater reduction was observed in double BDNF/NT4 mutants (Conover et al., 1995; Erickson et al., 1996). A severe loss of dopaminergic cells in the NPG observed in BDNF but not NT4 knockouts, affects the resting ventilation system (Erickson et al., 1996). Similar to BDNF heterozygotes, the NT4

knockouts show deficits in both long-term memory and late-phase LTP (L-LTP) (Xie et al., 2000). Recently, differences in trkB signaling were addressed with a targeted mutation in the Shc binding site that severely affects the NT4 dependent neuronal survival whereas the BDNF dependent populations were almost unaffected (Minichiello et al., 1998). Furthermore, when NT4 was knocked into the BDNF locus, the lethal phenotype on BDNF knockout was rescued (Fan et al., 2000). These mice, that express NT4 under BDNF promoter, were viable but markedly reduced in size and had abnormal cutaneous sensory function. Additionally, NT4 was able to support the survival of many BDNF dependent sensory neurons, and the formation of functional synapses was accelerated (Fan et al., 2000). Yet another observed difference between the trkB ligands is that NT4 is more effective than BDNF in counteracting the effects of monocular deprivation (Lodovichi et al., 2000). Taken together, both NT4 and BDNF signal via trkB receptor; however, the ligands are able to elicit distinct downstream responses.

## 3 AIMS OF THE STUDY

This experimental study was designed to elucidate the effects of increased neurotrophic actions in the adult central nervous system (CNS). Brain-derived neurotrophic factor (BDNF) is a neurotrophin regulating a variety of functions in the nervous system including development, maturation, plasticity and survival. BDNF mediates its primary functions via trkB tyrosine kinase receptor that is also widely expressed in the CNS. Previous genetic knockout studies have established a crucial role for adequate function of the BDNF/trkB system by showing the lethality of lacking either component. This study provides a new genetic model to study the effects of increased BDNF signaling in the adult nervous system by overexpressing the full-length trkB receptor in neurons.

The specific aims of this study were to:

1. Establish the transgenic mice line overexpressing the FLAG-trkB.TK+ genetic construct and distinguish the pattern of transgene expression

2. Characterize the effect of trkB overexpression on
    a. the expression of endogenous trkB and the ligand BDNF
    b. the receptor activity and downstream signaling pathways
    c. the expression of brain indoleamines

3. Elucidate the connection between the increased BDNF signaling and neuronal plasticity by examining the transgenic mice with
    d. behavioral testing
    e. electrophysiological parameters
    f. gene expression of plasticity related genes

# 4 EXPERIMENTAL PROCEDURES

All animal experiments were carried out with transgenic mice line overexpressing the full-length neurotrophin receptor trkB. The production and maintenance of experimental animals was done according to guidelines of the Society for Neuroscience and were accepted by the Experimental Animal Ethics Committee of the National Laboratory Animal Center, University of Kuopio, Finland. All mice experiments described below were performed on male heterozygous trkB.TK+ mice (TG) and their wild type (WT) littermates and samples of both genotypes were processed in parallel. The experimental animals were always used as adults (age between 2-8 months) except for the electrophysiology experiments that were performed on younger animals (age 4-12 weeks). Mice were housed in metal cages under standard animal room conditions (12:12 hour light cycle, ambient temperature of 23°C, standardized humidity) and they had free access to food and water. During this study, the mice were maintained in the animal facilities of the National Laboratory Animal Center, University of Kuopio and the National Public Health Institute, Kuopio, Finland.

## *4.1 Drug treatments*

In **publication III**, status epilepticus (SE) was induced by intraperitoneal (i.p.) injection of kainic acid (Tocris, 25/30/35 mg/kg) to WT and TG mice. To assess the acute effect of kainate to seizure threshold, mice were followed for 3 hours post-injection and sacrificed after 48 hours. For the chronic effect of kainate, mice were i.p. injected with 35 mg/kg of kainic acid to induce SE and sacrificed after 130-134 days. Control animals received a saline injection and were similarly handled. In **publication IV**, adult WT and TG mice received a single i.p. injection of either fluoxetine (20 mg/kg) or saline and were sacrificed after 3 hours.

## *4.2 TrkB.TK+ overexpressing mice*

The full-length trkB.TK+ cDNA (provided by Dr. Mart Saarma, University of Helsinki) was N-terminally tagged with FLAG-octapeptide (coding for *N*- AspTyrLysAspAspAspAspLys-*C*) that was placed between the signal peptide and the mature trkB *N*-terminus sequence. The synthesized Flag-trkB.TK+ cDNA was inserted into XhoI restriction site of the murine Thy-1.2 minigene cassette (provided by Dr. Pico Caroni, Friedrich Miescher Institute, Switzerland) and then cut using PvuI and EcoRI restriction enzymes. The final Thy-Flag-trkB.TK+ construct (**publication I**, figure 1a) was verified by sequencing (DNA-core facility, A.I.Virtanen Institute, University of Kuopio). Transgenic mice were generated by pronucleus injection of the purified construct into $CD_2F_1$ (BALB/c x DBA/2) hybrid embryos. Born males were analyzed for the

transgene expression by southern blot from short tailpieces. The transgenic lines were established by mating the transgenic male founders with $CD_2F_1$ females and the progeny was routinely identified by PCR.

### *4.3 Identification of transgenic mice with PCR*

Small pieces of tail or ear were digested in lysis buffer (200 mM NaCl, 20 mM EDTA, 40 mM Tris-HCl; pH 8.0, 0.5 % SDS, 0.5 % β-mercaptoethanol, 0.8 mg/ml proteinase K) overnight at + 60 °C. Genomic DNA was extracted by isopropanol precipitation, washed in cold 70 % ethanol and dissolved. PCR primers specific for Thy-1 (5'-CTC CCA CTT CCT TGG CTT-3') and trkB (5'-GCC CCA CGT AAG CTT CGA-3') were used to produce a 500 bp long fragment for identification of transgenic animals. The analysis was simple; the PCR-fragment was produced only by the transgenic samples and the wild type lanes were blank. Three separate tg-lines were produced; however, one line (UKU209) with strongest expression was selected for further use. Additionally, the expression profile in the selected line resembled the overexpression pattern in previously generated trkB.T1 mice under Thy-1 promoter control as well (Saarelainen, 2001).

### *4.4 Tissue processing*

#### 4.4.1 Fixation

##### *4.4.1.1 Nonradioactive in situ hybridization*

In **publication II**, the mice were deeply anesthetized with i.p. injection of pentobarbital (Mebunat, 60 mg/ml), perfused transcardially with cold RNase-free phosphate-buffered saline (PBS) followed by cold 4% paraformaldehyde (PFA) in PBS (RNase-free). Brains were removed and postfixed for 24 hours in the fixative at +4°C, cryoprotected in 20% sucrose/4% PFA/ PBS, frozen and stored overnight at -70°C. Coronal, 25µm thick sections were cut using a Leica CM 3000 cryostat and were stored as floating in the fixative at +4°C.

##### *4.4.1.2 Immunohistochemisty*

For the immunohistochemical analysis (**publication II**, additional data), mice were processed as above with some modifications. The perfusion solutions were not treated RNase – free, and the post-fixation step lasted for only 2 hours at +4°C. Further, coronal 25µm thick sections were cut throughout the brain using a sliding microtome (Leica SM 2000R) and stored as floating in tissue collecting solution (TCS; 30% ethylene glycol, 25% glycerol in 0.05 M sodium phosphate buffer) at -20°C until used.

In order to detect mossy fiber sprouting in **publication III**, WT and TG mice were perfused 130-134 days after induction of SE according to the Timm fixation protocol (Sloviter, 1982). Mice were deeply anesthetized with a mixture of sodium pentobarbital and chloral hydrate and

perfused first with 0.37% sulphide solution followed by 4% PFA. Further processing was performed as above.

### 4.4.2 Collection of fresh samples

#### *4.4.2.1 Radioactive in situ hybridization*

For the radioactive *in situ* hybridization in **publications I, II** and **III**, mice were anesthetized with $CO_2$, immediately killed by decapitation, and the brains were rapidly removed and frozen on dry ice. Coronal brain sections (14 µm) were cut using a Leica CM-3000 cryostat and collected on SuperFrost (Menzel-Glaeser, Germany) slides, fixed for 5 min in cold 4% paraformaldehyde, dehydrated and dried. Tissue samples and unfixed slides were stored at -70°C and fixed slides in 94% ethanol at +4°C.

#### *4.4.2.2 Northern blotting*

In **publication I**, the samples were collected for the mRNA expression analysis by northern blot. Mice of different ages (E17.5, P0.5, P5.5, P10.5, P15.5, P18.5, P28.5 and young adults (age approx. 2 months)) were quickly anesthetized with $CO_2$, rapidly decapitated and the brains were dissected. For the E17.5 sample, the mother was anesthetized, her neck dislocated and the unborn puppies were dissected out from the uterus for brain tissue collection. The cerebellum was removed and one cerebral hemisphere was collected into TRIzol reagent (Invitrogen, CA) and homogenized immediately. The other hemisphere was frozen on dry ice for possible later purposes.

#### *4.4.2.3 Western blotting*

In **publication I**, the protein expression levels were determined using western blotting. Hippocampi and a cortical area corresponding to motor and sensory cortices were dissected rapidly and collected into lysis buffer containing protease inhibitors and 2 mM sodium vanadate. Homogenized samples were incubated in rotation at +4°C for 20 min, centrifuged and the supernatant collected. Protein concentration was determined with DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). The supernatants were stored at –70°C if not processed immediately.

#### *4.4.2.4 ELISA*

In **publication I**, the BDNF protein levels of untreated WT and TG mice were measured. Hippocampal or cingulate cortex samples were carefully dissected, immediately transferred into cold homogenization buffer containing protease inhibitors and homogenized by sonication (Labsonic U, B.Braun Biotech Int.). Samples were centrifuged and stored at -70°C.

#### *4.4.2.5 HPLC analysis*

For the brain indoleamine measurement in **publication IV**, adult mice were injected with either fluoxetine or saline and decapitated after 3 hours. Rapidly dissected brain regions (hippocampus, the cingulate and prefrontal cortices) were dissected, weighed and frozen immediately on dry ice. Tissue samples were homogenized while still frozen in 10 volumes of 0.1 M HClO (containing 0.25 μM dihydrobenzylamine as an internal standard), centrifuged and used for HPLC analysis.

### *4.5 Northern blotting*

In **publication I**, the expression level of Thy-trkB.TK+ in adult brain lysates was studied by northern blotting. Also, the developmental pattern of transgene expression was determined from brains of animals between ages E17.5 and P28.5. For the RNA isolation, homogenates of one cerebral hemisphere were processed according the manufacturer's instructions. Total RNA was separated by agarose gel and detected using $\alpha$-[$^{32}$P]dCTP labeled cDNA probe recognizing the extracellular parts of trkB. Hybridized membranes were exposed to a Phosphor Screen (Molecular Dynamics, Inc., CA), scanned using the STORM 860 PhosphoImager (Molecular Dynamics, Inc.) and analyzed with ImageQuant (Molecular Dynamics, Inc.) software for trkB expression amounts. Transgenic expression was normalized against the endogenous trkB.TK+ signal.

