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SARI LÄHTEINEN

Brain-derived neurotrophic factor in the development of epilepsy

Doctoral dissertation

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Abstract

Epilepsy is one of the most common neurological diseases, but our understanding of the detailed cellular and molecular mechanisms underlying it remains incomplete. Several pathological changes typically occur in the epileptic brain, including neuronal loss, neurogenesis, neurite growth, and reorganization of extracellular matrix leading to changes in neuronal networks. Many of these features are normally regulated by neurotrophic growth factors, especially brain-derived neurotrophic factor (BDNF). Moreover, BDNF modifies both excitatory and inhibitory synaptic transmission in the brain. Therefore, BDNF signaling is an intriguing candidate for playing a role in seizures and epileptogenesis. However, its effects to the development of epilepsy are unknown, although the knowledge of the mechanisms underlying might allow better seizure control and possibly even prevention of epileptogenesis.

The aim of this study was to clarify the role of BDNF signaling in different phases of epilepsy. As a model, two transgenic mouse lines with opposite effects to BDNF signaling were used, and epileptogenesis was induced with kainate model of temporal lobe epilepsy. The severity of *status epilepticus*, development of epileptogenesis, and the severity of epilepsy were monitored with combined video-EEG recordings and histological markers were studied. Moreover, the mechanisms of BDNF action during epileptogenesis were assessed.

We extended the previous result by showing that BDNF signaling exacerbates both the severity of *status epilepticus* and the acute neuronal death. In the later phase, increased BDNF signaling promoted and decreased BDNF signaling reduced the development of epilepsy. Moreover, reduced BDNF signaling showed a disease-modifying role, alleviating the severity of epilepsy. BDNF signaling also increased its own synthesis during *status epilepticus*, and affected the expression of several functional groups during epileptogenesis; those include cytoskeletal and growth-related proteins, DNA repair, DNA recombination and neuronal death. This suggests that BDNF may regulate several significant processes via trkB receptors during the development of epilepsy. Furthermore, BDNF was shown to be a prominent player in all phases of epileptogenesis, and therefore it might be a useful target for the development of improved anti-epileptic drugs.

National Library of Medicine Classification: WL 385, WL 104
Medical Subject Headings: epilepsy, temporal lobe; seizures; status epilepticus; brain-derived neurotrophic factor; receptor, trkB; hippocampus; mice, transgenic; kainic acid; neurons; cell death; gene expression; oligonucleotide array sequence analysis; computational biology

Work like you don't need the money.
Love like you've never been hurt.
Dance like nobody's watching.
Live like it's Heaven on Earth.

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Abbreviations

BDNF	= brain derived neurotrophic factor
CA1-3	= hippocampal subfields 1-3 of pyramidal cell layer
EEG	= electroencephalogram
fl-trkB	= full-length trkB receptor
GAP-43	= growth associated protein 43
GABA	= γ-aminobutyric acid
IIP	= interictal spiking
KA	= kainic acid
Kif3a	= kinesin family member 3A
LTD	= long-term depression
LTP	= long-term potentiation
MAPK	= mitogen-activated protein kinase
mRNA	= messenger ribonucleic acid
NGF	= nerve growth factor
NMDA	= N-methyl-*D*-aspartate
NT-3	= neurotrophic growth factor 3
NT4/5	= neurotrophic growth factor 4/5
PI-3K	= phosphatidylinositol-3 kinase
PLCγ	= phospholipase C γ unit
SE	= status epilepticus
SOM	= self-organizing map
T1	= truncated trkB receptor isoform T1
Tg	= transgenic
TIMP3	= tissue inhibitor of metalloproteinase 3
TK+	= transgenic mice overexpressing full-length trkB
TK-	= transgenic mice overexpressing truncated trkB
TLE	= temporal lobe epilepsy
TrkB	= tropomyosin-related kinase B
Wt	= wild type
$BDNF^{+/-}$	= mice heterozygous for the BDNF gene
$BDNF^{-/-}$	= knockout mice lacking for the BDNF gene

LIST OF ORIGINAL PUBLICATIONS

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

I Lähteinen S., Pitkänen A., Saarelainen T., Nissinen J., Koponen E. & Castrén E. (2002) Decreased BDNF signaling in transgenic mice reduces epileptogenesis. *European Journal of Neuroscience*, 15:721-734

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

III Lähteinen S., Pitkänen A., Knuuttila J., Törönen P. & Castrén E. (2004) BDNF modifies hippocampal gene expression during epileptogenesis in transgenic mice. *European Journal of Neuroscience*, 19:3245-3254

IV Saarelainen T., Vaittinen S. & Castrén E. (2001) TrkB-receptor activation contributes to the kainate-induced increase in BDNF mRNA synthesis. Cellular and Molecular Neurobiology, 21: 429-435 *

In addition, some data published in abstract form is presented **

* Saarelainen, T., and Vaittinen, S., have equally contributed to the laboratory work.

** The data is obtained from collaboration with Prof. Antoine Depaulis (Université Joseph Fourier de Grenoble, France). For reference, see (Heinrich et al., 2003).

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1 Introduction

Epilepsy is one of the most common neurological disorders worldwide. Affecting 1-2 % of the population at some point in their lives and requiring considerable medical care, it has become significant also economically. Human temporal lobe epilepsy (TLE) is characterized by an initial insult, which is followed by a latent period before the initiation of spontaneous seizures and epilepsy diagnosis. Pathologic features of TLE include neuronal death, neurogenesis, gliosis and reorganization of the neuronal network. Although these features have been widely studied, our understanding of their functional consequences and molecular mechanisms still remains incomplete. Accordingly, both increased neuronal excitability and altered inhibition combined with neural network synchrony have been suggested as a cause for seizure generation and progression. More detailed knowledge of the molecular mechanisms would greatly help in designing anti-epileptic drugs with better seizure control and fewer side effects.

Many of the pathological features of epileptogenesis may be linked to processes normally regulated by neurotrophic factors. Neurotrophins, especially brain-derived neurotrophic factor (BDNF), regulates neuronal survival, maintenance, growth, neurogenesis, and morphology of neurons. These features may be linked to neuronal sprouting, death, and reorganization often associated with epileptogenesis. Moreover, the ability of BDNF to regulate both excitatory and inhibitory synaptic transmission makes it a tempting candidate to influence epileptogenesis and alter existing neuronal connections.

BDNF mediates its effects via trkB receptors. In addition, seizures upregulate both BDNF and trkB expression in the brain. Moreover, application of BDNF causes hyperexcitability both in normal and epileptic brain slices and it is suggested to be involved in s*tatus epilepticus* (SE). Some studies have also indicated that BDNF might participate in the development of epilepsy. However, those results have been controversial, a functional role in both promoting epilepsy and protecting from epileptogenesis.

The aim of this study was to clarify the role of BDNF in different phases of epileptogenesis, including SE, latent period, and the disease state. The molecular mechanisms of BDNF action were also studied in order to generate insight into brain processes during epileptogenesis.

2 Review of the literature

2.1 Neurotrophic growth factors

Neurotrophins are a family of growth factors initially identified by their ability to support neuronal survival (reviewed in Huang and Reichardt 2001; Huang and Reichardt 2003; Segal 2003). The family consists of at least four mammalian proteins, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophins 4/5 (NT-4/5), which mainly elicit actions in neural system, affecting both peripheral and central nervous systems. In addition to supporting neuronal survival, neurotrophins regulate the maintenance and differentiation of neurons as well as cell fate decisions and neuronal death (Miller and Kaplan 2001; Patapoutian and Reichardt 2001; reviewed in Miller and Kaplan 2003). Moreover, neurotrophins are important regulators of neuronal growth and morphology. Although neurotrophins were originally described as survival and growth factors, emerging evidence support their involvement in neuronal plasticity (reviewed in Aloyz et al., 1999; Schinder and Poo 2000; Lu 2003b). In fact, neurotrophin mediated adaptive regulation of excitatory and inhibitory signaling as well as changes in neuronal network reorganization are fundamental features of learning and memory.

Neurotrophins act via specific tyrosine kinase receptors called tropomyosin-related kinase (trk)-receptors (Barbacid 1994; Huang and Reichardt 2003). Although most neurotrophins can interact with several trk receptors, NGF preferentially binds to trkA, BDNF and NT 4/5 to trkB and NT-3 to trkC, respectively. The neurotrophin binding to trk receptor occurs with high affinity and initiates several alternative signaling cascades that convey the message to the targets (Patapoutian and Reichardt 2001). However, the first identified neurotrophin receptor was p75 that binds all mature neurotrophins with significantly lower affinity than trk-receptors (Hempstead 2002; Huang & Reichardt 2003). Recent results have now revealed a putative role of p75 in modifying neurotrophin signaling both independently and in collaboration with trk receptors (Beattie et al., 2002; Roux and Barker 2002; Kaplan and Miller 2003; Lu 2003a; Harrington et al., 2004). Altogether, neurotrophin signaling appears to be far more complicated than originally thought, influencing several different functions in the nervous system.

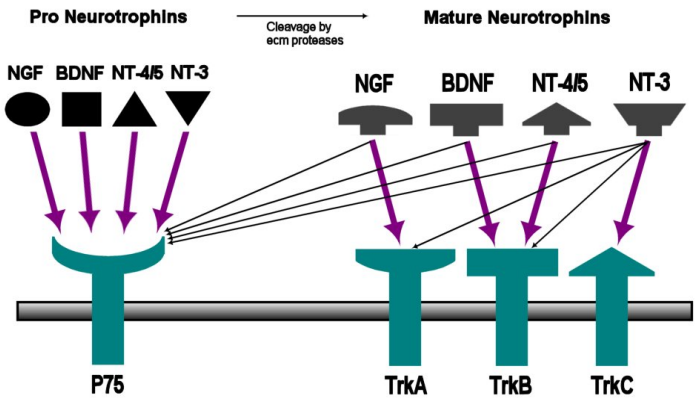

Fig.1. Neurotrophin binding to different receptors (adopted from Segal 2003).

2.2 Brain-derived neurotrophic factor

BDNF was first isolated from the brain in 1982 (Barde et al., 1982) and cloned in 1989 (Leibrock et al., 1989). The *BDNF* gene traverses through more than 40 kb of the genomic DNA, including at least five exons and four alternative promoters (Timmusk et al., 1993). Use of different 5' exons partly controls the tissue specificity of BDNF, because transcripts encoded by different exons are preferentially expressed in limited, although partly overlapping brain areas. The complexity of the BDNF gene is further increased by the alternative use of two polyadenylation sites independently of splicing and initiation of transcription, leading to coding of several different variants (Timmusk et al., 1993).

Mature BDNF is a 13.5 kDa secretory protein that is comprised of 120 amino acids from the carboxyterminal of the precursor, proBDNF (Mowla et al., 2001). It is abundantly expressed throughout the brain, with the most pronounced expression in the hippocampus, cerebral and cerebellar cortex, thalamic and hypothalamic nuclei, and striatum (Hofer et al.,1990; Nawa et al., 1995; Ivanova and Beyer 2001). More specifically, hippocampal expression is strongly seen in the dentate granule cells and to a lesser extent in the pyramidal neurons of CA1-CA3. Cortical areas expressing BDNF include frontal, piriform and entorhinal cortices and some amygdaloid nuclei. The expression is mainly neuronal, although astrocytes uptake and may even produce BDNF (Zafra et al., 1992). Furthermore, BDNF levels are developmentally regulated, being highest at P15 and then reducing slightly to reach the adult expression pattern (Ivanova and Beyer 2001).

Due to the abundant but selective neuronal expression of BDNF, it was proposed to be associated with neuronal activity. Indeed, excitatory stimulus, such as neuronal depolarization, increases BDNF mRNA expression in several brain regions (Zafra et al., 1990; Zafra et al., 1992). Similarly, excessive neuronal activity, such as limbic seizures,

enhance greatly the expression of BDNF (Zafra et al., 1990; Ernfors et al., 1991; Isackson et al., 1991; Humpel et al., 1993; Schmidt-Kastner et al., 1996a; Liang et al., 1998; for review, see Gall 1993). Besides increasing BDNF synthesis, neuronal activity induces transportation and accumulation of BDNF to neurites, where it is then activity-dependently released to the synaptic cleft (Wetmore et al., 1994; Blochl and Thoenen 1995; Goodman et al., 1996; Fawcett et al., 1997; for review, see Lu 2003b). Therefore, it is generally assumed that BDNF expression is tightly linked to neuronal activity.

2.2.1 Physiological roles of BDNF

BDNF mediates various divergent actions, such as neuronal survival, neurogenesis, cell death, neurite growth, connectivity, and plasticity. Moreover, physiological stimuli, such as light input in the eye, whisker stimuli or exercise, regulate the synthesis of BDNF. Therefore BDNF is able to convert physiological functions inducing neuronal activity into molecular and morphological changes in the nervous system.