### *4.6 In situ hybridization*

#### 4.6.1 Radioactive *in situ* hybridization

Radioactive *in situ* hybridization with antisense oligonucleotide probe was used to determine the mRNA expression of GAP-43, $\alpha$-CaMKII, CREB, *fos* family genes (*c-fos*, *fra1*, *fra2* and *fosB*) and *jun* family genes (*c-jun*, *junB*) (**publication II**). Furthermore, the mRNA expression of BDNF and the FLAG-octapeptide (**publications I, III**) and the NMDA receptor subunits NR1, NR2a and NR2b (unpublished data) were studied in transgenic and wild type mice. Oligoprobes were checked for mismatches or palindromes and the specificity to the desired gene was determined using public database searches (see table 1).

**Table 1: Oligonucleotide sequences used as probes for radioactive in situ hybridization**

| Gene | Specificity | Sequence (5'-3') |
|---|---|---|
| BDNF | mouse | GATTGGGTAGTTCGGCATTGCGAGTTCCAGTGCCT |
| α-CaMKII | mouse | TGCGAACGAGGACGCAGGGACCCTGGCCTGGTC |
| c- fos | rat | GCAGCGGGAGGATGACGCCTCGTAGTCCGCGTTGAAACCCGAGAA |
| c-jun | rat | CGGCTGCGAGGGAAAGGCCAGCCCGGCCGCGCCATAGGAGGGCGCCCC |
| CREB | mouse | TGGCTGGGCCGCCTGGATAACGCCATGGACCTGGA |
| FLAG | | GGCACTTGTCATCGTCGTCTTTGTAGTCGGCA |
| FosB | mouse | CATTTCCCCGAGACCGGCGCACTCCTGGGAGGCGGCGGCGGTGGGTGG |
| Fra1 | rat | CTGCTACTCTTTCGATGGGCTGAGGAACAAGGCTC |
| Fra2 | rat | TTCTCCGTCAGCTCACGTCGACGGTTCCGACACTT |
| GAP-43 | mouse/rat | TTCTTGGTCAGCCTCGGGGTCTTCTTTACCCTCAT |
| JunB | mouse/rat | GATGGTGGCCGTCGGGTATGAGCTCCCAGTCCCGACGGCGGTCCCGGA |
| NR1 | mouse | AACTGCAGCACCTTCTCTGCCTTGGACTCACGCTC |
| NR2a | mouse | AGAAGGCCCATGGGGAGCTTTCCCTTTGGCTAAGT |
| NR2b | mouse | GGGCCGCCTGGCTCTCTGCCATCAGCTAGGCACCG |

Oligonucleotides were 3'-end-labelled to specific activity of 1-5 x $10^7$ cpm/pmol by terminal transferase and α-[$^{33}$P]dATP (2000 Ci/mmol, New England Nuclear, Zaventem, Belgium). Hybridization was performed with 1-3 x $10^3$ cpm/µl of labeled probe in buffer containing 50% formamide, 10% dextran sulfate and 4 X SSC at 42°C overnight. Slides were subjected to serial washes of increasing stringency completed by rinses in ethanol. Finally, slides were air-dryed and exposed to Hyperfilm-βmax X-ray films (Amersham Biosciences) for 1-3 weeks and developed in Kodak D-19 developer. Quantitative data was obtained from 3-6 individual brains of both genotypes. Autoradiographs were quantified by using a video-based MCID/M4 image analysis program, version 3 (Imaging Research Inc.). The measured optical density was converted to radioactivity (nCi/g) using a standard curve acquired from the concurrently exposed $^{14}$C-microscale standards. The quantified brain regions were determined from the mouse brain atlas of Franklin and Paxinos (1997).

### 4.6.2 Non-radioactive *in situ* hybridization

In **publication II**, the distribution of the full-length trkB mRNA was determined using the digoxigenin-labeled cRNA probes for *in situ* hybridization. For the cRNA probe preparation, a plasmid containing a 996 bp cDNA insert recognizing the tyrosine kinase domain of trkB.TK+ (Genebank accession # M55291, corresponding to nucleotides 2093-3089 of rat trkB.TK+; gift by Dr. Mart Saarma, University of Helsinki) was linearized with the appropriate restriction enzyme,

purified, and stored at –20 °C. The linearized plasmid was labeled by *in vitro* transcription using the DIG RNA Labeling Mix (Roche, Germany) according to the manufacturer's instructions. The riboprobe was purified and the yield of labeled probe was estimated using the DIG-labeled Control RNA (Roche). The probe was stored at -70°C until use. Hybridization was performed under RNase-free conditions and corresponding sections of both genotypes were processed concurrently and developed for equal times. Floating sections were well rinsed to remove the storage solution. The probe penetration was improved with proteinase K- treatment and sections were acclimatized by prehybridization at +55°C for 60 min. Hybridization was performed in a solution containing prehybridization buffer, 10% dextran sulfate and 100 ng/ml of the DIG-labeled riboprobe at +55°C overnight. Sections were washed, incubated with the alkaline phosphatase conjugated anti-DIG antibody and the mRNA signal was detected with a chromogen solution containing nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) producing a blue/purple precipitate. Sections were washed overnight, mounted on slides using an aqueous mounting media and analyzed for the trkB.TK+ mRNA expression under light microscopy with an Olympus AX-70 microscope.

### *4.7 Western blotting*

In **publication I**, the expression of trkB protein in hippocampus and cortex was determined by western blotting from both TG and WT mice. In addition, the phosphorylation status of several tyrosine residues on trkB was studied. Samples with 0.5-1 mg of total protein were precipitated with wheat germ agglutinin for 2 hours at +4°C or immunoprecipitated overnight with 2 µg anti-PLC$\gamma$-1 or 4 µg anti-Shc at +4°C. Immunoprecipitates were collected with protein A-sepharose, washed intensively and boiled in sample buffer (2% SDS, 100 mM DTT, 10% glycerol, 0.05% bromophenol blue). The electrophoresis was run on 7.5% SDS-polyacrylamide gels and transferred electrophoretically (60 min, 400 mA) to 0.2 mm nitrocellulose membranes. The successful transfer was confirmed by Ponceau S staining (10-15 sec; 2 % Ponceau S, 30 % tricloroacetic acid and 30 % sulfosalicylic acid). Membranes were blocked and incubated overnight with the appropriate primary antibody. Membrane-bound immunoglobulins were detected with suitable HRP-conjugated secondary antibody and chemiluminesence visualization to X-ray films. For plain western blotting to detect the $trkB_{out}$, Akt/Ⓟ-Akt and MAPK/Ⓟ-MAPK signaling proteins, 60-80 µg of total protein lysate was loaded onto gels and processed further as above. Western blot films were quantified by using a video-based MCID/M4 image analysis software, version 3 (Imaging Research Inc.). Additionally, each blot had 3-6 samples of both genotypes and each experiment was repeated at least 3 times for each antibody with similar results. Individual blots were analyzed by comparing WT/TG samples within the same blot and combined results of all blots presented as % of wild type (mean± SD). Phosphotyrosine blots

(pY490, 4G10) were normalized against $trkB_{out}$ and other phosho-blots against the respective total protein (AKT, MAPK, Shc, PLC$\gamma$).

### *4.8 Immunohistochemistry*

Immunohistochemical stainings were used in **publication II** to study the protein expression of trkB.TK+, NPY, parvalbumin and calbindin. Additionally, a standard thionin staining was performed to specify the cellular structures and boundaries. For each antibody used, one set of sections (every $6^{th}$ cut section) was collected from the storage solution and rinsed overnight. The endogenous peroxidase activity was abolished by treatment in 1% hydrogen peroxidase and a blocking solution containing 10% normal serum reduced the non-specific binding. The primary antiserum contained 0.5% Triton X-100, 1% normal serum in TBS, pH 7.4 with the suitable primary antibody (see table 2). Incubation continued for 60 hours at +4°C with gentle rocking. Biotinylated secondary antibody with the avidin-biotin complex (Vector, A 1:100, B 1:100) and diaminobenzidine precipitation was used to visualize the antibody staining. Sections were washed overnight, mounted onto slides, dried overnight and coverslipped using xylene-based mounting media. Control experiments were performed in parallel with the regular immunohistochemistry protocol by omitting the primary antiserum. This step revealed a possible nonspecific labeling due to the secondary antibody or processing itself. The photomicrographs were taken with Olympus AX-70 microscope connected to ColorView II digital camera using analySIS® program (Soft Imaging System GmbH, Germany). Distribution of the immunoreactivity (ir) was analyzed microscopically under brightfield or darkfield optics. The analysis was performed blindly in regard to the genotype of the animals.

For neuropeptide Y (NPY), the number of immunoreactive neurons in the septal hilus was counted from three sections (150 µm apart) and the first section was chosen at the level 1.6 mm posterior to the bregma (according to the mouse brain atlas of (Franklin, 1997)). The total number of NPY ir-positive cells was counted manually and the area of hilus was determined as described previously (Lahteinen et al., 2002). The hilar NPY-positive cell counts were expressed as mean number of cells per $mm^2$. For Ca-binding proteins calbindin and parvalbumin, the density of immunoreactivity was scored in different regions of the septal hippocampus and neocortex. The density of cells, terminals and neuropil was expressed as +++ = high, ++ = moderate, + = low, - = absent.