2.2.1.1 Effect on neuronal survival and maintenance

BDNF was first described as a survival factor for certain neuronal populations. In cell cultures of CNS neurons, it supports the survival of retinal ganglion neurons (Johnson et al., 1986), basal forebrain cholinergic neurons (Alderson et al., 1990), substantia nigra dopaminergic neurons (Hyman et al., 1991; Knusel et al., 1991), cerebellar granule cells (Segal et al., 1992; Lindholm et al., 1993), and cortical neurons (Ghosh et al., 1994). The survival and branching of hippocampal dentate granule cells is also enhanced by BDNF and it promotes the survival of subventricular zone explant cultures (Kirschenbaum and Goldman 1995). In line with these results, reduced BDNF signaling in $trkB^{+/-}$ and $trkB^{+/-}/trkC^{+/-}$ mice decreases neuronal density in dentate granule and pyramidal CA1 cell layers of the hippocampus (von Bohlen und Halbach, et al., 2003). Moreover, $BDNF^{-/-}$ knockout mice show markedly increased apoptosis in the subventricular zone, in the hippocampal dentate gyrus and in the olfactory bulb (Linnarsson et al., 2000). Similarly, mice deficient of trkB receptors show elevated cell death (Minichiello et al., 1999b;). Apoptosis is gradually increased after birth of $BDNF^{-/-}$ mice, peaking at postnatal day 20 briefly before the death of the mice. This indicates the possible role of BDNF not only as a survival factor during development, but also in the maintenance of neurons. Furthermore, the evidence of neuronal BDNF dependence in subventricular zone, olfactory bulb, and hippocampal dentate gyrus suggest that BDNF might play a role in neurogenesis. Indeed, Linnarsson et al. (2000) proposed that BDNF may be required for the establishment of the second proliferative zone in

the dentate gyrus and may support the survival of new born cells in the subgranular zone. Similarly, chronic exposure of striatal neurons to BDNF or trkB promotes either stroke-induced neurogenesis or the survival of new born neurons (Gustafsson et al., 2003a; Gustafsson et al., 2003b). However, the effects of BDNF signaling to cell survival or maintenance can be converted into promotion of cell death depending on the intracellular pathways activated (Kim et al., 2002). This is supported by studies showing that excess BDNF exacerbates both SE- and stroke-induced neuronal damage in the brain (Rudge et al., 1998; Gustafsson et al., 2003a). Therefore, BDNF signaling regulated by the expression of different BDNF receptors and the posttranslational cleavage of BDNF control the divergent effects of BDNF on neurogenesis, cell survival, maintenance, and cell death.

2.2.1.2 Effect on neuronal morphology

Several studies with either wild type or gene-modified mice suggest that BDNF has a prominent effect on neuronal morphology. It modifies axonal and dendritic growth and complexity both *in vitro* and *in vivo*. Danzer and coworkers (2002) observed increased basal dendrite number and apical dendritic branching of dentate granule cells in hippocampal slice cultures transfected with BDNF. Similarly, dendrite length and complexity were increased in dentate granule cells of mice overexpressing BDNF (Tolwani et al., 2002, but see also Qiao et al., 2001). BDNF affects also the morphology of other brain areas, such as cortex. Neocortical neurons show decreased dendritic complexity both in conditional trkB knockout mice and in cell cultures from P0 $trkB^{-/-}$ mice (Gates et al., 2000; Xu et al., 2000). Furthermore, chimeric $trkB^{-/-}$ mice show delayed integration and differentiation of $trkB^{-/-}$ neurons into neuronal network (Gates et al., 2000).

In addition to dendritic changes, BDNF promotes branching of dentate granule cell axons and retinal ganglion axons (Cohen-Cory and Fraser 1994; Danzer et al., 2002). This effect can be blocked by anti-BDNF antibodies, which prevent axonal growth and reduce the complexity of axons (McAllister et al., 1999). Similarly, $trkB^{+/-}$ and $trkB^{+/-}/trkC^{+/-}$ mice suffer from increased axonal degeneration of both dentate granule cells and pyramidal neurons of layer CA3 (von Bohlen und Halbach et al., 2003). Moreover, changes in soma morphology have been reported in $trkB^{-/-}$ mice in neocortex, where decreased BDNF signaling caused cell bodies to grow rounder than in control mice (Xu et al., 2000). This evidence demonstrates that BDNF signaling regulates neuronal morphology and is essential in maintaining neuronal integrity.

Besides controlling neurite growth, BDNF regulates the fine structures of active neurons. It influences the formation, stabilization, and maintenance of spines, the major structures where synapses reside. Accordingly, BDNF promotes the formation of both excitatory and

inhibitory synapses and increases their maturation. (Martinez et al., 1998; Huang et al., 1999; for review, see Lu 2003b) BDNF also stabilizes newly formed synapses in response to neuronal activity and it may be able to stabilize synapses even in the absence of neuronal stimulus (for review, see Vicario-Abejon et al., 2002; Miller and Kaplan 2003). Therefore, BDNF presumably is one of the key players in the formation and stabilization of neuronal connections.

2.2.1.3 Effect on neuronal connectivity and plasticity

Depolarization of the cell membrane as well as stimulation parameters inducing long-term potentiation (LTP) regulate BDNF content in the brain (Zafra et al., 1990; Castren et al., 1992; Patterson et al., 1992). Since neuronal activity is the key regulatory element in neuronal plasticity, this supports the involvement of BDNF in activity-dependent plastic events. In fact, BDFN activity-dependently guides the formation of ocular dominance columns in the visual cortex, and it may play a role in learning and memory (Cabelli et al., 1995; Yamada et al., 2002a). Those functions require ability to modify synaptic transmission in the brain, as has been suggested for BDNF. Therefore, BDNF may mediate plastic changes in the neuronal connections, participating in the organization of the neuronal network under several physiological conditions.

LTP

Activity-dependent regulation and acute effects on neuronal connectivity suggest that BDNF may participate in LTP, a well-defined model of synaptic plasticity that is thought to model physiological changes occurring in the brain during learning and memory. In fact, exogenous BDNF enhances LTP in the normal brain, and blockade of BDNF signaling either with trkB-IgG or anti-BDNF antibody reduces LTP (Figurov et al., 1996; Kang et al., 1997; Chen et al., 1999; reviewed in Poo 2001). Impaired hippocampal LTP is also seen in knockout mice with reduced BDNF signaling (Korte et al., 1995; Patterson et al., 1996; Pozzo-Miller et al., 1999; Bartoletti et al., 2002), and the impairment can be rescued with BDNF application into the brain (Korte et al., 1996; Patterson et al., 1996), strengthening the evidence that BDNF participates in formation and maintenance of LTP. Defects in LTP at hippocampal CA1 synapse were also shown in trkB knockout mice; homozygous mice showed strong and heterozygous mice showed partial reduction in LTP (Minichiello et al., 1999a). Recently, the mechanism of BDNF action has begun to be clarified. Minichiello and coworkers (1999a; 2002) have studied the effects of different trkB receptor mutants to the formation and maintenance of LTP, revealing the significance of phospholipase γ (PLCγ) signaling pathways

in LTP. In line with this, the importance of voltage-gated Ca^{2+} channels in BDNF-mediated LTP has recently been demonstrated (Kovalchuk et al., 2002; Zakharenko et al., 2003).

In line with its involvement in LTP, BDNF is also increased during learning and memory (Hall et al., 2000). Blockade of BDNF signaling chemically or via overexpression of T1 in TK- mice (see 2.5) impairs learning and memory and TK+ mice with increased BDNF signaling (see 2.5) learn faster in the Morris watermaze test (Ma et al., 1998; Mu et al., 1999; Saarelainen et al., 2000b; Koponen et al., 2004). Intriguingly, forebrain-restricted $BDNF^{-/-}$ mice show severe deficits in specific forms of learning (Gorski et al., 2003a). Likewise, $trkB^{-/-}$ mice with strongly impaired LTP suffered from impaired learning in complex or stressful situations, whereas $trkB^{+/-}$ mice with partial reduction in LTP were behaviorally normal (Minichiello et al., 1999a; Vyssotski et al., 2002). These results propose the crucial involvement of BDNF/trkB signaling in LTP and possibly in learning and memory (Tyler et al., 2002a; for review, see Yamada et al., 2002a).

Excitatory synaptic transmission

As BDNF enhances LTP, it may also play a role in synaptic transmission. This was first demonstrated by showing that acute application of BNDF enhanced the strength of *Xenopus* neuro-muscular synapses (Lohof et al., 1993). A similar increase occurs also in the hippocampal excitatory synaptic transmission in neuronal cultures, brain slices, and adult rat brain after acute application of BDNF (Lessmann et al., 1994; Kang and Schuman 1995; Levine et al., 1995; Scharfman 1997; Messaoudi et al., 1998). Interestingly, BDNF seems to promote more profoundly immature-like synapses with low original synaptic strength than mature, strong synapses (Berninger and Poo 1999; Schinder et al., 2000). Reduced BDNF signaling in $BDNF^{-/-}$ mice lead to impaired high frequency synaptic transmission, which can be rescued with BDNF application. These mice further show decreased amount of docked synaptic vesicles and reduced expression of synaptic proteins (Pozzo-Miller et al., 1999). The BDNF-mediated increase in expression and phosphorylation of synaptic vesicle proteins has previously been associated with increased neurotransmitter release (Jovanovic et al., 1996; Takei et al., 1997; Jovanovic et al., 2000; Tyler et al., 2002b). Altogether, BDNF enhances excitatory synaptic strength, possibly by enhancing neurotransmitter release in the synapse.

Besides effects on neurotransmitter release, several studies suggest other methods of BDNF mediated excitatory synaptic transmission. First, BDNF enhances N-methyl-*D*-aspartate (NMDA) receptor-mediated currents, possibly by increasing the opening probability of the NMDA receptor (Jarvis et al., 1997) and raises the phosphorylation of NMDA subunits (Suen et al., 1997). Secondly, BDNF enhances neuronal excitability by increasing expression

of voltage-gated Ca^{2+} and Na^{2+} channels in the plasma membrane (McAllister et al., 1999). Thirdly, BDNF binding to trkB receptors are suggested to interact directly with $Na_v1.9$ leading to membrane depolarization and increased intracellular Ca^{2+} (Blum et al., 2002). Lastly, because of a very short latency from the BDNF release to the depolarization of the synapse, an exciting possibility of BDNF acting as a glutamate- like excitatory neurotransmitter has been proposed (Kafitz et al., 1999). All of these findings strongly support enhanced excitatory synaptic transmission by BDNF, and suggest possible mechanisms by which BDNF may affect LTP, learning, and memory.

Inhibitory synaptic transmission

BDNF is required for the maturation of inhibitory γ-aminobutyric acid (GABA)ergic synapses and the regulation of interneuron properties in the hippocampus (Berninger et al., 1995; Marty et al., 1996; Bartrup et al., 1997; Huang et al., 1999; Yamada et al., 2002b). However, BDNF reduces inhibitory synaptic transmission in hippocampal cell cultures and both evoked and spontaneous GABAergic currents are decreased in hippocampal slices via trkB receptor (Marty et al., 1996; Rutherford et al., 1997; Ikegaya et al., 2002). Indeed, BDNF knockout mice show decreased spontaneous neuronal network activity and increased inhibitory GABAergic currents (Olofsdotter et al., 2000; Henneberger et al., 2002). These effects are rescued with exogenous BDNF and are mimicked by a trk receptor antagonist, K252a, in wild type mice. These results indicate that BDNF increases spontaneous network activity by suppressing GABAergic inhibition via a trkB receptor mediated pathway. Recently, BDNF had been reported to suppress Cl^- -dependent fast inhibitory transmission by decreasing the expression of K^+/Cl^- cotransporter KCC2 (Rivera et al., 2002; Wardle and Poo 2003). This provides a possible mechanism for trkB-mediated depression of inhibition in the brain.

2.2.1.4 Association with cytoskeleton

BDNF involvement in LTP as well as regulation of neurite growth requires interplay with the cytoskeleton. Whereas neurite shafts are built of microtubules and associated proteins, which are relatively stabile after formation, growth cones and spines consist of rapidly changing actin networks. BDNF influences the rapid cytoskeletal rearrangements, such as membrane ruffling and protrusions, involving growth cones. Consistently, BDNF was recently shown to regulate F-actin changes mainly responsible for growth cone expansion (Gibney and Zheng 2003; Gallo and Letourneau 2004). Surprisingly, BDNF was also reported in the regulation of microtubule cytoskeleton in the active growth cones and synapses nearby (Reynolds et al., 2000; Gibney and Zheng 2003; Gallo and Letourneau 2004). Accordingly,

Smart et al (2003) reported BDNF in regulation of synaptic microfilaments and integrins, transmembrane proteins required for cell-cell, and cell-matrix interactions. The possible association of BDNF with other cytoskeletal components as well as with extracellular matrix components is currently unclear. Few suggestions on how BDNF affects the cytoskeleton have so far emerged, but at least trkB-mediated activation of Rho G-protein coupled protein is required for BDNF induced cytoskeletal effects. Src activation via trkB regulates endocytosis and a putative interaction of trkB with Abl tyrosine kinase provides a possible contact between BDNF signaling and cytoskeleton (Lanier and Gertler 2000). Although probably playing a marked role in the neurite growth and plastic organization of the neuronal network, the exact consequences of BDNF interactions with cytoskeleton as well as extracellular matrix still remain to be discovered.

2.3 TrkB receptor

The trk-family of receptors consists of three highly homologous cell surface receptor tyrosine kinases, trkA, trkB and trkC, which bind mature neurotrophins with high affinity (Barbacid 1994; Bothwell 1995; Lewin and Barde 1996). The structure of human *trkB* gene is exceptionally complex, consisting of 24 exons and spanning at least 590 kbp of the genome (Stoilov et al., 2002). Taking into account alternative promoters, splicing and different polyadenylation sites, altogether 100 different mRNAs and 10 proteins can be coded. However, only three trkB isoforms have been observed in humans, including the full-length receptor and two truncated variants, T1 and T-Shc, lacking intracellular kinase domain (Shelton et al., 1995; Stoilov et al., 2002). Similarly, three different isoforms are expressed in mice and rats; a full-length receptor and two truncated variants, T1 and T2, T1 corresponding to T1 are expressed in the human brain (Klein et al., 1989; Klein et al., 1990a; Middlemas et al., 1991 see Fig. 2).