**Table 2: Antibodies used for western blotting and immunohistochemistry**

| Antibody | Article | Application | Manufacturer | Dilution | Blocking | Secondary IgG | Detection | **Main measurement** |
|---|---|---|---|---|---|---|---|---|
| AKT | I | WB, IH | CST | 1:1000 | 5 % BSA | Goat anti-rabbit-HRP | CHE | Total Akt protein in HC and cortex |
| Calbindin (D-28K) | II | IH | SWant | 1:5000 | 10 % HS | Rabbit anti-mouse-B | DAB | Distribution of D-28k neurons in HC |
| Neuropeptide Y | II | IH | Incstar | 1:8000 | 10 % NGS | Goat anti-rabbit-B | DAB | The number of NPY neurons in DG |
| P42/44 MAPK | I | WB | Promega | 1:10000 | 5 % BSA | Goat anti-rabbit-HRP | CHE | Total MAPK protein in HC and cortex |
| Parvalbumin | II | IH | SWant | 1:10000 | 10 % HS | Rabbit anti-mouse-B | DAB | Distribution of PV neurons in HC |
| Ⓟ-AKT(Thr308) | I | WB | CST | 1:1000 | 5 % BSA | Goat anti-rabbit-HRP | CHE | Akt activation in HC and cortex |
| Ⓟ-p42/44 MAPK | I | WB | CST | 1:2000 | 5 % NFDM | Rabbit anti-mouse-HRP | CHE | MAPK activation in HC and cortex |
| Ⓟ-trkA (Tyr674/675) | I | WB | CST | | 5 % BSA | | CHE | TrkB activation at Tyr 706/707 |
| Ⓟ-SHC | I | WB | UBI | | | | CHE | Shc activation in HC and cortex |
| Ⓟ-trkA (Tyr490) | I | WB | CST | 1:1000 | 5 % NFDM | Goat anti-rabbit-HRP | CHE | TrkB activation at tyrosine 515 |
| Ⓟ-tyrosine (4G10) | I | WB | CST | 1:10000 | 3 % BSA | Rabbit anti-mouse-HRP | CHE | Tyrosine kinase activation in HC and cortex |
| PLC$\gamma$-1 | I | WB, IH | UBI | 1:1000 | 5 % NFDM | Rabbit anti-mouse-HRP | CHE | Total PLC$\gamma$-1 protein in HC and cortex |
| SHC | I | WB | UBI | 1:1000 | 5 % NFDM | Goat anti-rabbit-HRP | CHE | Total Shc protein in HC and cortex |
| TrkB in | II | IH | D.Kaplan (Univ. of Toronto) | 1:10000 | 10 % NGS | Goat anti-rabbit-B | DAB | Distribution of trkB.TK+ in HC and cortex |
| TrkB out | I | WB | D.Kaplan | 1:5000 | 5 % NFDM | Goat anti-rabbit-HRP | CHE | Total trkB protein in HC and cortex |
| TUC-4 | III | WB | Chemicon | 1:5000 | 10% NGS | Goat anti-rabbit-B | DAB | Newly born neurons in HC |

Abbreviations: B, biotinylated; BSA, bovine serum albumin; CHE, chemiluminesence; CST, Cell Signaling Technolygy; IH, immunohistochemistry; NFDM, non-fat dry milk; HC, hippocampus; HS, horse serum; NGS, normal goat serum; Ⓟ, phospho; PV, parvalbumin; UBI, Upstate Biotechnology; WB, western blo

4.8.1 Histology of kainate-induced acute and chronic effects

In **publication III**, the acute cell death was assessed with Fluoro-Jade B (Histo-Chem Inc.) that is directly binding to degenerating neurons. The damage was scored under fluorescence microscope as follows: 0, no damage; 1, <10% of cells dead; 2, 10-50% cells dead; 3, >50% cells dead. The chronic neuronal loss of hilar neurons was counted 130 days after kainate-induced SE from the cresyl violet-stained sections using a computer-assisted system (NeuroLucida morphometry system) and presented as a mean number of cells per hilus. Additionally, the number of immature neurons in the hilus was determined by TUC-4 immunohistochemisty. The immunoreactive neurons were plotted from three septal sections and the number of newly born neurons was counted in the granule cell layer, hilus and subgranular zone. The mossy fiber sprouting was detected according to a previously reported method of the Timm sulfide/silver protocol (Sloviter, 1982).

### *4.9 Enzyme-linked immunoassay (ELISA)*

In **publication I**, the BDNF levels in hippocampus and cortex of wild type and transgenic mice were determined using enzyme linked immunoassay according to a previously described method (Nawa et al., 1995). Ninety-six-well plates were coated with anti-BDNF antiserum (1: 2000, provided by Dr. H. Nawa, Niigata Univ., Japan) overnight at +4 °C, washed and blocked at room temperature for 4 hours with ELISA buffer (50 mM Tris-HCl, 300 mM NaCl, 0.1% Triton X-100, 1% BSA, 1% gelatine), pH 7.5. Sonicated hippocampal and cortical samples were diluted 3:1 into ELISA buffer and 100 µl of sample was incubated on coated plates overnight at ambient temperature. After washes, biotinylated high-affinity anti-BDNF-antibody (1:1800, provided by Dr. H. Nawa) was added and incubated overnight at ambient temperature. Detection was performed using avidin-β-galactosidase (1:5000) and 4-methylumbelliferyl- β-D-galactosidase (MUG) as substrate and fluorescent product was monitored by fluorometry (Multiwell Fluorometer HTS7000+, Perkin Elmer, Boston, MA) with 360 nm excitation and 440 nm emission wavelengths. BDNF concentrations were obtained from a standard curve, corrected for dilution factor and tissue weight and presented as pg/mg of tissue.

### *4.10 HPLC analysis of indoleamines*

In **publication IV**, HPLC was used to study the neurochemical changes in brain indoleamines expected to take place after antidepressant treatment. Adult mice were injected with either fluoxetine or saline and the animals were sacrificed after 3 hours. Homogenates containing the desired brain regions (hippocampus, cingulate and prefrontal cortices) were centrifuged and samples transferred to an HPLC autosampler (WISP 717, Waters) using a 20 µl injection volume. Separation was achieved on a Beckman C18 column (Ultraspere ODS, 25 X 0.4

cm) using separation buffer (0.1 M citric acid, 0.1 M sodium acetate, 7.5 % sodium octyl sulfate in methanol) and detected with gurad electrode analyzing the indoleamine levels in the processed samples.

### *4.11 Behavioral analysis*

In **publication I**, the wild type (n= 23) and trkB.TK+ (n= 22) mice were subjected to behavioral testing. Testing began at the age of 8 weeks and ended at the age of 14 weeks. The experiments were performed blindly with regard to the genotypes. The exact details of performed tests are published (**publications I and IV**). For this reason the tests are here explained more from a rationale viewpoint. The behavioral characterization was performed in collaboration with Dr. V. Voikar (University of Helsinki, Finland).

#### 4.11.1 Tests for motor functions

**Open field arena** is a well-characterized test for exploratory spontaneous activity. There a mouse is placed into a novel environment and is allowed to freely explore the arena for a short period of time during which the gross evaluation of locomotion and behavioral activity is carried out. In this study, mice were placed into a clear acrylic cage and the horizontal (distance traveled) and vertical (rearings) activity was measured during a 5-minute testing period.

Spontaneous alternation performance was assessed by a symmetrical **Y-maze**. Mice were subjected to the maze for 5 minutes with all three arms open and the number and sequence of the arms entered were recorded. The number of arms entered, number of rearings, and the alternation percentage were calculated.

For the evaluation of coordination and balance, we applied the **rota-rod** test. A mouse was placed into a motor-driven rotating cylinder where it must continuously walk in order to stay on the rod. Animals were tested on two consecutive days with increasing rod velocity (day1: 3 trials, 15 rpm; day2: 3 trials, 20 rpm) and 60 minutes interval between trials. The time to fall off was recorded and cut-off time was set at two minutes.

To evaluate the circadian rhythms and the locomotory behavior a **circadian wheel-running** test was performed. Female mice of both genotypes (12 WT; 12 TG) were individually housed into a cage attached to running wheel recording the hourly running time, speed, and distance. The test was continued for 23 hours a day (data acquisition daily during 10-11 a.m.) for 13 days.

### 4.11.2 Tests for sensory functions

The sensitivity of the paws to hot surface temperature was measured in the **hot-plate test**. A mouse was placed on a heated plate (+ 52°C) and a latency to lick or shake hind paw was measured as an indicator of discomfort.

The other sensory test used was the **von Frey hair-test** (n= 6 for both genotypes)**.** A mouse was confined to metal mesh platform and the von Frey hairs were used to poke the paw from below. At the pain threshold, a mouse flicks its paw away from the hair. The threshold was determined as the minimum hair stimulus to induce pulling out the paw.

### 4.11.3 Tests for emotional behaviors

To study the anxiety-like behavior in WT and TG mice, we used two different behavioral paradigms. **Light-dark exploration** test is an unconditioned test based on the innate tendency in mice to explore the novel environment but to avoid a brightly lit area. A transparent box is divided into two compartments: one brightly illuminated without a lid, and another painted black and covered with lid. An opening allowing a mouse to move between compartments is at the floor level. In the beginning of a 5-minute testing period, the animal was placed in the middle of the bright compartment facing opposite to the opening. The time spent in the lit compartment and the number of transitions between the two regions was measured.

**Elevated plus-maze** is an enhanced version of light-dark exploration combining the previously mentioned conflict of bright and dark areas with high and open runways. Here, at the beginning of a 5-minute test session, mice were placed in the center facing one enclosed arm. The following parameters were scored: latency to the first open arm entry, number of open and closed arm entries and time spent in different parts of the maze (open and closed arms, central platform). An arm entry was defined when a mouse entered an arm with all four legs. The percentage of the open arm entries and time on the open arms were calculated.

In **publication IV**, we used the **Porsolt forced swimming test** (FST) to estimate the depression-related behavior in WT (n= 17) and TG (n= 20) mice. The Porsolt swim task measures behavioral despair in a stressful and inescapable situation such as the water cylinder. In general, mice first swim actively looking for escape but after some time they get “depressed” and start to float. The time of immobility is measured as a depression-index. In this study, the mouse was placed for 6 minutes in the glass cylinder filled with ambient temperature water and the last 4 minutes were scored. Immobility was defined as passive floating, where the animal was motionless or doing only slight movements with tail or one hind limb, whereas activity was judged when the mouse was struggling, climbing or swimming using all four paws. This test was performed on two separate populations of mice and the results for both experiments were combined.

### 4.11.4 Tests for learning and memory

To study the learning and memory of transgenic and wild type mice, three independent tests were performed. **Cued and contextual fear conditioning** measures the ability of an animal to remember and connect aversive experience and environmental cues. Training on day 1 was performed in a constantly illuminated fear-conditioning box where a mouse was exposed to mild footshock paired with an auditory cue. The noise is the conditioning stimulus and the footshock is the unconditioned aversive stimulus. The total time of freezing (no movements except breathing for more than 5 sec) was scored as a behavioral parameter. Contextually conditioned fear was tested 24 and 48 hours after training in the fear-conditioning box without tone stimulation. The second phase, testing of the memory for the conditioning stimulus (tone) was performed 2 hours after first contextual memory test in a novel context. After 2 minutes of free exploration in the novel context the tone was applied and freezing was measured again.

The **Morris water maze** is a widely used and well characterized paradigm for measuring the learning and memory capabilities in rodents. It is a navigational task where an animal is placed into a pool and it has to swim to find a hidden platform using the cues provided by the environment. The animals were released to swim in random positions facing the wall and the time to reach the platform was measured. Training consisted of 18 trials (two training sessions of three trials, separated by a 3-minute interval between trials and 5 hours between training sessions) during which the platform remained in constant location. After three days (6 sessions), the platform was moved to the opposite quadrant for 2 days (4 sessions). The probe tests (duration 60 sec) with the platform removed were conducted 18 hours after training sessions 6 and 10, respectively. Swimming paths of the probe tests were recorded and analyzed by the water maze software (Columbus Instruments). The spatial memory was estimated by two parameters: 1) the number of annulus crossings at the target position and the corresponding locations in the left, right and opposite quadrants; 2) the time spent in the zone around the platform (covering 6.25% of the total maze area) and in corresponding zones of the three remaining quadrants. In addition, the swimming distances and the thigmotaxis were measured. Thigmotaxis was defined as time spent swimming within the outermost ring of the water maze covering 30.6% of the water maze area. After completing the spatial version of the water maze the platform was made visible in the quadrant not previously employed. The mice were tested in one session of three trials and the time to reach the platform was measured.