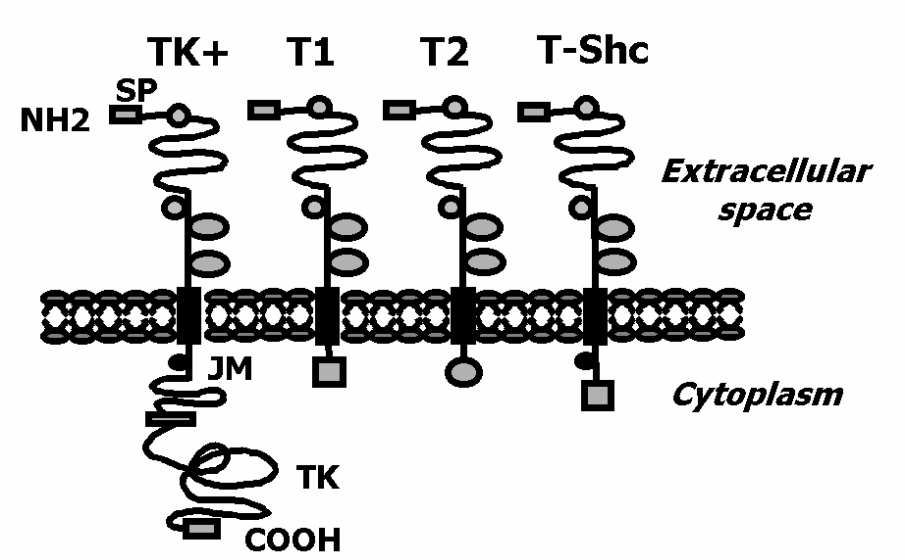

Fig.2 Different splicing variants coded by the *trkB* gene. Abbreviations: SP = signaling peptide, JM = juxtamembrane region, TK = tyrosine kinase region (adopted from A. Haapasalo)

Full-length trkB is a 145-kDa protein, showing 37 % similarity to trkA in the ligand-binding extracellular domain and 75 % similarity in the intracellular kinase domain (Middlemas et al., 1991). The receptor consists of a N-terminal signal peptide required for targeting of the receptor, N-glycosylated extracellular region responsible for ligand binding, transmembrane domain and cytoplasmic kinase domain initiating intracellular signaling cascades (Martin-Zanca et al., 1989; Middlemas et al., 1991; Schneider and Schweiger 1991). The intracellular part includes 10 conserved tyrosine residues, which may be autophosphorylated and serve as binding sites for PTB or SH2 domain containing proteins (Stephens et al., 1994). Full-length trkB is abundantly expressed in the brain, showing strong expression in the cerebral cortex and the hippocampus. More specifically, the strongest fl-trkB expression in the hippocampus is seen in the dendrites and postsynaptic densities of the pyramidal neurons of CA1 - CA3 and scattered neurons in the dentate hilus, while the expression in the dentate gyrus granule cells is lower (Schmidt-Kastner et al., 1996b; Wu et al., 1996; Drake et al., 1999). Although the strongest expression is seen in dendrites, fl-trkB is localized also to soma and axons, residing mainly in the intracellular vesicles in the absence of neuronal activity (Kryl et al., 1999; Haapasalo et al., 2002). It should be noted that fl-trkB expression is developmentally regulated, showing highest levels early in postnatal development and then reducing slightly to adult levels (Masana et al., 1993; Allendoerfer et al., 1994; Fryer et al., 1996).

2.3.1 Signaling pathways of trkB

BDNF is the primary ligand of trkB together with NT-4/5, but also NT-3 may bind to the receptor (Klein et al., 1991; Soppet et al., 1991; Squinto et al., 1991; Klein et al., 1992). Since NT-4/5 and NT-3 are markedly less expressed, most of the trkB signaling is probably mediated by BDNF. Ligand binding to the fl-trkB leads to dimerization of the receptor and autophosphorylation of the intracellular tyrosine residues of trkB (Huang and Reichardt 2001; Miller and Kaplan 2001; Patapoutian and Reichardt 2001; for review, see Huang and Reichardt 2003; Miller and Kaplan 2003; Segal 2003). At least five of the conserved tyrosine residues are phosphorylated following receptor activation, depending on the ligand and cell type (McCarty and Feinstein 1999). Phosphorylation of tyrosine analogous to trkA tyr-490 creates a binding site for Shc and FRS2 adaptor proteins, leading to the activation of Ras-mitogen-activated protein (MAP) kinase- and phosphatidylinositol-3-kinase (PI-3K)/Akt signaling cascades. Similarly, phosphorylation of tyrosine corresponding to trkA tyr-751 recruits and activates PLCγ. In addition to these, three tyrosine residues in the kinase domain may create binding sites to yet unidentified phosphotyrosine binding motif or SH2 domain containing proteins. Although the different cascades mainly convey distinct effects, they can interact with each other.

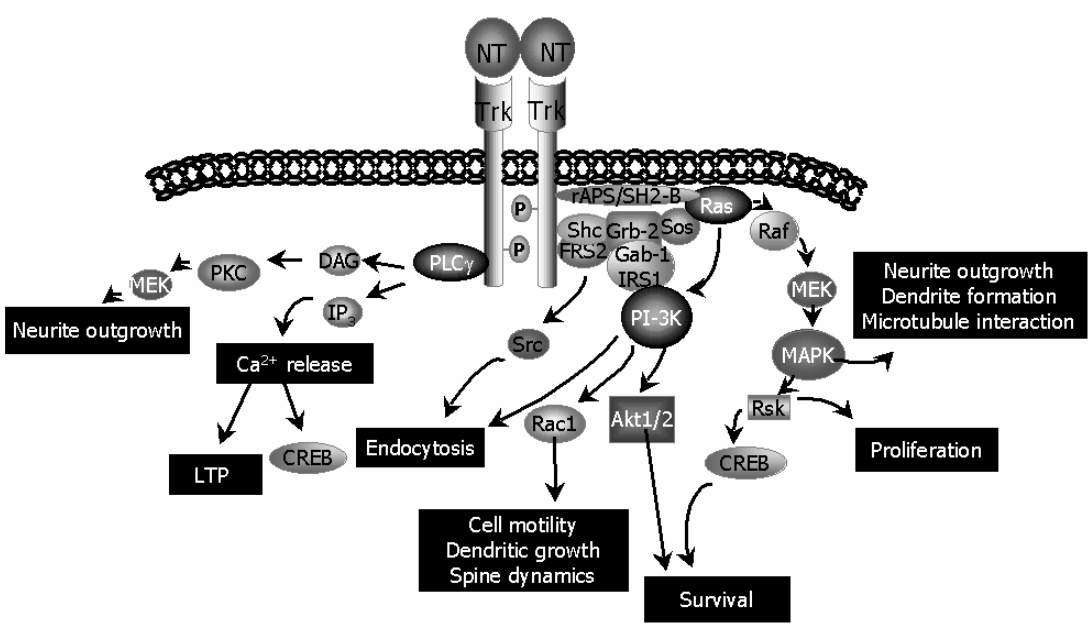

Fig.3. BDNF-induced signaling pathways elicited via fl-trkB receptor. Abbreviations: Akt = serine-threonine protein kinase, CREB = cyclic AMP response element binding protein, DAG = diacylglycerol, IP_3 = inositol-1,4,5-trisphosphate, LTP = long-term potentiation, MAPK = mitogen-activated protein kinase, NT = neurotrophin, PI-3-K = phosphatidylinosotol-3-kinase, PKC = protein kinase C, PLCγ = phospholipase Cγ, Ras = GTP-binding protein, Shc = adaptor protein with SH2 domain (modified from Kaplan and Miller 2000)

2.3.1.1 PLCγ pathway

TrkB activation recruits PLCγ binding to the intracellular tail of the receptor leading to the phosphorylation of PLCγ (Huang and Reichardt 2001; Patapoutian and Reichardt 2001; Huang and Reichardt 2003; Segal 2003). PLCγ further cleaves phosphatidylinositol-1,4,5-tris-phosphate to inositol-1,4,5-tris-phosphate (IP_3) and diacylglycerol (DAG). DAG may activate Ras-MAPK pathway, another trkB-induced signaling cascade, providing a mechanism for crosstalk between signaling cascades. Increase in IP_3, instead, leads to Ca^{2+} release from intracellular stores. The following increase in intracellular Ca^{2+} levels activate Ca^{2+} mediated signaling pathways, which for example activate cyclic AMP response element binding protein (CREB) transcription factor via calsium-calmodulin dependent kinases. Increased intracellular Ca^{2+} may also potentiate neurotransmitter release from synaptic vesicles (Lessmann 1998). Furthermore, because PLCγ is slightly sensitive to Ca^{2+}, concurrent neuronal activity may facilitate the activation of PLCγ pathway leading to enhanced neurotrophin effect. Therefore it is not surprising, that trkB-mediated activation of PLC γ is required for both initiation and maintenance of LTP (Minichiello et al., 2002). Thus, PLCγ-pathway allows activity-dependent modification of synapses and provides BDNF a mechanism to convey electrical activity to structural changes induced by LTP.

2.3.1.2 Ras-MAPK pathway

The Ras-MAP kinase pathway is initiated by the phosphorylation of tyr-490 in intracellular kinase domain, which creates a binding site for adaptor proteins (Huang and Reichardt 2003; reviewed in Miller and Kaplan 2003; Segal 2003). These adaptors seem to convey different signaling cascades, one leading to transient downstream activation promoting differentiation and prolonged survival of neurons whereas the other causes prolonged activation and induces proliferation. Although the Ras-MAPK pathway mainly regulates differentiation and proliferation of neurons, some of its targets are not associated with differentiation. For example, is linked to regulatation of receptor endocytosis. Furthermore, the Ras-MAPK pathway seems to convey ~20 % of the cell survival signals mediated by trkB receptor by regulating the activity of anti-apoptotic proteins. In conclusion, different adaptor molecules steer the effect of the Ras-MAPK pathway mainly to cell differentiation or proliferation, although other functions exist.

2.3.1.3 PI3K/Akt pathway

Ligand binding to trkB receptor activates PI-3K via alternative pathways. This activates downstream signaling cascades, which control the activity of proteins essential in modulating cell survival (Miller and Kaplan 2001; Huang and Reichardt 2003; Segal 2003). Indeed, in

many cell populations, 80 % of neurotrophin mediated cell survival signals are conveyed via PI-3K. The targets of the pathway include proapoptotic proteins, transcription factors controlling the expression of proapoptotic proteins and proteins inhibiting the promotion of cell survival. PI-3K may also indirectly suppress apoptosis by regulating inhibitor of apoptosis-protein family and caspase inhibitors. Besides regulating cell survival, PI-3K mediated signaling recruits signaling proteins to the plasma membrane, including trkB activated proteins controlling the behavior of F-actin cytoskeleton providing neurotrophins another route to mediate cell motility. Similarly, PI-3K is reported to regulate intracellular vesicle trafficking, mediating the effect of neurotrophin gradients to steer growth cones (Ming et al., 1999). PI-3K also conveys trkB-mediated effects to axon diameter and branching that are distinct from those mediated by Ras-MAPK pathway. Altogether, PI-3K/Akt cascades are mainly considered as a cell survival promoting pathway, although it does convey also other crucial signals.

In addition to the three well-known pathways, binding of c-Abl to the intracellular juxtamembrane region of trkA (and probably to other trk receptors as well) has been recently observed (Patapoutian and Reichardt 2001). The interaction is not dependent on tyrosine phosphorylation, and it has distinct effects to neuronal differentiation. Mutation of the region in trkA prevents the differentiation of PC12 cells without affecting to Ras-MAPK pathway. Moreover, trkB receptor can be activated by G-protein coupled receptors PAC-1 and adenosine receptor A2A (Lee and Chao 2001; Lee et al., 2002). Interaction of A2A with trkB, possibly via scaffolding proteins, leads to slow activation of trkB receptor and PI-3K. These additional activation mechanisms further increase the complexity of trkB-mediated signaling and highlight the importance of the control of signaling pathways.

2.3.2 Truncated trkB receptor

Truncated trkB receptors are ~95 kDa proteins with a ligand binding domain and transmembrane region identical to full-length receptor. Differing from the fl-trkB, they lack the intracellular kinase domain. Instead, T1 has a 23 amino acid long intracellular tail and the intracellular tail of T2 consists of 21 amino acids, where the last 11 and 9 amino acids, respectively, differ from full-length receptor and are unique for each isoform (Middlemas et al., 1991). Human T-Shc has an intracellular domain including Shc binding site, but lacking the kinase domain (Stoilov et al., 2002). Therefore, truncated receptors bind ligands as effectively as full-length receptor, but the normal signaling pathways are not activated. TrkB.T2 is weakly expressed in the brain and little is known about its functions as well as the functions of the T-Shc isoform. Instead, T1 is abundantly expressed in the brain showing both

neuronal and glial expression (Klein et al., 1990a; Klein et al., 1990b). In the hippocampus T1 is expressed both in the pyramidal neurons of CA1-CA3 and dentate gyrus as well as in GABAergic inhibitory interneurons of the hilus. Moreover, astrocytes express T1 in the hippocampus. In addition, similar to BDNF and fl-trkB, the expression of T1 is developmentally regulated. It is expressed at low levels in the prenatal brain, increasing to adult levels during late postnatal development (Masana et al., 1993; Allendoerfer et al., 1994; Fryer et al., 1996). In adulthood, T1 is the predominant trkB receptor in the brain, and the shift from fl-trkB to T1 as a predominant receptor form corresponds to decreased activation of fl-trkB receptor (Escandon et al., 1994; Knusel et al., 1994; Fryer et al., 1996).