Finally, as a third learning and memory paradigm we used **conditioned taste aversion**. This test pairs a pleasant taste with an injection of noxious agent such as lithium chloride. In this study, the mice were adapted to a specific drinking schedule (two 20- minute drinking sessions daily for two days) by presenting two bottles filled with tap water and the amount of liquid consumed was measured. On day 3, one bottle with saccharin solution (0.5%) was presented for 20 minutes. One hour after drinking the sweet solution, the mice were injected

with lithium chloride. The choice test was performed 48 hours after conditioning when bottles of both saccharin solution and tap water were available for 25 minutes. The aversion index was calculated as a percentage of consumed saccharin solution of the total fluid intake (saccharin + water). Suppression of saccharin drinking was considered as a measure of associative learning.

### *4.12 Electrophysiology*

For electrophysiological experiments in **publication I**, acute hippocampal slices were prepared from young adult mice. After 90 min recovery period, the measurements were carried out in a recording chamber with constant perfusion of artificial cerebrospinal fluid always kept at +32 °C and oxygenated (5% $CO_2$/95% $O_2$). Field excitatory postsynaptic potentials (fEPSP), evoked by stimulation of the Schaffer collateral afferents, were recorded in the CA1 *stratum radiatum*. Extracellular recording microelectrodes (2-10 MΩ) were filled with 150 mM NaCl. Baseline stimulation frequency was 0.05 Hz and pulse duration 0.1 ms. Stimulus intensity was adjusted to evoke half-maximal fEPSP amplitude. Input-output data obtained at different stimulus strengths was collected 15 min before and 2 hr after LTP induction. Paired pulse facilitation was examined 15 min before and 2 hr after LTP induction by stimulation at 20-, 40-, 60-, 80-, 100-, 150- and 200-ms interpulse intervals. LTP was induced with a 100 Hz tetanic stimulation for 1 second during which the pulse length was doubled. The slope of fEPSP was used as an indicator of synaptic efficacy and was calculated between 20% and 80% of the maximal amplitude. The level of LTP was measured as a percent (%) increase of the fEPSP slope, averaged at a 5-min interval 130 minutes after the tetanus and compared to the averaged baseline fEPSP slope. The LTP program (www.ltp-program.com) (Anderson and Collingridge, 2001) was used for data acquisition and analysis. Electrophysiological experiments were performed in collaboration with Dr. R. Riekki (University of Helsinki, Finland).

### *4.13 Statistical analysis*

The behavioral data (**publication I**) are presented as mean ± SEM. The data were analyzed by one-way or repeated measures ANOVA with genotype as an independent variable together with Newman-Keuls as a *post hoc* test when appropriate. The data on western blots (**publication I**) are presented as mean ± SD and were analyzed by one-way ANOVA together with Bonferroni as a *post hoc* test. The mRNA expression analysis in **publication II** and the electrophysiological data are presented as mean ± SEM and the statistics are calculated using t-test.

# 5 RESULTS

## 5.1 Phenotype of mice overexpressing the full-length trkB receptor

Transgenic mice overexpressing the full-length trkB receptor (trkB.TK+) were first subjected to certain preliminary observations on their general abilities and health by comparing their performance on the wild type littermates. Heterozygous transgenic mice used throughout this study possessed normally long lifespan, were viable and fertile. They were indistinguishable from the wild type littermates by general appearance; there were no visible structural malformations, their fur was in standard shape, they had whiskers and apparently normal hearing and vision (unpublished observations, E.K.). Also, the feeding behavior resembled the wild type mice because the litters developed equally and there was no difference in the weight of the adult mice (unpublished observation, E.K, S.L.). The social activities observed in the home cage environment revealed regular grooming and nesting behavior and no abnormal aggressive conduct was observed among the male mice groups. The sense of touch was assessed similar in both genotypes with von Frey hairs (unpublished observation, E.K., T.S.) and the nociceptive functions were shown identical to WT littermates in the hot plate test (**publication I**, table 1).

The motor coordination assessed by rota-rod showed identical latencies to fall (**publication I**, table 1) and the righting reflexes were identical for both genotypes (unpublished observation, E.K.). Similar locomotory functions were confirmed in a voluntary wheel running test that also showed no alterations in daily activity periods between genotypes (E.K., unpublished information). Testing the general exploratory, spontaneous activity in the open field and Y-maze tests established no difference between the genotypes (**publication I**, table 1).

The histological analysis by thionin staining did not reveal any major changes in lamination and morphology of brain (unpublished observation, E.K.). Finally, an immunohistochemical study of the calcium binding proteins parvalbumin and calbindin revealed similar distribution and intensity of immunoreactivity for these proteins in hippocampus and cortex of the wild type and trkB.TK+ mice (unpublished observation E.K.). Taken together, the gross structure of the CNS and the basic behavioral parameters were not affected by trkB.TK+ overexpression.

## 5.2 Expression of the transgene

To address the question of transgene expression during fetal and early postnatal life of trkB.TK+ overexpressing mice, we carried out Northern blot studies using a probe complementary to trkB extracellular domains. The transgenic overexpression was determined against the endogenous trkB.TK+ expression at each time point studied. The first sign of

transgene mRNA was observed at postnatal day 10 after which the expression increased gradually and reached the maximum level by postnatal day 20 and remained stable thereafter (**publication I**, figure 1b). The distribution of the FLAG-tagged transgene in adult TG mice was assessed with *in situ* hybridization using FLAG- specific oligoprobe. Strong transgene expression was observed in the hippocampal pyramidal neurons, dentate granule cells and on cerebral cortex, particularly in layers II, III, V and VI (**publication I**/figure 1c, **publication III**/figure 1a). Likewise, thalamus, amygdala and cerebellum showed clear, although a slightly lower intensity of trkB.TK+ transgene expression but in striatum no transgenic trkB.TK+ was present. As expected, the wild type sections showed no hybridization signal (**publication I**/figure 1c). In all brain areas expressing the transgenic trkB.TK+ the expression appeared to be restricted to neuronal cells (unpublished observation, E.K.). Transgenic trkB expression under Thy-1 promoter was sustained for at least one year at the levels comparable to young adulthood. In summary, the Thy-1 directed transgene expression initiated postnatally and was strongest in the cerebral cortex and hippocampus

Although the role of increased trkB.TK+ in the peripheral nervous system was not the main target in the current work, we have addressed it briefly. Samples of peripheral ganglia (superior cervical ganglia;SCG, nodose ganglia;ND, dorsal root ganglia;DRG) were dissected, their RNA isolated and transcribed to cDNA with RT-PCR. Finally samples were subjected to conventional PCR with transgene-specific primers (see 4.3). Results showed mild expression in the nodose and dorsal root ganglia whereas SCG had no transgene expression (unpublished observation E.K., T.S.). Additionally, by *in situ* hybridization with FLAG-oligoprobe on the DRG and spinal cord, we found neuronal low abundance transgene expression (unpublished observation E.K., T.S., S.B.). Altogether these preliminary observations confirmed to us that the role of trkB.TK+ overexpression is most relevant to address in the adult central neurons.

### *5.3 Expression and localization of the full-length trkB mRNA*

To analyze whether the transgene expression had altered the trkB mRNA distribution, we performed a non-radioactive *in situ* hybridization using a probe specifically recognizing the trkB kinase domain. The endogenous trkB.TK+ signal in the WT mice was compared with the hybridization results of the TG mice. In the WT mice, the endogenous trkB.TK+ mRNA was expressed in the layers II-VI in all subdivisions of the neocortex (**publication II**/figure 1). In the hippocampal formation, the trkB.TK+ mRNA expression was expressed in the cell bodies of the pyramidal CA1- CA3 field neurons and in the dentate granule cells. In the TG mice, the full-length trkB receptor mRNA expression was clearly increased in all cortical layers but most prominently in layer V cell bodies. Additionally, strongly elevated expression was observed in hippocampal CA1-CA3 and dentate granule cell bodies and amygdala (**publication II**/figure 1). In other brain regions, the mRNA expression was equally strong and similarly localized between

genotypes. The distribution of the endogenous trkB.TK+ in WT mice was comparable to trkB localization previously published in rat. Taken together, the increased trkB.TK+ expression did not redistribute the mRNA signal into novel areas in hippocampus and cortex. Furthermore, the locations of increased trkB.TK+ transcript in TG mice corresponded to areas expressing the flag-tagged transgene construct (see above).

## *5.4 Expression of the full-length trkB protein*

### 5.4.1 Distribution of the trkB.TK+ immunoreactivity

To analyze the trkB protein distribution within the hippocampus of the wild type and trkB.TK+ overexpressing mice, we performed an immunohistochemical analysis using an antibody specifically recognizing the intracellular domain of the full length trkB receptor (trkB*in*). Using this antibody the truncated trkB receptor protein was not detected and did not interfere with the analysis. In comparison to WT littermates, the TG mice displayed increased immunoreactivity in cortical regions, most highly in layer V pyramidal somata and apical dendrites (**publication II**/figure 2). In the TG hippocampus, the full-length trkB immunoreactivity was mostly increased in the dentate gyrus. Altogether, the trkB protein in TG mice was expressed essentially as the endogenous trkB protein, but with elevated levels.

### 5.4.2 Amount of the trkB.TK+ receptor protein

To assess the levels of trkB.TK+ protein in WT and TG mice, biochemical analysis was performed on cortical and hippocampal tissue extracts using trkB specific antibodies (**publication I**/figure 2, for antibodies see table 2). Western blot results showed dramatically elevated levels of the full-length trkB receptor protein (gp145TrkB) in TG mice on both hippocampus and cortex when compared to WT littermates. Levels of the truncated trkB receptor protein (gp95TrkB) were unaltered between genotypes. Also, no change in trkB receptor maturation was observed in transgenic mice (unpublished observation, E.K.).

The basal activation status of the trkB.TK+ receptor was further characterized using phosphotyrosine-specific antibodies (**publication I**/figures 2-4). In TG mice, we observed markedly increased trkB phosphotyrosine levels in both hippocampus and cortex by two different phospho-specific tyrosine kinase/trk antibodies (4G10 and Y490, see table 2 in experimental procedures) thus confirming that transgenic trkB.TK+ receptor was phosphorylated *in vivo*. Also, the trk autophosphorylation site, Y706/707 in trkB, showed increased tyrosine phosphorylation in TG mice (unpublished observation, E.K.). Increased levels of trkB receptor protein and phosphorylation were sustained up to at least 18 months; further ages were not studied (unpublished observation, E.K.). In conclusion, in TG mice, the total trkB protein and trkB phosphorylation are increased in comparison to WT littermates.