Because the intracellular tail of T1 is very short, mainly passive roles for T1 have been proposed. It is thought to inhibit the fl-trkB and, indeed, after ligand binding T1 can dimerize with full-length receptor inhibiting its autophosphorylation (Haapasalo et al., 2002). As hypothesized, no signal transduction can be elicited from the T1 – fl-trkB dimer. The mechanism was first suggested when coexpression with T1 receptor inhibited fl-trkB in *Xenopus* oocyte and hippocampal cell culture (Eide et al., 1996; Li et al., 1998). Moreover, microinjection of T1 decreased cell survival in trigeminal ganglion (Ninkina et al., 1996). These results prove that truncated T1 receptor can prevent the activation of fl trkB by a dominant negative action when coexpressed in the same cell. The strong expression of T1 in glial cells has also lead to a proposal that T1 might function as a ligand trapper limiting the availability and spread of BDNF or NT-4/5 (Biffo et al., 1995; Fryer et al., 1997; Rubio 1997; Alderson et al., 2000). Glial cells might therefore also present the ligands to fl-trkB in order to balance the local ligand concentration. Thus, T1 regulates the activation and function of fl-trkB by a dominant negative inhibition and ligand trapping.

Although short, the intracellular tail of T1 has been proposed to participate in signal transduction. Baxter et al. (1997) suggested that T1 might participate in slow intracellular signaling, because BDNF application increased the release of acidic metabolites in a cell line expressing only T1 isoform. Only recently Rose et al. (2003) demonstrated that brief BDNF application to astrocytic culture elicits an intracellular signaling cascade mediated by T1 independently of fl-trkB. Binding of BDNF to T1 leads to PLCγ activation and Ca^{2+} release from intracellular stores and simultaneously, Ca^{2+} entry to astrocytes from the extracellular space. The increase in intracellular Ca^{2+} causes a slow Ca^{2+} current that spreads along the astrocyte network. Interestingly, the presence of T1 on mice lacking fl-trkB exacerbates cellular damage in comparison to mice lacking all trkB isoforms (Luikart et al., 2003). As astrocytes modulate the activity of neurons and other types of glial cells, these results suggest

that BDNF signaling via T1 receptor may be a conceivable mediator of neuron-glia interaction or regulator of neuronal excitability in the adult brain.

2.3.3 Localization of trkB isoforms

Although T1 is predominantly expressed in glial cells, neuronal coexpression with fl-trkB occurs (Rudge et al., 1994; Armanini et al., 1995; Wetmore and Olson 1995). Both isoforms are distributed throughout the neuron, showing expression in both axonal and somatodendritic compartments (Kryl et al., 1999). Instead, the subcellular localization of isoforms differs. In unstimulated neurons, T1 mainly resides in the plasma membrane, whereas fl-trkB is mainly located in intracellular vesicles (Du et al., 2000; Haapasalo et al., 2002). Moreover, coexpression of T1 with fl-trkB in the plasma membrane leads to decrease in the amount of full-length receptor on the cell surface (Haapasalo et al., 2002). Electrical stimulation or neuronal activation rapidly recruits fl-trkB to the cell surface without evident changes in T1 expression (Du et al., 2000; Kingsbury et al., 2003). Similarly, brief application of BDNF leads to transport of fl-trkB to the plasma membrane, whereas longer exposure to BDNF causes down-regulation of the full-length receptor from the cell surface (Haapasalo et al., 2002). This activity-dependent relocalization of the fl-trkB probably is mediated by increased intracellular Ca^{2+} levels via opening of voltage-gated Ca^{2+} channels (Du et al., 2000; Kingsbury et al., 2003). Interestingly, both *BDNF* and *trkB* promoters contain Ca^{2+} responsive CRE elements, suggesting that intracellular Ca^{2+} levels may play a role in enhancing BDNF signaling in active neurons (Shieh et al., 1998; Tao et al., 1998; Tao et al., 2002; Kingsbury et al., 2003).

2.4 Regulation of BNDF/trkB signaling

BDNF/trkB signaling is an essential regulator of several biological functions, and is therefore carefully regulated in the brain. Regulation involves the production and expression of BDNF and trkB, the specificity and activation of the receptor and the intracellular signaling that is elicited (Poo 2001; for review, see Lu 2003a; Lu 2003b). As discussed above, T1 regulates the expression and activation of the fl-trkB receptor and the influence of a common neurotrophin receptor, p75, which brings additional complexity in the regulation of BDNF/trkB signaling that will be discussed later.

Expression of both BDNF and trkB are tissue- and cell type specific, and the availability of trkB finally determines which BDNF actions are elicited. Tissue-specific expression is controlled by several different promoters expressed in limited tissues (Timmusk et al., 1993).

Intriguingly, an absence of a short insert in the extracellular juxtamembrane region of trkB makes the receptor specific to BDNF, whereas the presence of this insert allows also binding of other trkB ligands, NT-4/5 and NT-3 (Strohmaier et al., 1996; Boeshore et al., 1999). Besides the ligand, the mode of activation and content of signaling proteins in the target cell define the signaling pathways elicited.

Transcription of BDNF and trkB is controlled by neuronal activity via increased intracellular Ca^{2+}; both genes contain CRE and/or CaRE1 elements activated by binding of Ca^{2+} responsive CREB or CaRF transcription factors, respectively, leading to initiation of transcription. Besides translation in the soma, BDNF mRNA was suggested to be translocated to dendrites for local translation (Aakalu et al., 2001; Steward and Schuman 2001). Indeed, BDNF mRNA is transported to dendrites, and the transport is facilitated by neuronal activity and BDNF release (Tongiorgi et al., 1997; Righi et al., 2000; Simonato et al., 2002). It is not clear whether BDNF is targeted to active synapses or trapped by them, but in both cases the result is local, activity-dependent translation of BNDF.

BDNF protein is packed into vesicles and transported to correct cellular compartments, unless translated on site. Unlike most growth factors, BDNF release from vesicles is controlled by neuronal activity and similarly to transportation, BDNF is able to induce its own release (Canossa et al., 1997; Kruttgen et al., 1998). It should be noted that also proBDNF is transported and released to extracellular space (Heymach et al., 1996; Mowla et al., 1999; Mowla et al., 2001). Some studies have even suggested that the proregion of BDNF may be crucial for protein transportation, because valine 66 to methionine (val66met) mutation in the proregion impairs BDNF transportation (Egan et al., 2003). The extracellular release of proBDNF provides another dimension in the BDNF signaling, since uncleaved proBDNF mediates, via p75 receptor, actions different from those elicited via trkB (Hempstead 2002). Therefore the regulated cleavage of proBDNF by extracellular matrix proteases increases the complexity of the signaling cascade.

As already discussed, availability of fl-trkB in the plasma membrane is regulated by both neuronal activity and T1. The presence of fl-trkB on the cell surface is required for the activation and internalization of the receptor complex after ligand binding. Exceptionally, during internalization trkB remains active and bound to both BDNF and several signaling proteins recruited to the kinase domain (Grimes et al., 1996; Howe et al., 2001). This signaling endosome is required for trkB signaling and the carrying of the message to the cell nucleus. Altogether, the development of the complex and multi level regulation of BDNF/trkB signaling proposes a crucial role of BDNF in both the developing and adult brain.

Similarly, the association to several neurological diseases highlights the importance of the regulation of BDNF signaling.

2.5 Animal models of BDNF/trkB signaling

Several animal models have been produced to study BDNF signaling. First, BDNF signaling has been decreased by knocking out BDNF or trkB gene from the mouse genome. However, most $BDNF^{-/-}$ and $trkB^{-/-}$ mice die soon after birth, and only a small fraction survives for 2-4 weeks (Klein et al., 1993; Ernfors et al., 1994; Jones et al., 1994; Korte et al., 1995; Minichiello and Klein 1996). $BDNF^{-/-}$ mice that survive exhibit reduced size and weight, impaired motor behavior, coordination and balance as well as localized neuronal loss. Moreover, impairment of LTP induction has been observed (Korte et al., 1995). However, a conditional knockout $trkB^{-/-}$ mice survive, exhibiting cortical degeneration, dendritic retraction and neuronal death after onset of the knockout (Xu et al., 2000). On the contrary, $BDNF^{+/-}$ mice are viable and fertile, and do not expresses gross abnormalities in behavior (Ernfors et al., 1994; Snider 1994; Gorski et al., 2003b). Similar to $BDNF^{-/-}$ mice, heterozygous BDNF knockout mice show impaired LTP and learning (Korte et al., 1995; Patterson et al., 1996; Linnarsson et al., 1997; Gorski et al., 2003a). Furthermore, increased inhibition in the dentate granule cell layer has been reported (Olofsdotter et al., 2000). Also $trkB^{+/-}$ mice are viable and fertile showing hyperactivity, reduced exploration and impaired learning, as seen also in $BDNF^{+/-}$ mice (Minichiello et al., 1999b; Zorner et al., 2003). BDNF signaling can be reduced also by overexpression of T1 receptor isoform in neurons (TK- mice; Saarelainen et al., 2000b). As other mice with decreased BDNF signaling, TK- mice show impaired long-term memory, although no changes in LTP are seen (Saarelainen et al., 2000b).

Increased BDNF signaling has been produced by overexpression of BDNF (Causing et al., 1997; Croll et al., 1999; Huang et al., 1999), but no marked phenotype has been seen in any of the mouse lines. However, BDNF overexpression increases seizure severity to KA induced seizures, promotes maturation of cortical inhibition, and enhances dendritic complexity in the cortex (Croll et al., 1999; Huang et al., 1999; Tolwani et al., 2002). BDNF signaling can also be enhanced by increasing the expression of fl-trkB receptor in neurons (TK+ mice; Koponen et al., 2004), which reduces the possibility to induce downregulation of the signaling due to continuous BDNF expression. Also, increased BDNF signaling enhanced learning and memory, as described in other comparable models. To study the function of trkB receptor more carefully, targeted mutations to specific tyrosines have been made. Surprisingly, mutation of Shc-binding site in trkB causes only a mild phenotype, without

affecting LTP (Minichiello et al., 1998; Korte et al., 2000; He et al., 2002; Postigo et al., 2002). Instead, mutation to PLCγ site leads to severely impaired LTP induction, indicating the significance of PLCγ signaling in learning and memory (Minichiello et al., 2002).

2.6 p75 neurotrophin receptor

The first identified neurotrophin receptor was p75, which binds all mature neurotrophins with similar, although low, affinity in comparison to trk receptors (Rodriguez-Tebar et al., 1990; Rodriguez-Tebar et al., 1992; for review, see Kaplan and Miller 2000; Dechant and Barde 2002; Hempstead 2002). After discovery of the trk family, it was mainly believed to passively present ligands to trkB and enhance trk signaling. Recently, however, new evidence of a p75 role has emerged. Now it is widely accepted that p75 may both oppose and augment trk signaling, that coincident activation of trk receptors modifies the signaling of p75 and that p75 has both trk-dependent and trk-independent actions.

First, p75 plays a role in ligand discrimination of trkB by reducing its affinity to non-cognate ligands such as NT-4/5 and NT-3 and may increase trkB affinity to BDNF. Second, p75 is involved in axonal growth via the cytoskeletal affecting protein Rho (Walsh et al., 1999a; Walsh et al., 1999b; Yamashita et al., 1999). In the absence of neurotrophin binding, p75 activates Rho, which in turn inhibits axonal elongation. The binding of neurotrophin to p75 abolishes p75 mediated Rho activation both *in vitro* and *in vivo*. Third, BDNF may affect inhibitory synaptic transmission via p75 (reviewed by Dechant and Barde 2002). It has been reported to promote inhibitory synaptic transmission in between sympathetic neurons and cardiac myocytes *in vitro* (Yang et al., 2002). *In vivo*, p75 does not seem to affect hippocampal LTP, but hippocampal LTD may be impaired in $p75^{-/-}$ mice. Fourth, in the absence of trkB signaling in sympathetic neuron culture, p75 effectively promotes, and is required for, neuronal apoptosis (Bamji et al., 1998; Roux and Barker 2002). Especially, p75 seems to cause cell death following neuronal injury and it is increased after seizures (Roux et al., 1999). In fact, p75 is upregulated in degenerating hippocampal pyramidal neurons and the blockade of its activity by anti-p75 antibody reduces cell death without affecting seizure behavior (Yi et al., 2003). Activated trk receptors silence p75 mediated apoptotic signaling, but does not interfere with p75-mediated survival signaling collaborating with trkB. Surprisingly, it was discovered that proforms of neurotrophins bind to p75 receptor with high affinity causing only limited activation of trk receptors. Therefore, the balance between mature and proneurotrophins may regulate the activation of signaling cascades either via trk receptors or p75. In conclusion, the contradictory effects of neurotrophin signaling are

controlled by a complex regulation of trk and p75 receptors as well as the balance between pro- and mature neurotrophins.

2.7 Epilepsy

Epilepsy is the second most common neurological disorder after stroke, affecting ~1 % of the population worldwide (Hauser 1997). It is described as transient and recurrent abnormal electrical activity in the brain leading to expression of spontaneous seizures. Despite the treatment with modern antiepileptic drugs, 25-50 % of patients continue to have seizures after epilepsy diagnosis. Recurrent seizures, in turn, may lead to decline in cognitive function in a subpopulation of patients, and the decline may progress in response to uncontrolled seizures (Pitkänen and Sutula 2002; Pitkänen et al., 2002b) In the future, increased life expectancy is predicted to enhance the appearance of epilepsy, since incidence of epilepsy is again increased in elderly people. Since the time that patients live with epilepsy is expected to lengthen simultaneously with increased life expectancy, good seizure control is very important for both the patients and the community.

Epilepsy is commonly classified by five different axes. These include ictal phenomenology, seizure type, epilepsy syndrome, etiology and possible impairment caused by seizures (Engel and International League Against Epilepsy (ILAE). 2001). Seizure types are divided to self-limited and continuous seizures, and further to focal and generalized seizures. In TLE, secondarily generalized self-limited seizures are the most common seizure type. The different seizure types may differentially influence the development of pathological features, since seizure severity probably regulates neuronal damage, but this issue remains to be further studied.

TLE is the most common symptomatic epilepsy. It is assumed to begin with an initial insult, such as head trauma, SE, stroke or brain inflammation (Mathern et al., 1996). After the initial insult, the brain of a subset of the patients undergoes molecular and structural reorganization, leading to changes in neuronal networks and finally emergence of spontaneous epileptic seizures (see Fig. 4). This time is called the latent period, which ends with the appearance of spontaneous recurrent seizures and epilepsy diagnosis. Simultaneously, abnormal interictal spiking activity may be detected in the EEG recordings. Because of differences in the latent period, the development of epilepsy may take in humans anything from months to tens of years. So far, the risk factors are not well known, and the risk for epileptogenesis cannot be predicted for patients, although it might greatly help in determining those in the need of alleviating or preventive treatment when it becomes available.

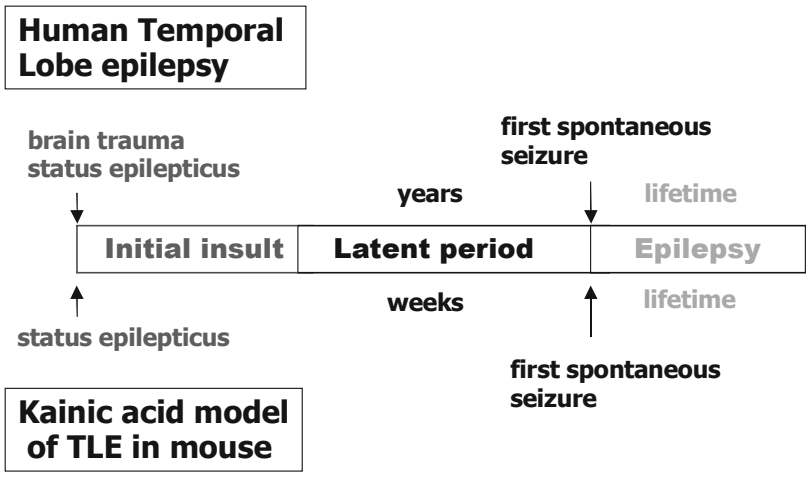

Fig.4 Epileptogenesis in human TLE and KA model of TLE

2.7.1 Pathophysiology of epilepsy

An initial insult, such as SE, induces several molecular and structural changes in the brain. These include at least both acute and delayed cell loss, neurogenesis, gliosis, axonal and dendritic sprouting, inflammation and changes in the extracellular matrix and plasma membrane leading to neuronal network reorganization (Pitkänen and Sutula 2002; Scharfman 2002; for review, see Lukasiuk and Pitkänen 2004). Most of the above mentioned changes are not only limited to the time shortly after the brain damaging insult, but do continue during epileptogenesis. Similar changes are seen in the brain of TLE patients as well as in animal models of epileptogenesis. Epileptic changes are most studied in the hippocampus, a cortical structure often damaged in epilepsy (see Fig. 5a). However, similar changes may also occur in the other brain areas, and similar mechanisms as in the hippocampus may underlie the changes also in other structures.

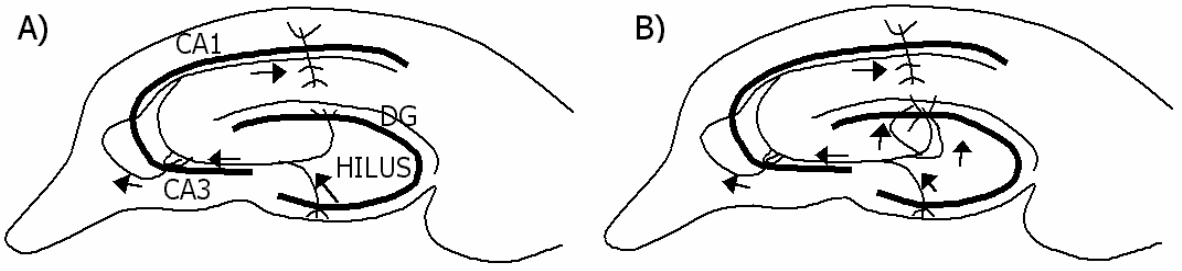

Fig.5 Hippocampal neuronal circuitry in the normal (a) and epileptic (b) brain. Arrows represent the direction of the information flow. Abbreviations: CA1-CA3 = hippocampal subfields, DG = dentate granule cell layer.

Neuronal loss is a prominent feature of the epileptic brain (Dam 1980). It is evident in several brain regions both in animal models as well as in the human brain. It has been long debated whether neuron loss is a cause or consequence of the seizures, but several studies have provided evidence that even brief seizures can cause neuronal death (Cavazos and Sutula 1990; Cavazos et al., 1994). Moreover, increased seizure severity promotes neuronal death in the hippocampus, although not all seizures seem to damage neurons (Mathern et al., 1996; Mathern et al., 2002). Indeed, some neurons are more vulnerable to seizures than others; rodent hippocampal pyramidal neurons of both CA3 and CA1 as well as subpopulations of hilar neurons, such as mossy cells, show marked death after seizures, whereas dentate granule cells are highly resistant to excitotoxicity (see Fig.5). However, in humans, single seizures cause only very limited damage, and the neuronal loss becomes evident only 20-30 years after the onset of epilepsy.

Neurogenesis is another typical feature for epileptic hippocampus (Bengzon et al., 1997; Gould & Tanapat 1997; Gray and Sundstrom 1998; Nakagawa et al., 2000; Parent 2002). Unlike other SE-induced changes, it is limited to the subventricular zone and the hippocampal subgranular zone. Several seizure types induce proliferation of progenitor cells, leading to increased immature granule cell number in the hippocampus. Immature granule cells do differentiate and mature into dentate granule cells, mainly locating to the granule cell layer (Markakis and Gage 1999; Scharfman et al*.,* 2000). The maturing granule cells are thought to integrate into the granule cell network and form synaptic connections similar to old granule cells, possibly replacing lost neurons and affecting neuronal network reorganization (Markakis and Gage 1999; Scharfman et al., 2000). However, after seizures some granule-cell like ectopic neurons are found next to CA3 region in the hilus, where they abnormally connect to the hilar – CA3 network (Scharfman et al., 2000).

Another remarkable morphological alteration seen in the human epileptic brain is increased and/or abnormal sprouting of neurons (Sutula et al., 1989; Houser et al., 1990). The classic example is sprouting of the hippocampal granule cell axons, mossy fibers, to the inner molecular layer of the dentate gyrus (see Fig 5b). Here, dentate granule cell axons normally projecting to hilar interneurons and CA3 pyramidal neurons sprout through granule cell layer and target the dendrites of the granule cells. Mossy fiber sprouting is first seen 1-2 weeks after seizures and it progresses throughout the life targeting to both granule cells and the GABAergic hilar neurons (Sutula et al., 1988; Davenport et al., 1990; Okazaki et al., 1995; Kotti et al., 1997; Morin et al., 1999). Although participation of the new born neurons has been suggested, mossy fibers seem to mainly originate from mature granule cells. Especially, axonal sprouting is a widely studied phenomenon, but the functional consequences of the

reorganization are not clear. The formation of excitatory loop increasing seizure susceptibility and promoting epileptogenesis has been proposed. In contrast, sprouting may also lead to restoration of synaptic contacts and altered inhibition in the hilus.

Besides axonal sprouting, also dendritic remodeling occurs in the epileptic brain. Apical dendrites of the hippocampal dentate granule cells enlarge, increasing the possibility to the formation of positive feedback loop in the hippocampus. Moreover, seizure-induced formation of new basal dendrites projecting from granule cell layer to hilus creates another new target for sprouting mossy fibers (Spigelman et al., 1998; Ribak et al., 2000).

Gliosis is a prominent, although less studied phenomenon in the epileptic brain. Seizures lead to a marked rise in glial cells, indicated by the increased expression of glial proteins (Hansen et al., 1990; Steward et al., 1991). Simultaneously, glial cells undergo morphological alterations possibly linked with functional changes in glia. Especially, changes in the expression of membrane channels and receptors, such as ion channels, gap junctions, and glutamate receptors, may be linked to altered electrical properties of the glial network (Steinhauser and Seifert 2002; Seifert et al., 2004). Since the glial network regulates neuronal function via buffering extracellular ion levels, balancing local neurotransmitter and growth factor concentrations and regulating pH, glial cells may have a noteworthy role in controlling influences of the extracellular milieu, for example to neuronal excitability.

Recently, many of the SE-induced molecular changes have begun to be unveiled in more detail with the microarray technique. Although the lists of genes with altered expression from different studies are quite divergent, similar functional groups are revealed; during SE and early epileptogenesis, genes responding to cellular stress and neuronal injury are differentially regulated in comparison to controls (Zagulska-Szymczak et al., 2001; Becker et al., 2002; Becker et al., 2003; Lukasiuk et al., 2003; Lukasiuk and Pitkänen 2004). Only a few studies have concentrated on epileptogenesis, revealing altered expression of genes involved in synaptic plasticity, axonal growth, signal transduction, gliosis and regulation of cell cycle, correlating with cellular repair and regrowth later after initial insult (Hendriksen et al., 2001; Becker et al., 2003; Lukasiuk et al., 2003; Lukasiuk and Pitkänen 2004). In fact, overlapping gene expression profiles of genes involved in axonal growth and neurogenesis have been observed between neuronal development and epileptogenesis a week after SE suggesting the re-expression of developmentally regulated genes during epileptogenesis, which may be involved in the reorganization of neuronal networks (Elliott et al., 2003). Moreover, changes in cytoskeletal organization and extracellular matrix as well as cell adhesion and metabolism have been observed during epileptogenesis (Lukasiuk et al., 2003). The highest number of altered genes is seen a few days after SE and the number decreases gradually (Becker et al.,

2003). Furthermore, the gene expression patterns between animals in the latent phase and animals having epilepsy is markedly divergent, possibly due to the alterations caused by spontaneous seizures (Becker et al., 2003; Lukasiuk et al., 2003). These findings suggest that epilepsy is a progressive disease affected by multiple genes underlying several functional processes. The clarification of these processes might help to understand the development of epilepsy.

2.7.2 Animal models of epilepsy

To better understand the development of epilepsy and to study antiepileptic drugs, several animal models mimicking the disease have been developed. It should be noted that most animal models only simulate certain features of epilepsy, instead of representing the disease as seen in humans. For instance, seizure models inducing a limited number of seizures can be used to study the effects of single or repeated seizures instead of the progression of epilepsy. On the contrary, either chemical or electrical SE-inducing models more carefully mimic human epilepsy starting with initial insult. Both systemic and localized application of chemicals is used to induce SE, as well as both continuous and intermitted sustained electrical stimulation.

2.7.2.1 Chemoconvulsants; Kainate model

Kainate is a widely used glutamate agonist leading to depolarization of neurons and overexcitation of the central nervous system (Mathern 1999; Ben-Ari and Cossart 2000). It induces seizures and sustained SE in response to a single injection, producing acute neuronal injury similar to that seen in TLE patients (Schwob et al., 1980; Ben-Ari 1985; Mathern 1999). After systemic kainate injection, seizures quite often begin from the hippocampus spreading to other brain regions, whereas focal kainate injection determines the origin of the seizures (Ben-Ari et al., 1981; Lothman and Collins 1981). Similarly, cellular responses vary slightly between systemic and focal application (Zagulska-Szymczak et al., 2001). After the initial insult, a latent period follows as in human TLE and recurrent spontaneous seizures occur afterwards. Therefore, the time course of epileptogenesis resembles that of the medical history of human patients and many other aspects similar to human disease are seen (Mathern 1999). As a drawback, especially systemic injection of kainate may dose-dependently lead to notable mortality, and biological variation in responses may be noticeable. However, due to its simplicity, kainate is a popular method to induce SE leading to the development of the disease closely resembling human TLE.

2.7.2.2 Electrical stimulation

Electrical stimulation is another method to interfere with brain function, leading to either development of single seizures or SE. Sustained intermitted or continuous electrical stimulation is used to induce the development of self-sustained SE resembling that induced by chemoconvulsants. After a latent period, animals express spontaneous recurrent seizures representing characteristics similar to TLE (Mathern 1999). Accordingly, both the early and late neuronal death is seen mainly in the same regions as in epileptic patients (Mathern 1999). Self-sustained SE avoids the possible neurotoxic effects of chemical convulsants, and allows the specification of the seizure focus unlike systemic chemoconvulsants. Moreover, stimulation with electricity enables variations in the stimulus intensity and frequency, which can be used to modulate the development of epilepsy. However, electrical stimulation is technically more demanding and cannot overcome the problem of biological variation. Therefore electrical induction of self-sustained SE is a good, although demanding protocol to induce disease state resembling human TLE.

Another electrical stimulation model used to induce seizures is kindling, where repeated administration of initially subconvulsive stimulus leads to progressive intensification of seizure activity and finally to convulsive seizures. Like human epilepsy, kindling induces progressive neuronal loss in the hippocampus and amygdala as well as axonal sprouting (Mathern 1999). Spontaneous recurrent seizures, a hallmark of epilepsy, are however, rarely induced except after a large number of stimulations (Mathern 1999; Scharfman 2002). Although widely used as an epilepsy model, it may therefore better model the controlled formation of abnormal neuronal plasticity in the brain.

It should be noted that most animal models have been first developed in rats and have only later been applied to other animals, such as mice, cats and primates. Therefore, although chemical and electrical models of epilepsy are well defined in rat, much less is known of their function and usage in other animals. Moreover, different models may cause slightly different changes in different species, not to mention variations between laboratories using models. Those differences complicate the comparison of results from animal studies to human epilepsy and to studies using other animal models.