### 5.4.3 TrkB.TK+ downstream signaling

As the level of activated trkB receptors was increased in TG mice (**publication I**/figure 2), we further analyzed the three main downstream signaling pathways initiating from trk receptor. The Shc/MAPK pathway showed no changes in the total protein expression or phosphorylation in either cortex or hippocampus in TG mice (publication I/figure 3a,b). Next, the PI3K/Akt pathway was examined by studying the levels of Ⓟ-Akt and Akt proteins. In contrast to Shc/MAPK pathway, here we observed a 25 % reduction in the total Akt protein whereas the expression of Ⓟ-Akt was unaltered in TG mice (**publication I**/figure 3c). Moreover, the reduction in Akt total protein on hippocampus and cortex was confirmed by immunohistochemical staining (unpublished observation, E.K.). Finally, the PLCγ expression was dramatically regulated by trkB.TK+ overexpression. The total PLCγ protein was markedly reduced in TG mice but the phosphorylation of PLCγ was still induced over twofold above the WT levels (**publication I**/figure 4). Again, the reduction of total PLCγ protein was immunohistochemically verified (unpublished observation, E.K.). In summary, the increased trkB.TK+ expression regulated the PLCγ and Akt downstream pathways, but not the Shc/MAPK pathway.

## *5.5 BDNF expression in transgenic mice*

To establish whether trkB.TK+ receptor overexpression had affected the expression of the ligand BDNF, the mRNA and protein amounts were determined by *in situ* hybridization and ELISA assay. There was no difference in BDNF mRNA amount in hippocampus (**publication I**/figure 5) or other brain regions (unpublished observation, E.K.). Similarly, the BDNF protein expression was not significantly regulated by transgene expression when assessed using ELISA assay (**publication I**/figure 5).

## *5.6 Effect of increased trkB.TK+ signaling on plasticity-related molecules*

### 5.6.1 mRNA expression of inducible transcription factors

Immediate early genes are robustly and rapidly induced upon various neuronal stimuli. In order to determine if the increased trkB.TK+ signaling had affected the mRNA expression of inducible transcription factors of the *fos* (*c-fos*, *fosB*, *fra-1*, and *fra-2*) and *jun* (*c-jun*, *junB*) families was studied by in cortex and hippocampus. The expression of *c-fos* was upregulated by trkB overexpression whereas the expression of *fra-2* was downregulated in the same areas (**publication II**/figure 3, table 1). However, the *fosB* and *fra-1* mRNA expressions were unaltered in all regions analyzed (**publication II**/table 1).

The expression of *c-Jun* and *junB* mRNAs was unaltered in cortex whereas in the hippocampal CA1 field *junB* expression was markedly induced in TG mice (**publication II**/figure

3, table 1). Finally, in the striatum a trend of reduced *junB* expression was observed (**publication II**/figure 3).

### 5.6.2 mRNA expression of α-CaMKII, GAP-43 and CREB

The trkB/BDNF downstream signaling coincides with a number of molecules implicated in molecular plasticity. Here we analyzed the effects of increased trkB.TK+ signaling on the expression of α-CaMKII, GAP-43 and CREB mRNAs. In the trkB.TK+ overexpressing mice, the α-CaMKII mRNA expression was reduced in hippocampal and cortical subfields (**publication II**/figure 4b). In addition, α-CaMKII expression was reduced in the TG piriform cortex, amygdaloid nuclei and thalamus. Moreover, the expression of growth –associated protein GAP-43 mRNA was significantly induced in the transgenic mice cortex (**publication II**/figure 4d). Additionally, hippocampal CA1 and CA3 fields, thalamus and amygdaloid complex expressed elevated levels of GAP-43 mRNA (**publication II**/fig 4d). In the dentate granule cells of both genotypes, the GAP-43 mRNA was undetectable. Surprisingly, the transcription factor cAMP response element binding protein (CREB) was not affected by the increased level of trkB.TK+ receptor (**publication II**/figure 4c). Instead, some transgenic thalamic regions show increased CREB mRNA signal (**publication II**/figure 4c).

### 5.6.3 NPY protein

Neuropeptide Y (NPY) is a peptide neurotransmitter suggested to play a role also in memory processing. The number on NPY immunoreactive neurons was determined in the hilus of the wild type and trkB.TK+ overexpressing mice. The density of NPY immunoreactive cells in septal hilus was increased in trkB.TK+ mice compared with the WT mice (**publication II**/figure 5a). The density of NPY immunoreactive fibers in the dentate molecular layer was similar in wild type and trkB.TK+ mice (**publication II**/figure 5b).

### 5.6.4 Brain monoamine proteins

As the behavioral testing revealed reduced depression-related behavior in TG mice, we determined the neurochemical changes of brain monoamines within hippocampus and two cortical areas. The basal concentrations of 5-hydroxytryptamine (5-HT) and norepinephrine (NA) were increased in TG mice whereas the levels of tryptophan were significantly reduced in the TG hippocampus in comparison to WT littermates (**publication IV**/ table 1). A similar trend of reduced tryptophan amount was observed in TG cortex. The amount of 5-HIIA, a serotonin metabolite, was instead similar in both genotypes. Fluoxetine injection caused an increase in serotonin concentrations in every region examined on both genotypes; however, the changes were subtler in the transgenic mice. In summary, the increased trkB.TK+ signaling results

neurochemically in a situation mimicking the antidepressant treatment by increasing hippocampal NA and 5-HT concentrations.

## *5.7 Effect of increased trkB.TK+ signaling on behavioral parameters*

As the trkB/BDNF signaling system is suggested to modulate a wide variety of behavioral parameters in rodents, we address this issue in trkB.TK+ mice by performing a battery of behavioral tests.

### 5.7.1 Learning and memory

The Morris water maze is a widely recognized paradigm to evaluate learning and memory in rodents. The task requires spatial navigation to find the hidden platform using visual cues and the latency to find the platform is criteria for successful learning. The initial latency to locate the hidden platform was similar in both WT and TG mice and importantly, both groups learned to find the platform (**publication I**/figure 6a). The first probe test confirmed better spatial navigation strategy in TG mice (**publication I**/figure 6b), because they crossed the platform location more often and swam in the near vicinity of the platform longer than the WT mice. During the reversal learning, the initial escape latencies increased similarly in both genotypes but again the TG mice learned the new task significantly faster than WT mice (**publication I**/figure 6a,c). A second probe test did not reveal any difference in the preference to the new target zone. During both probe tests, the swimming distances were similar between genotypes (**publication I**/figure 6d). Finally, the TG mice were significantly less thigmotactic during the first probe test (**publication I**/figure 6e).

In the fear conditioning task, both groups displayed similar levels of freezing in the pre-conditioning phase. However, when returned into the conditioning context, the TG mice exhibited significantly higher amount of freezing 48 hours after training than WT littermates (**publication I**/figure 7a). The freezing in the conditioning context 24 hours after training and in the presence of conditioned stimulus in novel context did not differ between the groups (**publication I**/figure 7a).

The last learning and memory test in the battery was the taste aversion conditioning. During training, there was no difference in the drinking pattern between the groups. However, the trkB.TK+ mice appeared to display significantly stronger taste aversion by suppressing their saccharin drinking pattern to 7.2 ± 2.5 % of total fluid intake whereas the WT mice still drank saccharin solution 20.3 ± 4.0 % of the total fluid consumed (**publication I**/figure 7b). The results suggest that TG mice learned the saccharin-lithium association more quickly that WT mice.

### 5.7.2 Anxiety-like behavior

Behavioral characterization further established reduced anxiety-like behavior in trkB.TK+ mice (**publication I**/table 1). In the elevated plus maze, trkB.TK+ mice entered open arm significantly faster, made more entries into the open arms of the maze and stayed there for longer time than WT mice. Importantly, the locomotor activity in this test was similar for both genotypes. Furthermore, in the light-dark exploration test the TG mice made significantly more transitions between two compartments than WT mice although the time spent in the lit compartment did not differ.

### 5.7.3 Depression-related behavior

Finally, to assess possible depression-related behavior, WT and TG mice were subjected to the Porsolt forced swimming test. During the last four minutes of testing, trkB.TK+ mice displayed significantly reduced immobility as compared to WT mice (37.8 ± 4.3 vs. 59.6 ± 3.6 % respectively, $F_{1,35}=14.2$, $p<0.01$) (**publication IV**/ figure 1), thus suggesting reduced depression-related behavior.

In conclusion of the behavioral studies, the mice with increased trkB.TK+ signaling exhibit enhanced learning and memory in three separate learning paradigms, reduced anxiety-like behavior in the elevated plus maze and diminished depression-related behavior in the swim test.

## *5.8 Effect of transgene on electrophysiological properties*

As a cellular model of learning and memory, we studied the LTP in Schaffer collateral-CA1 synapses. The basal synaptic transmission and responsiveness analyzed by the input-output curve in transgenic mice was indistinguishable from that in WT littermates both before and 2 hours after the LTP induction (**publication I**/figure 8a). A form of short-term plasticity, paired pulse facilitation (PPF) was similar in both TG and WT mice both before and after the LTP induction (**publication I**/figure 8b). Despite these, HFS induced significantly lower level of LTP in trkB.TK+ mice and the attenuation of LTP during experiment was more pronounced in transgenic mice (**publication I**/figure 8c). Finally, the synaptic responses to HFS were similar in both genotypes (**publication I**/figure 8d). Taken together, the basal synaptic transmission in trkB.TK+ mice is intact; however the HFS-induced LTP appears attenuated.

## *5.9 Increased trkB.TK+ signaling in epileptogenesis*

A systemic kainic acid administration was used to study the role of enhanced BDNF signaling during SE, epileptogenesis and epilepsy. Determination of seizure threshold revealed

that in comparison to WT mice, trkB.TK+ mice showed no dose-dependent response to increasing kainate concentrations but their response appeared saturated already by the lowest dose used in this study (20 mg/kg). This observation could point towards lowered seizure threshold in trkB.TK+ mice. In addition, the TG mice showed increased number and severity of seizures and prolonged duration of SE. The acute neuronal damage 48 h after the initial insult was increased in trkB.TK+ mice hippocampus and amygdala although the earlier 24 h-timepoint showed similar cell death in both genotypes (**publication III**/table 2, figure 2). Altogether, the increased BDNF signaling aggravates the responses to sudden hyperexcitation and exacerbates the consequent cellular damage in the acute phase.

The long-term effects of increased BDNF signaling to the development of epilepsy were assessed 4.5 months after SE. In contrast to the acute effects, both genotypes showed similar cell death in the hilar neurons (**publication III**/table 3). Interestingly, the counting revealed increased septal hilar neuron number in saline-treated TG mice in comparison to WT mice. Additionally, even if both genotypes showed increased hilar neurogenesis in response to kainate, no differences were observed between genotypes in either saline or kainate injected groups (**publication III**/table 3, figure 3). In addition, increased mossy fiber sprouting in response to kainate was clearly seen in WT mice, whereas sprouting in trkB.TK+ mice was only modestly increased; however there was no significant difference between genotypes. Finally, the epileptic activity during the combined video-EEG monitoring was similar in both genotypes (**publication III**/table 4).