2.8 BDNF in epilepsy

Many mechanisms associated with pathophysiology of epilepsy are regulated by BDNF in the healthy brain. First, regional cell death is prominently seen in the epileptic brain and BDNF crucially contributes to neuronal maintenance and survival as well as cell death during normal conditions. Second, both axonal and dendritic changes as well as synaptogenesis are

observed after seizures, and BDNF is well known to regulate neuronal morphology and growth. This may further lead to neuronal reorganization clearly seen in the epileptic brain. Third, neurogenesis has been recently reported in the epileptic hippocampus, and BDNF is suggested to either increase neurogenesis or the survival of new born neurons at least in the subgranular zone of the hippocampus. Fourth, glial cells are thought to play an important role in regulating neuronal functions, and truncated trkB was recently proposed to regulate electrical properties of the astrocyte network. Moreover, the ability of BDNF to enhance excitatory synaptic transmission and decrease inhibition in active synapses may be involved in the spread and development of epileptiform activity in the neuronal network. Based on these common findings, BDNF has been proposed as one of the key players both during initial insult, latency period, and epilepsy.

In fact, seizures increase the expression of both BDNF mRNA and protein in several animal models of epilepsy (Zafra et al., 1990; Ernfors et al., 1991; Isackson et al., 1991; Gall 1993; Humpel et al., 1993; Schmidt-Kastner et al., 1996a; Liang et al., 1998; reviewed in Jankowsky and Patterson 2001). Increased expression is seen most prominently in the hippocampal pyramidal cells, dentate granule cells and hilar neurons, as well as in neurons of both superficial and deep cortical layers. BDNF is also upregulated in piriform and entorhinal cortex and in the amygdala. Strikingly, the expression pattern is almost similar in all animal models, supporting a conserved BDNF response to epileptic seizures. Even individual afterdischarges are sufficient to induce increase in BDNF content, although the extent of the increased expression is related to the severity and duration of seizures (Ernfors et al., 1991; Isackson et al., 1991; Bengzon et al., 1993; Rudge et al., 1998; Mhyre and Applegate 2003). Upregulation of BDNF mRNA is first seen 30 min after the initiation of seizures and the expression returns to baseline in ~6 h (Zafra et al., 1990; Dugich-Djordjevic et al., 1992; Dugich et al., 1995). The temporal pattern of BDNF protein upregulation is slower; increased immunoreactivity is first seen at 3 h after seizures and it peaks at 24 h, staying transiently elevated for 48 h (Nawa et al., 1995; Goutan et al., 1998; Katoh-Semba et al., 1999; Poulsen et al., 2002). The second peak of BDNF expression emerges 2 weeks after seizures, corresponding to increased synaptogenesis at that time (Goutan et al., 1998; Nadler 2003). This increase is localized to primary dendrites, nucleus and perisynaptic extracellular space, reflecting BDNF release from neurons, but axonal immunoreactivity is hardly seen (Goutan et al., 1998). The proposed long term increase of BDNF expression possibly leads to morphogenetic changes in the adult neurons (Suzuki et al., 1995). TrkB receptor is also regulated by seizure activity. Both acute spatial and temporal expression resemble closely that seen in BDNF, although the net change is more modest (Bengzon et al., 1993; Humpel et al.,

1993; Merlio et al., 1993; Dugich-Djordjevic et al., 1995). Interestingly, the total expression of fl-trkB is not markedly affected, but the synthesis of truncated trkB receptor is increased from day 3 on, possibly in astrocytes due to gliosis (Goutan et al., 1998; Rudge et al., 1998). However, activation of fl-trkB receptors is enhanced especially in the hippocampal mossy fibers after kindling and KA-induced seizures (Aloyz et al., 1999; Binder et al., 1999a; He et al., 2002). Convincingly, increased expression of both BDNF and trkB has been observed also in the brain of TLE patients (Murray et al., 1994; Mathern et al., 1997; Takahashi et al., 1999; Murray et al., 2000; Zhu and Roper 2001).

2.8.1 BDNF in seizures and SE

The effect of BDNF to excitability, seizures and SE has been studied with several models. Acute application of BDNF to normal hippocampal slices increases excitability and prolonged intrahippocampal BDNF infusion produces spontaneous seizures and abnormalities in the cortical EEG (Scharfman 1997; Scharfman et al., 2002a). Similarly, application of BDNF to slices from the epileptic rat hippocampus containing mossy fiber sprouting enhances synaptic transmission from mossy fibers to the inner molecular layer and provokes spontaneous bursts of dentate granule cells, possibly generated in sprouted axon plexus (Scharfman et al., 1999). BDNF enhances excitatory transmission also in the epileptic human hippocampus with no marked effect to inhibitory transmission (Zhu and Roper 2001). Moreover, after chronic BDNF infusion animals exhibited faster progression of the SE in response to pilocarpine, and exogenous BDNF excacerbates acute cellular damage at the CA1 pyramidal cell layer (Rudge et al., 1998; Scharfman et al., 2002b). Accordingly, transgenic mice overexpressing BDNF show increased seizure severity in response to KA treatment in comparison to wt controls (Croll et al., 1999). This evidence strongly support the participation of BDNF/trkB signaling in the response to enhanced neuronal activity, seizure generation, and the severity of SE.

2.8.2 BDNF in epileptogenesis

Most epilepsy-related neuronal changes take place during epileptogenesis, including delayed neuronal death, axonal sprouting, and neurogenesis leading to neuronal network reorganization. Although all these may be affected by BDNF, the long-term effects of BDNF to epileptogenesis have remained poorly studied. However, Kokaia et al. (1995) reported, that $BDNF^{+/-}$ mice showed suppressed develoment of kindling in comparison to wild type (wt) controls, although the maintenance of kindling was not affected. Similarly, the development

of kindling was reduced in wt mice after blocking BDNF signaling with trkB-IgGs, which harvests BDNF from the extracellular space (Shelton et al., 1995; Binder et al., 1999b; He et al., 2002). On the other hand, controversial results have been obtained, suggesting that chronic BDNF infusion delays kindling (Larmet et al., 1995; Osehobo et al., 1999; Reibel et al., 2000) and the application of BDNF antisense oligonucleotide aggravates seizures in hippocampal kindling (Reibel et al., 2000). Although kindling may not be the ideal model for epiletogenesis, these results allude to the involvement of BDNF/trkB signaling in the development of epileptogenesis, but the effect is controversial.

2.8.3 BDNF in the disease state

The role of BDNF in epilepsy has remained unresolved. No animal studies have been performed to clarify the possible role of BDNF in the aggravation of spontaneous epileptic seizures or in modifying the disease otherwise. However, BDNF expression is altered in the epileptic human brain (Murray et al., 1994; Mathern et al., 1997; Takahashi et al., 1999; Murray et al., 2000; Zhu and Roper 2001) and a polymorphism of the non-coding region of BDNF gene may be a risk factor for epilepsy; the allele 240T is increased in patients with partial epilepsy in comparison to control population (Kanemoto et al., 2003). BDNF may play a role in the plastic changes induced by recurrent spontaneous seizures, therefore possibly leading to changes in the neuronal network. These changes may favor the formation and strengthening of excitatory and/or inhibitory synapses, leading to altered balance between excitatory and inhibitory currents. Therefore, BDNF may either promote or suppress epileptogenesis and modify the outcome of epilepsy.

2.8.4 Molecular mechanisms of BDNF in the development of epilepsy

The possible molecular mechanisms of BDNF signaling in epileptogenesis are mainly unknown. Activity-dependent regulation of BDNF/trkB signaling may be important, possibly leading to hyperexcitation of synapses activated by the seizure activity. In fact, excess engagement of the mechanism mediating LTP has been speculated to cause hyperexcitability in the brain. Furthermore, BDNF has been suggested to regulate its own release in normal conditions (Canossa et al., 1997; Kruttgen et al., 1998), which might increase the effect of seizure activity during epileptogenesis. The main pathway involved in BDNF-mediated LTP is initiated by the engagement of PLCγ, but the function of this pathway in epileptogenesis remains to be clarified (Minichiello et al., 2002). Instead, the effect of Ras-MAP kinase pathway, the main cascade mediating BDNF-induced survival signals, in the development of

kindling was assessed. Surprisingly, mutation in the trkB receptor Shc binding site caused no alteration in the development of kindling, suggesting that other signaling cascades are responsible for epileptogenic changes (He et al., 2002). Unraveling the functions and mechanism of BDNF action would be helpful in understanding epileptogenesis, and might guide the development of medication preventing epileptogenesis as well as better antiepileptic drugs.

3 AIMS OF THE STUDY

Epilepsy is a neuronal disorder affecting ~1 % of the population worldwide. Although medication exists, many patients still suffer from drug resistant seizures throughout their lives. Invention of more effective drugs, or ideally, preventing epileptogenesis, would therefore be of great benefit. For that, we need more detailed knowledge of the molecular mechanisms underlying the disease. The hallmarks of epilepsy in the brain are cell death, neuronal sprouting, and abnormal neuronal network reorganization. BDNF is a neurotrophic factor involved in neuronal maintenance and plasticity in the adult brain. Therefore it is suggested to play a role in epileptogenesis, but whether BDNF signaling protects from or promotes epileptogenesis has remained controversial. The aim of this study was to clarify the role of BDNF and its receptor, trkB, in different developmental phases of epilepsy. More specifically, this study aims to:

1) apply a rat kainate model of temporal lobe epilepsy and EEG follow-up to mice

2) elucidate how decreased or increased BDNF signaling affects epileptogenesis
   a. does BDNF signaling modify status epilepticus
   b. does BDNF signaling modify the latent phase
   c. does BDNF signaling modify the severity of epilepsy
   d. does BDNF signaling modify histopathology

3) elucidate how BDNF elicits its actions during epileptogenesis
   a. which processes may be involved
   b. does BDNF regulate its own signaling

4 Materials and methods

4.1 Animals

Two heterozygous transgenic mouse lines were utilized; one overexpressing truncated trkB (TK-), the other overexpressing full-length trkB (TK+) receptor. Transgenic mice were produced by pronucleus injection of N-terminally FLAG-tagged cDNA in Thy-1.2 expression cassette into hybrid CD2F1 (BALB/c x DBA/2) embryos (for detailed description, see Saarelainen et al., 2000a). Thy 1.2 directs the expression of the transgene into neurons, so that the effects seen probably do not originate from glial cell action. Transgenic mice were viable and fertile, and they were indistinguishable from their wild type littermates without biochemical methods. The presence of the transgene was verified by PCR from the tail tissue. Both mouse lines show altered trkB mediated signaling; decreased activation of trkB receptor in TK- mice (Saarelainen et al., 2000a) and increased trkB activation in TK+ mouse line (Koponen et al., 2004).

For the experiments, heterozygous transgenic male mice were mated with wild type (wt) CD_2F_1 females to produce both transgenic and wt control mice from the same litters. Adult male mice (age 8-10 weeks) were used in all experiments. Animals were housed in controlled conditions (constant temperature 22±1^{0}C, humidity 50-60%, 12:12 light-dark cycle). Standard food pellets and water were freely available. All animal procedures were performed in accordance with the guidelines of the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were accepted by the Experimental Animal Ethics Committee of the University of Kuopio.

Intrahippocampal KA injections and the quantification of the development of epileptogenesis were performed in collaboration with Prof. Antoine Depaulis (Université Joseph Fourier de Grenoble, France) and were therefore not approved by the Experimental Animal Ethics Committee of the University of Kuopio.

4.2 Expression of transgene

Verification of the transgene expression was done by PCR. Small pieces of tail tissue were lysed in lysis buffer (200mM NaCl, 20 mM EDTA, 40 mM Tris-HCl, pH 8.0, 0.5% SDS, 0.5% β-mercaptoethanol, 0.8 mg/ml proteinase K) and centrifuged. Genomic DNA was precipitated from the lysate with isopropanol, washed with ethanol and dissolved in water. PCR primers recognizing thy-1.2 (5'-CTC CCA CTT CCT TGG CTT-3') and trkB (5'-GCC CCA CGT AAG CTT CGA-3') producing a 500 bp nucleotide were used to probe the transgene.

The transgene expression in the brain structures in mRNA level was quantified with *in situ*- hybridization method (**I-II, IV**). *In situ* hybridization with oligonucleotide probe recognizing the flag-tag in the transgene was performed as described earlier (Koponen et al., 2004). For both mouse lines, the oligonucleotide probe (5' GGCACTTGTCATCGTCGTCTTTGTAGTCGGCA 3'; DNA-core facility of the A. I. Virtanen Institute, University of Kuopio, Finland) was labeled with α-[^{33}P]-dATP (2000 mCi/mmol, New England Nuclear, Boston, MA) by terminal deoxynucleotidyl transferase (MBI Fermentas, Vilnus, Lithuania) and hybridized with postfixed sections. Afterwards, sections were treated with RNase A (Sigma, 20 μg/ml) solution, washed, dehydrated and exposed to Hyperfilm β-Max (Amersham, Buckinghamshire, England) for 12 days.

In article **IV**, mouse BDNF expression was studied with antisense riboprobe (X55573, nucleotides 1-927) that was labeled with α-[^{33}P]-UTP (1250 Ci/mmol, New England Nuclear) by T7 polymerase. After postfixation, sections were hybridized with labeled cRNA probe and processed further as above. To quantify the differences in the expression levels, ^{14}C-standards (American Radiolabelled Chemicals Inc.) were exposed to the film together with the probed sections (IV).

The mRNA expression was quantified with a MCID M4-image analysis system (Image Research Inc.). In article **IV**, the autoradiographic density of BDNF-recognizing probe was separately analyzed from hippocampal CA1 and CA3 subfields and dentate granule cell layer. Optical densities of BDNF expression were converted to μCi/g by using ^{14}C-standards. The statistics were calculated with Mann-Whitney *U*-test.

Transgene expression at protein level was verified with western blotting (**I-II, IV**). Tissue lysis, trkB protein precipitation, western blotting, and detection were performed essentially as described earlier (Aloyz et al., 1999). Briefly, total trkB protein was precipitated with 50 μl wheat germ agglutinin (Pharmacia, Uppsala, Sweden) from homogenized tissue and detected with anti-trkBout antibody (dilution 1:5000 (Knusel et al., 1994)) recognizing all forms of trkB. As a secondary antibody goat anti-rabbit HRP (dilution 1:5000, Boehringer Mannheim Biochemicals, Mannheim, Germany) was used and trkB was visualized with enhanced chemiluminescence (Amersham, Arlington Heights, IL).

4.3 Systemic kainate model of TLE

To monitor EEG activity, epidural screw electrodes were implanted into the skull of anaesthetized animals. Operations were made either before (**I-III**) or after SE (**II-III**).

Animals were fixed to the Kopf stereotaxic equipment and self-made measure electrodes were attached to the drill holes bilaterally over the frontal cortex 1 mm anterior and 1 mm lateral to the bregma. Similarly, a reference electrode was placed 1 mm posterior and 1 mm lateral to the bregma and a ground electrode over the cerebellum. Electrodes and pedestal were fixed to the skull with dental acrylate. Antibiotic (Tribrissen vet. 40 mg/kg, Mallinckrodt Veterinary Ltd., England) was given to the mice isc. prior the operation and 24h after the first injection (**II-III**). Animals were allowed to recover for two weeks after the operation before further steps.

Status epilepticus was induced to mice with i.p. injection of kainate (20 – 40 mg/kg) at the age of 8-10 weeks. The development of SE was monitored with combined video-EEG system for at least 24 h (**I-III**, (Nissinen et al., 2000)) and visually observed for 3-4 h by two observers blinded with regard to the genotype (**I-IV**). Latency to the first seizure and the number and severity of behavioral seizures during the follow-up were observed in all experiments. Moreover, the total duration of SE was calculated. Behavioral seizure severity was classified according to a modified Racine's scale (Racine 1972): 1, head nodding, staring; 2, unilateral forelimb clonus; 3, bilateral forelimb clonus; 4, loss of postural control: falling, jumping, wobbling, rolling etc. An animal was considered to have developed a status epilepticus if it was still seizing 2 h after induction of SE, that is, mice had had seizures on average for 90 min. Control animals received an i.p. injection of saline instead of KA and their EEG was recorded in the end of the study.

The development of epilepsy was followed with the same video-EEG system as above. All long-term EEG recordings were performed in several periods to monitor the possible progress of the disease (**I-II)**. In all experiments, mice were randomly assigned to four groups that were evenly recorded for 24h / day during the recording periods. In Article **I**, the follow-up of the first experiment was conducted in two parts (7 + 4 days) from 6^{th} to 13^{th} weeks after SE and the follow-up of the second experiment was extended in three parts (4 + 4 + 2 days) from 11^{th} to the 20^{th} week after induction of SE, consisting of 11 and 10 days of EEG recording for each animal group, respectively. In Article **II** the follow-up was performed in 3 parts (4 + 4 + 2 days) between 13^{th} and 18^{th} weeks following the induction of SE. In Article **III**, we performed continuous follow-up of all KA treated animals for two weeks, starting 15 min before induction of SE and continuing until the perfusion of the animals. Saline treated control mice were recorded in the end of the follow-up period.

4.4 Tissue processing

4.4.1 Fixation

4.4.1.1 For immunohistochemistry:

In Articles **I-II**, the animals for immunohistochemistry were anesthetized with a mixture of sodium pentobarbital and chloral hydrate. The mice were then transcardially perfused with saline and 4% paraformaldehyde, unless TIMM perfusion was used. The brain postfixed in 4% paraformaldehyde. After cryoprotection in 20% glycerol with 0.02 M potassium phosphate buffered saline, brains were blocked, frozen, and stored in –70^{o}C until sectioning. The brains were cut with a sliding microtome in 1-to-5 (**I**) or 1-to-6 (**II**) series and stored either in 10% formalin or in tissue-collecting solution (30% ethylene glycol, 25% glycerol in 0.05 M sodium phosphate buffer) at – 20^{o}C.

4.4.1.2 For TIMM staining method:

In Articles **I-II**, animals of the long-term follow-up were perfused according to TIMM fixation protocol (Sloviter 1982). Mice were deeply anaesthetized with a mixture of sodium pentobarbital and chloral hydrate and thereafter perfused first with 0.37% sulphide solution (5 ml/min, 10 min) and then with 4% paraformaldehyde (5 ml/min, 15 min). The brains were processed further as above.

4.4.1.3 For *in situ*-hybridization and western blotting:

Animals were anesthetized with CO_2 and the brain was rapidly removed from the skull. For *in situ*-hybridization, the brain was immediately frozen on dry ice. Coronal sections were cut with a Leica CM 3000 cryostat, mounted onto SuperFrost/Plus slides and stored at –80^{o}C until use. For western blotting, both hippocampi were dissected out and stored on dry ice until homogenization (articles **I-II, IV).**

4.4.2 *Histological processing*

Standard histochemical stainings, Thionin (I) and Cresyl violet (II), were used to assess cellular boundaries and neuronal damage in the brain.

Mossy fiber sprouting was studied with TIMM staining as described by Sloviter (1982), **I-II**).

4.4.3 Immunohistochemistry

4.4.3.1 Neuropeptide Y

To detect possible changes in the expression of Neuropeptide Y (**I**), a series of sections were stained immunohistochemically with a polyclonal antibody recognizing Neuropeptide Y

(dilution 1:8000; #821295, Incstar, Stillwater, MN,). Secondary antibody was a biotinylated goat anti-rabbit IgG (1:200, BA-1000, Vector, Burlingame, CA) and the peroxidase activity was visualized with diaminobenzidine.

4.4.3.2 TUC-4:

To assess the birth of new neurons in hippocampus (**II**), sections were stained immunohistochemically with a polyclonal antibody recognizing TUC-4 (dilution 1:5000; AB5454, Chemicon International Inc., Temecula, CA) that is localized in mitotic neurons. As secondary antibody, a biotinylated goat anti-rabbit IgG (1:200, BA-1000, Vector**,** Burlingame, CA) was used, and the peroxidase activity was visualized with diaminobenzidine.

4.4.3.3 Fluoro Jade B:

To detect acute cell death after KA induced SE (**II**), a series of sections were stained with Fluoro-Jade B (Histo-Chem Inc., Jefferson, AR) as described before (Schmued *et al.,* 1997). Fluoro-Jade is a fluorescent marker that binds to degenerating neurons.

4.5 Data Analysis

Immunohistochemical stainings and TIMM-stained sections (**I-II**) were analyzed with Leica DMRD light microscope. Since both sides of the brain were identical, the data analysis was done for one side only. Instead, neuronal damage and mossy fiber sprouting varied along the septotemporal axis of the hippocampus, and therefore septal and temporal ends were analyzed separately. All analyses were performed blindly with respect to the genotype and treatment of the animals.

4.5.1 Cellular damage

Acute cellular damage (**II**) was assessed from Fluoro Jade B stained sections. Analysis was performed under a Leica DMRD fluorescent microscope throughout the hippocampus. The damage was scored semi-quantitatively as follows: 0, no damage visible; 1, <10 % of cells died; 2, 10-50 % of cells died; 3, >50 % of cells died.

To assess the chronic neuronal damage (**I-II**), 3 septal (300 μm apart) and 2 temporal (150 μm apart) sections from each case were included in the analysis. Septally, the first section was chosen at the level where the suprapyramidal and infrapyramidal blades of the granule cell layer form a continuous band [according to the mouse brain atlas of Franklin and Paxinos, (1997) at AP level 1.4-1.6 mm posterior to the bregma]. Temporally, the first section

was taken at the level at which the granule cell layer has an "oval" shape [AP level 3.4-3.6 mm posterior the bregma, Fig. 3C (Franklin and Paxinos 1997)].

The sections were inspected according to a systematic random sampling scheme so that counts were derived from a known and representative fraction of the hilus. The motorized stage of the microscope system was under computer control (Neuro Lucida morphometry system, MicroBrightField, Colchester, VT), and the hilar fields in every histological section were surveyed at evenly spaced x-y intervals (septal: 100 by 100 μm; temporal 120 x 120 μm). For each x-y step, cell counts were derived from a known fraction of the total area by using an unbiased counting frame (septal: 20 by 20 μm; temporal 25 x 25 μm). Glial cells, identified by size and cytological characteristics, were excluded. Statistical analysis of the mean areas of the hilus showed that the mean hilar area in epileptic wt animals was significantly larger than in wt vehicle-injected mice. Therefore, the hilar cell counts are expressed as mean number of cells per hilus rather than as neuronal densities per unit area.

4.5.2 New born neurons

To analyze the distribution and number of new born neurons in the hippocampal hilus (**II**), theTUC-4 immunoreactive somas were plotted from three septal sections with a computer-aided digitizing system (Minnesota Datametrics, St. Paul, MN). To show the layer specific distribution of new born neurons, the analysis was done separately in three regions of the hilus: the granule cell layer, the subgranular layer, and the hilar region.

4.5.3 Sprouting

The density of sprouting was analyzed in each TIMM-stained section along the septotemporal axis of the hippocampus, and separately from septal and temporal ends (**I-II**). At the septal end, sprouting was assessed from the "tip", "mid", and "crest" portions of the granule cell layer by starting at the level at which the suprapyramidal and infrapyramidal blades of the granule cell layer form a continuous band of cells. More temporally, sprouting was scored in the dorsal mid-portion and ventral mid-portion of the dentate gyrus where the granule cell layer of the septal and temporal ends becomes fused and forms an easily identifiable and standardized “oval-shaped” layer. The density of mossy fiber sprouting was scored according to Cavazos et al. (1991): Score 0, no granules; Score 1, sparse granules in the supragranular region and in the inner molecular layer; Score 2, granules evenly distributed throughout the supragranular region and the inner molecular layer; Score 3, almost a continuous band of granules in the supragranular region and inner molecular layer; Score 4,

continuous band of granules in the supragranular region and in the inner molecular layer; Score 5, confluent and dense laminar band of granules that covers most of the inner molecular layer, in addition to the supragranular region. The mean of the scores in all septal sections and the dorsal mid and ventral mid temporal portions were calculated and used in the statistical analysis.

4.6 Hippocampal KA-induce SE

All results from intrahippocampal KA-induced SE presented here have been produced in collaboration, and the experiments were performed by C. Heinrich in Strasbourg, France (Heinrich et al., 2003).

4.6.1 Surgery

All mice were anaesthetized with chloral hydrate (400 mg/kg, i.p.) and placed in a stereotaxic frame in flat skull position. Stainless steel cannula (outer diameter :0.28 mm) connected to a 0.5 µL microsyringue (Hamilton, Bonaduz, Switzerland) via PE20 tubing containing distilled water, was filled with a 20 mM solution of KA in 0.9% sterile NaCl and implanted in the right dorsal hippocampus (AP = -2, ML = -1.5, DV = -2 mm with bregma as reference). Mice were then injected with 50 nL of the KA solution (i.e.,1 nmol) over 1 min using a micro-pump (CMA/100, Carnegie Medicin, Stockholm, Sweden) operating the microsyringe. At the end of the injection, the cannula was left in the hippocampus for an additional 2-min period to avoid reflux along the cannula track. In addition, control mice were injected with 50 nL of 0.9 % sterile NaCl under the same conditions.

After the intrahippocampal injection, a bipolar electrode was implanted in the injected hippocampus at the same coordinates as the injection site. Each mouse was also equipped with three monopolar electrodes placed over the left and right frontoparietal cortices and over the cerebellum (reference electrode). These monopolar electrodes, were inserted in the skull so that only the tip (0.2 mm) protruded onto cortical or cerebellar tissue. All electrodes were fixed on the skull with cyanoacrylate and dental acrylic cement. The animals were then allowed to recover from surgery before being placed in the EEG recording chamber.

4.6.2 Follow-up of epileptogenesis.

EEG activities were recorded using a digital acquisition computer-based system (Coherence, Deltamed, France; sampling rate 200 Hz) in freely moving mice placed in

Plexiglass test cages in a Farraday cage. All EEG sessions were performed in the afternoon at fixed hours during the resting phase of mice as follows: mice were first habituated to their test cage for 1- 2 h, and were then recorded for 2-3 h. EEG monitoring was performed to characterize the development of epileptogenesis. To this aim, a recording of hippocampal and cortical activities was performed in all mice every two days after the kainic acid injection until the mice were sacrificed. Control mice were recorded two or three times after a saline injection. In all experiments, the last EEG recording was monitored on the decapitation day of each mouse. A referential setup was used in which cortical and hippocampal electrodes were referenced with an electrode placed over the cerebellum. This setup allowed analysing EEG activity in different derivations after the acquisition phase. Digitally recorded EEG were analysed with the same software as for acquisition by browsing the EEG manually on a computer screen. Bipolar hippocampal and cortical derivations were used for EEG analyses.

4.6.2.1 Hippocampal recurrent discharge quantification.

The occurrence of spontaneous recurrent epileptic discharges was quantified in wt (n= 7), TK+ (n= 7) and TK- (n= 7) mice. Mice were first recorded as described above until the occurrence of spontaneous recurrent seizures. They were then recorded, three weeks after KA injection, for six consecutive days 1 hour per day. The number and duration of spontaneous recurrent hippocampal discharges were calculated for each animal from each EEG recording. Hippocampal discharges were defined as follows: two hippocampal spikes belonged to the same discharge if the time period between these two spikes was < 1s. We decided to count any hippocampal discharge that lasted for at least 5s. For each mouse, the mean number of hippocampal discharges recorded for 1 hour, as well as the mean duration of time spent on epileptic discharge during 1 hour were calculated for the six day follow-up. Epileptogenesis after an intrahippocampal kainate injection in mice was quantified as follows: the EEG recordings between the hippocampal bipolar electrodes located in the injected hippocampus and between the left and right frontoparietal cortical electrodes were compared to classify focal, secondarily generalized and immediate generalized seizures. EEG was classified to: score 1, hippocampal hyperactivity without organized spikes; score 2, hippocampal spikes; score 3, short hippocampal discharge; score 4, long hippocampal discharge; score 5, hippocampal recurrent seizure; score 6, Hippocampal secondary generalized seizure; score 7, immediate generalized seizure.