# 6 DISCUSSION

## *6.1 Methodological considerations*

### 6.1.1 The use of hybrid mice strain in behavioral analysis

Significant variability in behavioral scores exists between different mouse strains (Crawley, 2000; Crawley et al., 1997). Most reliable results are achieved if the chosen breeder strain is an average performer on the behavioral tests of interest. In this study, the $CD_2F_1$ mouse hybrid (BALB/c x DBA/2) was selected as a breeding strain for the transgenic trkB.TK+ overexpressing mice. At the time of trkB.TK+ mice generation, the hybrid strain was easily available and it had a good reputation as a breeder. Furthermore, choosing a hybrid line breeder was considered a safe choice as the exact plan for the biochemical and behavioral testing was not available at the time. Judging afterwards, the choice was relatively good. Although using hybrid strain complicated the genetic background, it was overcome by using wild type littermates as the control group in all biochemical and behavioral testing. This was accomplished by always breeding heterozygous transgenic males with wild type $CD_2F_1$ females. Furthermore, as both parental lines had impairments in their performance on specific tests, the behavioral analysis probably profited for the use of hybrid line. The DBA/2 inbred strain for example, is a poor performer in both water maze and contextual fear conditioning paradigms of learning whereas BALB/c performance is much better in those tests (Crawley et al., 1997; Owen et al., 1997). Similarly, BALB/c is resistant to kainate-induced cell death whereas DBA/2 is more vulnerable (Ferraro et al., 1995; Royle et al., 1999). Altogether, the role of background genes should be carefully evaluated before choosing the parental strain keeping in mind the testing paradigms of interest. For help, extensive literature exists on the differences observed between inbred mouse strains in various behavioral paradigms (see Crawley, 2000 and references therein).

### 6.1.2 The use of Thy1.2 promoter and FLAG-tag in the construct

The pan-neuronal Thy1.2 promoter used in the current study, directs the trkB.TK+ transgene expression into postnatal neurons with highest expression in the cerebral cortex, hippocampus, amygdala and cerebellum. Indeed, similar Thy1.2-driven transgene expression has been described in previous reports (Caroni, 1997; Luthi et al., 1997; Saarelainen et al., 2001; Wisden et al., 2002). In this study, at least two specific advantages were gained by the use of the Thy1.2 promoter. First, the increased trkB.TK+ expression is restricted into neuronal cells. The full-length trkB receptor is predominantly expressed in neurons, therefore by using Thy1.2 promoter, we avoided the confounding glial trkB expression. Instead of trkB.TK+, the truncated trkB might have its own specific signaling role in glial cells as recently reported (Rose et al.,

2003). In this study, we did not specifically address the possibility of non-neuronal Thy1.2-driven expression; however, it has not been previously observed (Caroni, 1997). Second, increased trkB.TK+ expression is initiated only during the second postnatal week. Even if trkB augmentation during development is not expected to be as deleterious as the lack of trkB, the possibility of disturbing the neuronal development is excluded by using the Thy1.2-driven overexpression. In support of the previous, we did not observe any structural malformations in the nervous system of trkB.TK+ mice.

As a part of our transgene construct we included a tagging molecule, the FLAG-octapeptide, between the signal peptide and trkB N-terminus sequences for specific follow-up of the transgene expression. Indeed, *in situ* hybridization with the FLAG-specific oligoprobe revealed the transgenic mRNA distribution clearly. However, the protein localization caused difficulties. Despite extensive trials and methodological modifications, we were not able to see any FLAG-specific signal in transgenic immunohistochemical stainings due to an unknown reason. As a result, the protein expression analysis in trkB.TK+ mice became more complicated and needed a constant comparison with the wt littermates. Besides FLAG-tag, other possible transgene tagging molecules would have been myc, lacZ, or fluorescent markers like GFP. The advantage especially in case of GFP would be the detection without any histology.

### *6.2 BDNF/trkB system in genetically modified mice*

Despite the above mentioned difficulties, we were able to conclude that the trkB.TK+ protein was increased most in the hippocampal dentate gyrus and cortical layer V pyramidal neurons in transgenic mice. However, the possibility exists that the more subtle and specified alterations in the trkB.TK+ protein expression might have escaped our notice. In agreement, however, both these regions displayed high expression of the FLAG-tagged mRNA by *in situ* hybridization. The protein distribution of the full-length trkB has been less addressed in the literature (Dolci et al., 2003; Drake et al., 1999) although the total trkB receptor protein in nervous system is widely described (Cabelli et al., 1996; Fryer et al., 1996; Wu et al., 1996). Localization data on the EM-level provides evidence for differential localization of the trkB isoforms in rat hippocampus: the full-length trkB is localized mostly into axons, terminals and dendritic spines whereas the truncated trkB is localized mainly in somata and dendritic shafts (Drake et al., 1999).

Several BDNF overexpressing mice are reported to date, however to our knowledge only mice mutants overexpressing trkB receptors are the currently discussed trkB.TK+ mice and trkB.T1 mice we previously generated (Saarelainen et al., 2003; Saarelainen et al., 2000a; Saarelainen et al., 2000b; Saarelainen et al., 2001). The BDNF approaches have used either a more general β-actin promoter (Croll et al., 1999; Qiao et al., 2001) or a region-specific αCaMKII

and Keratin K14 promoters for transgene expression (Huang et al., 1999a; LeMaster et al., 1999). The β-actin driven overexpression leads to an average of 30 % increase in BDNF protein both in brain and to various peripheral organs, which is however decreased by the age of nine months (Croll et al., 1999). Accordingly, the increased general activity and a deficit in the passive avoidance observed in BDNF mutants are diminished when BDNF levels go down. The forebrain-specific αCaMKII promoter activates BDNF transgene expression in postnatal neurons and reaches the maximum by 5 weeks (Huang et al., 1999a). Interestingly, the BDNF overexpression has no effect on the trkB receptor levels (Huang et al., 1999a). In accordance, our study observed no regulation of BDNF levels by constitutively increased receptor expression. However, the importance of properly working BDNF/trkB signaling during development and neonatal life is clearly demonstrated by the lethality of trkB (Alcantara et al., 1997; Klein et al., 1993; Minichiello and Klein, 1996) or BDNF null mutants (Ernfors et al., 1994; Jones et al., 1994). Since then, the genetic techniques have evolved rapidly, so the early lethality can be avoided, and the role of BDNF/trkB in adult nervous system can be addressed. Indeed, targeted trkB mutations in the cytoplasmic adaptor binding sites have clarified the role of individual trkB downstream signaling pathways in survival, synaptic plasticity and epileptogenesis (He et al., 2002; Minichiello et al., 2002; Minichiello et al., 1998). Moreover, conditional mutations (Baquet et al., 2004; Gorski et al., 2003a; Gorski et al., 2003b; Ming et al., 1999; Rios et al., 2001; Xu et al., 2000a; Xu et al., 2000b) or gene replacement studies (Agerman et al., 2003; Fan et al., 2000) have addressed the role of BDNF/trkB system in functional and structural maintenance of neural networks.

## *6.3 The physiological effects of increased trkB signaling*

### 6.3.1 TrkB.TK+ overexpression selectively activates downstream signaling

According to our results, a relatively modest trkB.TK+ mRNA overexpression achieved results in significant enhancement of trkB protein expression and individual downstream signaling cascades. More specifically, in TG mice we observed strong phosphorylation of the Y515 docking site responsible of binding the Shc adaptor protein, but curiously, neither Shc itself nor the downstream MAP-ERK signaling partners were regulated on the protein level. Quite recently, it was suggested that Shc-site activation is required for full trkB activation and association of PLCγ1 with trkB (Minichiello et al., 1998; Postigo et al., 2002). In the current study, we have observed increased Y515 phosphorylation in combination with increased levels of phosphorylated tyrosines Y706/707 of the trkB activation loop and increased PLCγ1 activation thus suggesting full receptor activation in trkB.TK+ mice. Nevertheless, the lack of regulation on MAPK pathway is slightly surprising since it has important regulatory functions in many activity-dependent neuronal events (Grewal et al., 1999; Segal, 2003). Then again, the Shc-site dependent trkB

signaling is not apparently required for some forms of neuronal plasticity (Korte et al., 2000). Interestingly, the Shc-site was suggested to be more important for NT4-derived signaling than for BDNF (Minichiello et al., 1998). In the current study, the role of NT4 in trkB.TK+ overexpressing mice was not addressed.

In contrast to unregulated MAPK pathway, we observed increased activity of the PI3K-Akt signaling pathway in TG mice. Although the phosphorylated Akt was expressed at similar levels in both genotypes, in TG mice the total Akt protein amount was 25 % lower. One feasible explanation would be a compensatory downregulation of total Akt protein for keeping the active Akt within normal range. But, as the major PI3K/Akt pathway initiates from the Y515 and is downstream of Shc, which was not regulated by trkB.TK+ overexpression (see above), another explanation for Akt regulation is required. However, there appears to be alternative routes to PI3K regulation that bypass the Shc/Ras pathway. First, phosphorylated tyrosines in the trk activation loop can directly employ the adaptors rAPS and SH2-B (Qian and Ginty, 2001; Qian et al., 1998). Upon activation, the recruited adaptors are reported to associate with Grb2/SOS/Ras signaling mediators and eventually with PI3K. Second, in cortical neurons the insulin receptor substrate (IRS)-1 is tyrosine phosphorylated in response to BDNF and in turn activates PI3K (Yamada et al., 1997). Finally, overexpression of Src-homology phosphatase (Shp2) in cortical neurons is reported to increase the BDNF-dependent PI3K activation (Takai et al., 2002). Whatever role these mechanisms may play in our transgenic model, if any, needs further assessment before more definite conclusions.

Activation of PLC$\gamma$ is a direct consequence of trk activation since the enzyme binds directly to the phosphotyrosine residue of trk kinase. Indeed, we observed markedly increased PLC$\gamma$ activation in trkB.TK+ mice. Since the total PLC$\gamma$ protein was significantly reduced, systemic compensatory actions are probably occurring here also; however, the buffering capacity is overloaded as the PLC$\gamma$-1 activation is persistently increased. The issue of continuously increased receptor phosphorylation is intriguing, since evidence suggests that a rapid pulse of ligand is the most effective stimulus for receptor activation. What would be the possible mechanisms used for a longterm increase in receptor phosphorylation? Obviously, one reason could be the mere number of receptors on the membrane. An increased number of receptors in TG mice would result in increased phosphorylation as we observe it on the western blot. On the other hand, in culture conditions short NGF pulses on PC12 cells induce sustained PLC$\gamma$ phosphorylation but only transient Shc phosphorylation, thus suggesting a role for PLC$\gamma$ pathway in long-term receptor activation (Choi et al., 2001). Recently, a novel trk-interacting protein ARMS was proposed to have a role in sustained NT signaling via MAP kinase (Arevalo et al., 2004). Additionally, the role of specific tyrosine phosphatases in controlling the trkB receptor downstream signaling remains unexplored. The tyrosine phosphatase Shp2, for

example, is reported to exhibit receptor-specific actions in regulation of PI3K activity (Zhang et al., 2002). Yet, one unexplored possibility is the ligand-independent receptor activation. If the TK+ receptors on plasma membrane are locally concentrated, the dimerization and *trans*-phosphorylation could happen ligand-independently. In conclusion, *in vivo* overexpression of full-length trkB receptor produces higher levels of activated receptors and selectively regulates the downstream signaling pathways.

### 6.3.2 TrkB.TK+ overexpression in molecular plasticity

BDNF signaling is a prerequisite for induction and maintenance of hippocampal LTP. Indeed, intrahippocampal application of BDNF induces slowly a long-lasting synaptic strengthening whereas BDNF application paired with a weak electric pulse rapidly elicits LTP (Kovalchuk et al., 2002; Messaoudi et al., 2002). In addition, LTP induction increases both BDNF release and trkB activation (Gooney and Lynch, 2001). Conversely, reduction in trkB receptor protein impairs LTP (Minichiello et al., 1999; Xu et al., 2000a). Recently, signaling via trkB-PLC$\gamma$1 pathway, but not via Shc-Ras-ERK pathway, was demonstrated to be crucial for hippocampal synaptic plasticity (Minichiello et al., 2002). Rather surprisingly, in the current study the trkB.TK+ mice where the PLC$\gamma$1 activation is increased, we observe attenuated hippocampal LTP. One possible explanation for the apparent controversy could be the LTP-occlusion. Indeed, if the synaptic activity is already enhanced, it cannot be further potentiated by normal LTP-inducing stimuli (Bliss et al., 2003). Then again, the addition of BDNF is not necessarily beneficial in terms of LTP. Although re-expression of BDNF rescues the LTP phenotype in BDNF knockout mice, the addition has no LTP-enhancing effect in wild type mice (Korte et al., 1996; Kramar et al., 2004; Patterson et al., 1996). In fact, the virus-mediated BDNF gene transfer in wild type slices actually reduces the potentiation in comparison to untreated ones (Korte et al., 1996). Additionally, the BDNF overexpressing mice display inconsistent LTP induction in response to tetanic stimulation, possibly due to an interfering effect of hippocampal hyperexcitability observed during stimulation (Croll et al., 1999). Although we did not observe regulated BDNF protein levels in hippocampal homogenates, the possibility of regional alterations in BDNF concentration and whatever contribution it may have on CA1-LTP, cannot be excluded.

Besides BDNF, genetic disruption of genes such as CaMKII, CREB, calcium-binding proteins calbindin and calretinin, GAP-43 or NMDA receptor subunits NR2A and NR1, all disrupt LTP induction in CA3 $\rightarrow$ CA1 synapse (Bourtchuladze et al., 1994; Molinari et al., 1996; Routtenberg et al., 2000; Sakimura et al., 1995; Schurmans et al., 1997; Silva et al., 1992; Tsien et al., 1996). In the current study, we observed no changes in either gene expression of CREB or protein expression of calcium-binding proteins parvalbumin and calbindin in TG mice hippocampus where attenuated LTP was observed. Furthermore, gene expression of the NMDA receptor

subunits NR1, NR2A and NR2B was not regulated whereas the α-CaMKII mRNA was about 15 % reduced in TG mice. From the literature, αCaMKII and CREB are both fundamentally important for the plastic changes occurring during LTP and learning (Silva, 2003; Silva et al., 1998; Soderling, 2000). *In vitro* evidence that links trkB-PLCγ signaling to activation of CaMKIV, αCaMKII and CREB was recently provided (Minichiello et al., 2002). However, the protein phosphorylation is essential for plasticity via both CREB and αCaMKII (Giese et al., 1998; Glazewski et al., 2000; Gonzalez et al., 1999; Sheng et al., 1991). Therefore, our results describing the transcriptional regulation of CREB, αCaMKII and NMDA receptor subunits are not directly comparable with the above literature cited but would require protein level studies for complementation. However, the altered activation of the PLCγ signaling pathway is a likely candidate modulating the LTP responses observed in trkB.TK+ mice.

*Fos* and *jun* family transcription factors are members of the immediate early genes (IEGs) that are robustly induced after various neuronal stimuli. In order to affect gene expression, the *fos* and *jun* family members heterodimerize to form the AP-1 complex that binds to regulatory elements in target genes. Like BDNF expression, several IEGs are regulated by neuronal trauma, drug administration, LTP, and learning (Herdegen and Leah, 1998; Kovacs, 1998; Lindvall et al., 1994). In the current study we report the upregulation of *c-fos*, *fra-2* and *jun-B* transcripts as a consequence of long-lasting trkB activation. However, traditionally the IEG induction is considered rapid and transient. Within the *fos* family, *c-fos* and *fosB* are the most acute members, whereas the *fra1* and *fra2* expression is more constitutive and could accumulate into the nucleus (Kovacs, 1998). The prolonged expression of IEGs is suggested to occur via mRNA stabilization or sustained activation of second messenger systems (Herdegen and Leah, 1998). These second messengers, such as $Ca^{2+}$ or calmodulin, would stimulate CREB phosphorylation that consequently would promote IEG induction. Uniquely, c-fos gene detects changes in Ca2+ levels via two distinct mechanisms (Bading et al., 1993; Ghosh et al., 1994). Calcium influx through the L-type, voltage-gated $Ca^{2+}$ channels (VSCCs), induces c-fos expression after CREB phosphorylation via cAMP response element (CRE). Alternatively, $Ca^{2+}$ signals via NMDA receptor activate the MAPK pathway and regulate c-fos expression through the serum response element (SRE). Interestingly, the spatial aspects of $Ca^{2+}$ signaling could influence the c-fos expression as the CRE and SRE are suggested to respond to distinct $Ca^{2+}$ pools (Ginty, 1997). However, the role of this mechanism in trkB.TK+ mice has not been addressed. Furthermore, frequent stimulation could desensitize the IEG responses as repeatedly stressed animals exhibit reduced c-fos activation in amygdala and cortex (Kovacs, 1998; Melia et al., 1994). Finally, certain chronic responses could be mediated by the long-lived *fosB* splice variant ΔFosB, which regulates the formation of AP-1 complex and further gene expression; however, we did not detect fosB regulation in transgenic mice (Nestler et al., 1999).

GAP-43 is increased in regions that are growing axons or remodeling connections (Skene, 1989). Furthermore, the induction of GAP-43 expression is considered a sign of increased plasticity as during LTP or learning (Namgung et al., 1997; Routtenberg et al., 2000; Young et al., 2000). GAP-43 is suggested as a mediator of BDNF-derived modifications of axonal plasticity (Elmer et al., 1996; Klocker et al., 2001; Kobayashi et al., 1996). In agreement with our hypothesis of increased neuronal plasticity in trkB.TK+ mice, we observed elevated GAP-43 mRNA expression in hippocampus. The GAP-43 mRNA levels, however, are modified by transcription regulation though the AP-1 complex (Weber and Skene, 1998) as well as by NGF-dependent stabilization of the GAP-43 mRNA half-life (Irwin et al., 2002). Therefore, the possible contribution of factors other than increased BDNF signaling in GAP-43 mRNA regulation should be acknowledged. Finally, the NTs are suggested to regulate expression of neuropeptides such as NPY. Indeed, BDNF administration induces long-lasting increase in NPY expression whereas the BDNF null mice show reduced levels of NPY in the cerebral cortex and hippocampus (Croll et al., 1994; Jones et al., 1994; Nawa et al., 1993; Nawa et al., 1994). Blockade of trk kinase activity prevents the BDNF-induced NPY induction *in vitro*, while reduced trkB signaling *in vivo* has no effect on the hilar NPY protein (Lahteinen et al., 2002; Marty and Onteniente, 1999). In the present study, we observed an increased density of NPY-immunopositive cells within hilus as a consequence of increased BDNF signaling. Altogether, our results suggest a role for BDNF signaling in the modulation of hippocampal NPY expression.

### *6.4 Behavioral effects of increased trkB.TK+ signaling*

The physical appearance often reveals many aspects of the general health of genetically modified mice. TrkB.TK+ mice displayed no obvious differences in comparison to WT littermates. Indeed, their body weight, posture, gait, home cage activities were identical to WT mice. Additionally, vision, hearing, and eyeblink reflexes were assessed during the daily care routines and were determined normal. General locomotory and spontaneous activity, pain threshold and sense of touch, were all similar to WT littermates. Interestingly, increased spontaneous activity has been repeatedly observed in all BDNF mutants (Kernie et al., 2000; Lyons et al., 1999; Monteggia et al., 2004; Rios et al., 2001), except one (Gorski et al., 2003a; Monteggia et al., 2004). Hyperlocomotion and impulsivity was reported also for the trkB-CRE and trkB-Shc mutants (Minichiello et al., 1998; Minichiello et al., 1999; Zorner et al., 2003). Although largely unknown, factors like serotoninergic abnormalities, inner ear defect or aggravated stress response are suggested as the origin for the hyperactive behavior (Lyons et al., 1999; Minichiello et al., 1998; Zorner et al., 2003).

### 6.4.1 TrkB.TK+ overexpression facilitates learning

The trkB/BDNF system has been strongly implicated in hippocampus-dependent learning (Tyler et al., 2002; Yamada et al., 2002). Indeed, both BDNF expression and trkB activity are increased in response to learning (Hall et al., 2000; Kesslak et al., 1998; Ma et al., 1998; Mizuno et al., 2000). In accordance, reduction of BDNF or trkB constantly produces learning deficits (Gorski et al., 2003a; Linnarsson et al., 1997; Minichiello and Klein, 1996). In agreement, the present study demonstrates the increased trkB.TK+ receptor activity in postnatal neurons ultimately promotes memory processes. Specifically, trkB.TK+ mice displayed better memory acquisition and faster learning in the Morris water maze task assessing hippocampus-dependent spatial memory. The fear conditioning task is often used to complement the water maze task in the assessment of learning. Indeed, trkB.TK+ mice behavior in this test supported the finding of improved memory. Finally, the conditioned taste aversion represents a totally different learning task, where aversive stimulus is learned to be paired with a certain taste. TrkB.TK+ mice developed stronger aversion against the paired taste, therefore suggesting better associative learning. Altogether, these three independent measures suggested that increased trkB.TK+ receptor signaling improves memory. Our results agree with studies reporting spatial learning deficits in mice lacking either trkB or BDNF and in mice overexpressing the truncated trkB.T1 receptor (Gorski et al., 2003a; Linnarsson et al., 1997; Liu et al., 2004; Minichiello and Klein, 1996; Saarelainen et al., 2000b). The contextual fear conditioning is dependent on both hippocampus and amygdala. In trkB.TK+ mice, the transgene is expressed also in the amygdaloid nuclei although we have not addressed that region more specifically. In agreement, it was recently reported that BDNF/trkB signaling in the amygdala is necessary for fear conditioning response (Liu et al., 2004; Rattiner et al., 2004). Furthermore, by using multiple learning tests we avoided the possible confounding effects that could be misinterpreted as altered learning ability if only one specific test was used. Indeed, the learning inability of trkB-CRE mice occurring in laboratory circumstances was found to largely depend on the reduced behavioral flexibility in mutants when observed in a more naturalistic environment (Vyssotski et al., 2002). Thigmotaxis, the excessive wall swimming in the water maze task, is a primitive escape strategy rodents adopt initially when placed into a pool and correlates with diminished behavioral flexibility. Interestingly, trkB.TK+ mice exhibited less thigmotactic behavior as the WT littermates, suggestive that they had no difficulties in adopting new behavioral strategies. Excessive thigmotaxis, as observed in trkB-CRE mice, can prevent the successful performance of spatial learning paradigms. Finally, the addition of BDNF itself does not apparently improve learning and memory (Croll et al., 1999; Pelleymounter et al., 1996). With respect to our results, it might be suggested that the addition of a receptor is more physiological way to increase signaling-dependent learning than mere addition of the ligand itself. Indeed, the chronic BDNF exposure on cortical neurons leads to reduced trkB.TK+ mRNA and protein levels,

and enhanced trkB internalization (Frank et al., 1996; Haapasalo et al., 2002; Knusel et al., 1997).

Synaptic plasticity has a central role in learning and memory, and LTP is considered as a principal cellular mechanism of learning (Bliss et al., 2003). Furthermore, a variety of factors, such as αCaMKII, NMDA receptors, AMPA receptors or Erk signaling, are suggested as mechanisms responsible for the synaptic changes occurring in learning. Interestingly, BDNF/trkB signaling pathways converge with many of these mechanisms and regulate their expression thus supporting the role of BDNF signaling in memory processes (Tyler et al., 2002). However, direct correlation between measured LTP and observed behavioral responses are not always achieved. Indeed, a deficit in potentiation does not necessarily impair learning (Frisch et al., 2000; Nosten-Bertrand et al., 1996; Sprengel et al., 1999; Zamanillo et al., 1999) and similarly, learning deficits occur without LTP impairment (Kaksonen et al., 2002; Motro et al., 1996; Pavlov et al., 2002; Saarelainen et al., 2000b). The obvious discrepancy between these two measurements of learning is not surprising, however. In contrast, most likely the mechanisms behind plasticity are more complex, and the behavioral response represents a sum of several simultaneous processes that ultimately lead to formation of memory traces.

### 6.4.2 TrkB.TK+ overexpression reduces anxiety

BDNF is suggested to have a significant role in the pathophysiology of psychiatric disorders, like depression (Russo-Neustadt, 2003; Sen et al., 2003; Sklar et al., 2002). In the current study, we observed that mice with increased BDNF signaling exhibited lower anxiety-like behavior. Indeed, in the light-dark exploration test the trkB.TK+ mice made more transitions between the light and dark compartments. Furthermore, TG mice entered the open arms of the elevated plus maze more readily than WT mice, thus indicating reduced anxiety. In agreement, conditional BDNF knockouts exhibit increased anxiety (Rios et al., 2001). Further behavioral data suggests that transgenic trkB.TK+ mice exhibited less depression-related behavior in the forced swim test. In this paradigm, the time spent inactive in a water tank is interpreted as depression-related behavior. Indeed, the saline-injected TG mice exhibited significantly reduced immobility in comparison to WT mice. Recently, trkB-CRE knockout mice were reported to display reduced immobility in the forced swim test, however the behavioral response in these mice may be confounded by other factors (Vyssotski et al., 2002; Zorner et al., 2003). Interestingly, a preliminary experiment performed on a smaller set of mice revealed that if the antidepressant fluoxetine was injected prior to the swim, there was no clear behavioral correlate in TG mice (unpublished data, E.K.). In contrast, the fluoxetine-injected WT mice displayed reduced immobility (although not statistically significant) in comparison to their saline-injected performance, as expected. Remarkably, the neurochemical studies on hippocampal monoamines support the observed behavioral data. In untreated transgenic mice, the levels of hippocampal

5-HT and NA were increased in comparison to WT mice. Curiously, 5-HT levels measured in TG mice hippocampus appear similar to WT levels after fluoxetine injection. In contrast, fluoxetine administration has no visible effect on the hippocampal 5-HT levels in TG mice whereas the WT mice respond to antidepressant as expected. In general, the neurochemical changes in TG mice are exciting. Tryptophan, a precursor for 5-HT, is reduced in TG mice whereas 5-HT is increased. The tryptophan levels are strongly modulated by dietary tryptophan, however, in our case both genotypes were fed with same food pellets. On the other hand, the 5-HT accumulation observed in TG mice could result from a blockade of 5-HT reuptake mechanism. Antidepressants like fluoxetine, inhibit 5-HT reuptake mechanism through serotonin transporter (SERT), therefore resulting in serotonin accumulation as observed in WT mice upon fluoxetine injection. Another possibility for 5-HT accumulation could be defective catabolism. However, the degradation appears to work normally as the levels of the primary metabolite 5-HIAA are similar in both genotypes and respond similarly to antidepressant treatment. Our results, however, do not provide a specific answer for the speculations above, but would require additional experiments.

The combined behavioral and biochemical data in our study would suggest that increased trkB signaling *per se* results in a situation comparable to antidepressive drug treatment. In support of this, exogenous BDNF administration produces behavioral responses similar to antidepressive drugs (Shirayama et al., 2002; Siuciak et al., 1997), whereas mice either lacking BDNF or overexpressing trkB.T1, exhibit no behavioral response in the swim test (Monteggia et al., 2004; Saarelainen et al., 2003). Moreover, trkB activation induced after antidepressant treatment, is not observed in trkB.T1 overexpressing mice (Saarelainen et al., 2003). Taken together, increased trkB .TK+ activation produces behavior similar to antidepressant treatment therefore suggesting a role for BDNF/trkB signaling in the antidepressant drug actions.

### *6.5 The role of BDNF/trkB signaling in epilepsy*

Epileptic activity provokes axonal reorganization in hippocampus, namely mossy fiber sprouting. In addition, BDNF activity-dependently regulates the axonal morphology and the trkB receptor is activated in dentate granule cells and mossy fibers after kindling (Binder et al., 1999; Elmer et al., 1997; He et al., 2002). However, in this study we were not able to see significant differences in the extent of kainate-induced mossy fiber sprouting between trkB.TK+ and wt mice. Indeed, the existing data on the contribution of BDNF on mossy fiber sprouting is controversial. In line with our results, there is no apparent association of mossy fiber sprouting with BDNF signaling (Bender et al., 1998; Lahteinen et al., 2002; Qiao et al., 2001). In accordance, both acute and chronic intrahippocampal BDNF infusions failed to modulate kindling-induced axonal sprouting (Xu et al., 2004). In a recent study, local hilar BDNF application induced mossy fiber sprouting whereas global addition did not, therefore suggesting

the local actions of BDNF (Koyama et al., 2004). Altogether, these results suggest that BDNF signaling as such is not sufficient to induce the characteristic axonal reorganization during epilepsy.

BDNF application enhances synaptic transmission, increases neuronal excitability, and facilitates LTP. Consequently, mice overexpressing BDNF show increased seizure severity in response to kainic acid (Croll et al., 1999), whereas mutant mice heterozygous for BDNF have higher seizure threshold (Kokaia et al., 1995). Furthermore, epileptogenesis is reduced either by scavenging BDNF with trkB receptor bodies (Binder et al., 1999) or in mice with reduced BDNF signaling (Lahteinen et al., 2002). In the current study, we observed more severe SE in mice with enhanced BDNF signaling. In addition, the acute cellular death was enhanced in transgenic mice, suggesting that BDNF is not a pro-survival factor during the acute phase of the insult. In contrast, we did not observe a difference between genotypes either in the chronic cell death or in neurogenesis 4.5 months after the initial insult. Likewise, the epileptogenesis and severity of epilepsy were similar between genotypes. Altogether, our results suggest that the increased trkB receptor activity present in transgenic mice is crucial for the seizure generation and in defining the rapid cellular responses after an excitotoxic event, but is not regulating the chronic phase of the disease.

The role for elevated BDNF in epileptogenesis has yielded conflicting results suggesting both augmented seizure severity (Croll et al., 1999; Scharfman et al., 2002) and inhibition of seizures (Larmet et al., 1995; Reibel et al., 2000a; Reibel et al., 2000b). Interestingly a recent study demonstrated that the BDNF administration protocol had a crucial impact on behavioral outcome, and activation of trkB in a kindling-model (Xu et al., 2004). There, a continuous BDNF infusion inhibited the development of behavioral seizures and decreased trkB activation. Certainly, the trkB receptor amount on the cell membrane is regulated by both BDNF and neuronal activity (Haapasalo et al., 2002; Meyer-Franke et al., 1998), therefore alterations in the trkB cell surface expression could modulate the responses during epileptogenesis. Indeed, new data on BDNF and trkB conditional knockout mice confirm that sufficient trkB expression and signaling is required for epileptogenesis in kindling (He et al., 2004). Taken together, our data (Lahteinen et al., 2003; Lahteinen et al., 2002) together with literature cited here, suggest that reduction in trkB signaling appears to be more detrimental for the development of epilepsy than merely altered levels of BDNF.

## 7 SUMMARY

The current series of studies was undertaken to elucidate the consequences of overexpressing the full-length neurotrophin receptor trkB in adult central neurons. Transgenic trkB.TK+ overexpressing mice displayed normal general health, locomotion, sensory modalities, and spontaneous activity. Transgenic mice were subjected to a series of biochemical, behavioral and physiological tests that can be summarized as follows:

I. In hippocampus and cortex the overexpressed trkB.TK+ was continuously activated as demonstrated by strong phosphorylation of the three trkB receptor tyrosines. Receptor overexpression specifically regulated downstream signaling of the PI3K/Akt and PLC$\gamma$-1 pathways whereas the Ras/MAP kinase pathway was not regulated. Finally, the levels of trkB ligand, BDNF, were not affected by trkB.TK+ overexpression.

II. Behaviorally, the trkB.TK+ overexpression produced a specific learning enhancement in the water maze paradigm that was further confirmed by two other learning tests. Further characterization of emotional behaviors revealed reduced anxiety-like behavior in trkB.TK+ mice.

III. In contrast to behavioral data, increased trkB signaling attenuated the hippocampal long-term potentiation that is considered as a cellular level measure of learning.

IV. TrkB.TK+ overexpression regulated genes implicated in neuronal plasticity. Specifically, the mRNA expression of GAP-43 and $\alpha$CaMKII was regulated whereas CREB gene expression was unaltered. Furthermore, trkB.TK+ overexpression changed the expression of *fos* and *jun* family members.

V. TrkB.TK+ mice exhibited reduced depression-like behavior in the forced swim test. Accordingly, neurochemical analysis showed increased hippocampal serotonin levels.

VI. Increased trkB.TK+ signaling modulated the developmental phases of epilepsy. TrkB.TK+ mice exhibited lowered seizure threshold and more severe seizures during SE. The acute neuronal damage was increased in trkB.TK+ mice whereas long-term cell death was not changed. The rate of epileptogenesis and severity of epilepsy were not regulated by trkB.TK+ overexpression.

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