4.7 KA-induced changes during epileptogenesis

The gene expression pattern was studied in the epileptogenic phase in both transgenic mouse lines and wild type mice in comparison to untreated animals with the same background (**III**). Three KA treated and three untreated animals per genotype were used; that is altogether 6 different groups were assigned: 1) TK- c, 2) TK- KA treated, 3) wt c, 4) wt KA treated, 5) TK+ c, 6) TK+ KA treated. SE was induced and monitored as described previously. KA treated animals were continuously monitored with video-EEG monitoring system to verify that they remained in the epileptogenic phase, i.e. that no spontaneous seizures occurred before perfusion.

4.7.1 Sample preparation

Mice were sacrificed 12 days after induction of SE and the brain was rapidly removed from the skull. Both hippocampi were dissected out and separately homogenized to Eurozol reagent (EuroClone, Wetherby, UK). mRNA from one hippocampus was extracted according to manufacturers instructions and treated with Dnase (Ambion, Austin, Texas). The quality of DNA was verified with agarose gel electrophoresis before probe preparation.

4.7.2 *Probe preparation*

Probes were prepared according to instructions of the Atlas™ PowerScript™ fluorescent labelling kit (Clontech, Palo Alto, CA). Shortly, mRNA from all three animals of each group was pooled in equal amounts and pools were reverse transcribed to cDNA. Fluorescent dyes Cy3 and Cy5 (Amersham Pharmasia, Uppsala, Sweden) were coupled to cDNA from control and KA treated mice, respectively, for each genotype and purified with Qiaquick PCR purification kit (Qiagen GmbH, Hilden, Germany).

4.7.3 Hybridisation & fluorescence detection

Control and KA-treated samples from the same genotype were hybridised together to a Atlas Glass Mouse 1.0 microarray chips of 1090 verified cDNA sequences (Clontech) overnight. Microarray chips were washed and dried and the intensity of each label was detected by ScanArray5000 microarray scanning equipment (GSI Lumonics/Perkin Elmer, Boston, MA). Results of each genotype were confirmed by preparing and hybridising a new probe as previously, except that this time control samples were labelled with Cy5 and KA treated samples with Cy3. That is, two microarray chips were hybridised for each genotype with dye swapping. Fluorescent intensities were quantified with QuantArray software (GSI

Lumonics) using the histogram quantification method. Besides genes of interest, each chip also included several negative and positive controls, controls for reaction efficacy, specificity, and orientation markers.

4.7.4 Data analysis

For gene expression analysis, data was loaded to GeneSpring software (Silicon Genetics, Redwood City, CA). Data was normalized with GeneSpring normal options and the level of altered expression was set to > 1.25x or < 0.8x (25 % change in expression). Genes with altered expression were named as candidate epileptogenesis related genes. Only genes, which we were able to quantify from both microarrays of one genotype, were considered reliable, and in case of changed expression, similar change in both arrays was required.

Each gene is represented with a gene expression profile, which is composed of ratios of gene expression levels in KA treated versus control mice in each genotype (TK-, wt, TK+). That is, each gene expression profile contains three data points, one from each genotype. For cluster analysis, the data was loaded to Visual Data neural network based software (Visipoint Ltd., Kuopio, Finland) to generate self-organizing map (SOM) based on the gene expression profile (Törönen et al., 1999; Nikkila et al., 2002; Kaski et al., 2003) SOM was organized to 64 nodes (8x8 grid), each node containing genes with a similar gene expression profile. Furthermore, the neighborhood function of SOM places nodes with the most similar gene expression profiles closest to each other and the distinct expression profiles to opposite sides of the grid.

Genes were annotated with Gene Ontology (Ashburner et al., 2000) descriptions to categorize genes according to their biological role (functional class) and the annotations were used to evaluate the biologically relevant groups of similarly regulated genes during epileptogenesis. Only those Gene Ontology descriptions appearing at least 4 times in the microarray data set were included in the further analysis. It should be noted that a gene may belong to several functional classes, so that classes may be partially overlapping.

VisualData software was used to visualise the distribution of functional classes in the generated SOM (see figures 1 and 2). We took advantage of the neighborhood function of the SOM and grouped together neighboring nodes to clusters where genes belonging to a given functional class were overrrepresented, in other words, present more often that would be expected by random sampling. The statistical significance of overepresentation of a given functional class in the cluster was analysed by Fisher's exact test (Wu et al., 2002; Draghici et al., 2003; Hosack et al., 2003; Hosack et al., 2003; Segal et al., 2003).

Candidate epileptogenesis-related genes showing altered gene expression in KA treated mice in comparison to control animals were further classified into subgroups based on Gene Ontology descriptions. The annotation revealed overrepresentation of several functional classes and the statistical significance of the overrepresentation of functional classes among candidate epileptogenesis-related genes was calculated with Fisher's exact test. Therefore, both changes in single candidate epileptogenesis-related genes and functional classes of candidate epileptogenesis-related genes were analyzed.

4.7.5 Real time PCR

Some candidate epileptogenesis - related genes were selected for confirmation with real time PCR. First, mRNA from each mouse was separately transcribed to cDNA with standard RT procedure. Second, cDNA from three mice per group were pooled for the verification similarly to microarray analysis. Gene specific primers were designed with Oligo Software (Molecular Biology Insights, Cascade, CO, USA) and ordered from TAG Copenhagen (Copenhagen, Denmark). The primer sequences used were as follows:

GAP-43up 5'-AGAAGAAGGGTGAAGGGGATG-3',
GAP-43lo 5'-AGAAGAAGGGTGAAGGGGATG-3',
KIF3Aup 5'-ACTCCAGTCCCTGACAAGAAG-3',
KIF3Alo 5'-TCATCAGAGACTGACGCAGAC-3',
TIMP3up 5'-GCTACCACCTGGGTTGC-3',
TIMP3lo 5´-GGCGTAGTGTTTGGACTGATAG-3´.

ABI Prism 7700 Sequence detector (Applied Biosystems) with ABI Prism Sequence Detection System (SDS) 1.9.1 software (Applied Biosystems) was used for running with SYBR Green master mix (Applied Biosystems) and Uracil-DNA N-glycosylase (UNG) enzyme (0.1U, MBI Fermentas, Vilnius, Lithuania. Control samples were run to create standard curve for each gene, and crossing point (CP)-values of treated samples were compared to that. Samples were run as duplicates or triplicates and repeated at least twice. The specificity of RT-PCR was controlled with melting curve analysis using SDS Dissociation curves 1.0 software (Applied Biosystems) and agarose gel electrophoresis.

4.7.5.1 Data analysis

Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as a housekeeping gene to normalize the gene expression in treated samples. The fold changes in gene expression of the treated sample in comparison to control were calculated with the equation

$$(E_{target})^{\Delta CP(target(control-sample))}/(E_{ref})^{\Delta CP(ref(control-sample))}$$

E = real time PCR efficiency of one cycle in the exponential phase

CP = crossing point; the point where the fluorescence of the gene of interest overcomes background fluorescence

ΔCP = change in crossing point between control and KA treated sample

by using REST software (Pfaffl 2001). Statistic was calculated by SPSS 9.0 for Windows by comparing the difference in gene expression of treated sample versus control in both housekeeping genes and genes of interest. The difference between the gene of interest in comparison to the housekeeping gene was then statistically estimated by Kruskall-Wallis nonparametric test and Mann-Whitney *U* post-hoc test.

5 RESULTS

5.1 Transgene expression

Transgenic mice overexpress the transgene mRNA (truncated trkB receptor in TK- and full-length trkB receptor in TK+) in neurons in several brain regions. The transgene expression is most prominent in the hippocampus, cortex, thalamus and the basal nucleus of amygdala. More specifically, hippocampal expression is seen in the pyramidal cells of the CA1- CA3 subfields and in the dentate granule cells and the cortical expression is strongest in the pyramidal cells of layers II-III and IV. The protein expression level in the hippocampus is ~2.5-fold in TK- and 18-fold in TK+. Both tg mouse lines have altered BDNF signaling via fl-trkB receptor due to transgene expression. TK- mice show decrease in the fl-receptor phosphorylation, whereas TK+ mice have increased receptor phosphorylation and PLCγ activation, but no changes in Shc, MAPK or Akt phosphorylation were detected (Saarelainen et al., 2000a; Koponen et al., 2004).

5.2 Applying KA-model and EEG follow-up to mice

To apply the well-defined rat KA model of TLE to mice, the acute response to KA was first tested. The optimal KA dose in CD_2F_1 (BALB/c x DBA) hybrid strain turned out to depend on the manufacturer, ranging from 25 mg/kg to 32 mg/kg in comparison 9-12 mg/kg in rat. Moreover, operated mice with cortical screw electrodes tolerated less KA than naive animals, possibly due to breakage of the blood-brain barrier, and female mice seemed to be more sensitive to KA induced excitotoxicity. As also observed in rats, increase in the room temperature enhanced the effect of KA in mice. The procedure of electrode implantation was also fine-tuned to fit for mice. Smaller screw electrodes were prepared, the recovery from anesthesia and operation examined and extra heat added to the operation stage to keep mice viable during the operation. Moreover, the need of antibiotics and heat during recovery were tested. Lastly, the EEG-recording system was adjusted to be suitable for mice. For example, EEG cables and the monitoring cage were redesigned to suit smaller mice (mean weight ~30g) instead of rats (mean weight ~200g). This was of particular significance to prevent injuries or impairment of the mice during the long recording periods. Moreover, video monitoring of the mice required some extra work in comparison to rats, which both are bigger and express more distinct seizures.

5.3 Effect of decreased BDNF signaling in epileptogenesis; systemic KA (I)

The effect of decreased BDNF signaling to epileptogenesis was studied by using transgenic (tg) mice overexpressing truncated trkB (TK- mice). The effect to the development of epilepsy was studied separately in three phases: SE, epileptogenesis, and epilepsy. As a tool, the i.p. kainate model of temporal lobe epilepsy was used. To assess the role of BDNF in acute excitotoxicity, seizure threshold and the development and severity of SE were monitored. The effects of BDNF signaling to the development of epilepsy were studied with a long-term study starting from the 6^{th} week after SE to 13^{th} week after SE. The study was repeated with another set of animals and the follow-up was extended occurring from the 11^{th} to 20^{th} weeks after SE. Both experiments revealed amazingly similar results even though the number of mice developing epilepsy was low. Finally, the putative disease-modifying role of BDNF was clarified by examining epileptiform activity in the brain and analyzing spontaneous seizures both based on the EEG data and the behavioral scoring.

5.3.1 Decreased BDNF signaling during SE

Seizure threshold of tg and wt mice was tested with five doses of KA ranging from 20 to 40 mg/kg. Latency to the first seizure, duration of SE, the number of seizures during SE, and the behavioral severity of seizures were defined as parameters for quantifying the SE. At higher doses, wt mice suffered from higher mortality during SE in comparison to tg mice. However, the latency to the first seizure and the duration of SE were similar in both genotypes. Furthermore, the number and behavioral severity of seizures during SE did not differ in wt and tg mice, although in later studies a trend towards milder response to KA in tg mice has been observed (unpublished data). Altogether, these results suggest that decreased BDNF signaling may not have a great impact on acute excitotoxicity in the brain.

5.3.2 Decreased BDNF signaling during the latent period

Spontaneous seizures are the hallmark of epilepsy. However, the presence of abnormal spiking activity between seizures, interictal spiking activity, is closely associated to epilepsy in human patients. During the long-term follow-up, both spontaneous seizures and interictal spiking activity were analyzed from the mice; in the first experiment, 33% (4) of wt mice and 31% (5) of tg mice developed epilepsy according to the presence of either spontaneous seizures or interictal spiking activity. In the second experiment, 38% (5) of wt mice and 8% (1) of tg mice developed epilepsy.

The presence of interictal spiking activity in EEG epochs was analyzed in both experiments. In the first experiment, all epileptic animals showed interictal spiking activity. However, TK- mice showed marked reduction in the frequency of the interictal spiking (IIP present in 13% of quantified epochs in tg mice vs. 61% in wt mice). In agreement with this, in the other experiment, none of the tg mice exhibited interictal spiking activity in comparison to the presence of interictal spiking activity in 10% of the epochs quantified in 4 wt mice.

During the follow-up of the first experiment, 3 of 12 wt (25%) and 1 of 16 tg (6%) mice experienced spontaneous seizures. That is, 3 of 4 epileptic wt mice developed spontaneous seizures, whereas only 1 of 5 tg mice suffered from epileptic seizures. Similarly, in the second experiment, 3 of 13 wt (23%) and 1 of 12 tg (8%) mice experienced spontaneous seizures. Furthermore, the latency to the first spontaneous seizure was considerably longer in tg mice in the first experiment (50±5 days in wt mice, 84 days in tg mouse). In the second experiment, the latency to the first seizure could not be reliably detected, because of the late beginning of the monitoring session. However, the suggested later onset of spontaneous seizures does not merely indicate delayed epileptogenesis, since the percent of epileptic animals was not increased after the expanded follow-up in the second experiment (31% after 13 weeks, 8% after 20 weeks). In conclusion, tg mice showed reduced epileptogenesis after systemic KA injection in comparison to wt animals, as suggested by decreased interictal spiking and fewer tg mice developing spontaneous seizures in comparison to wt mice, although the number of epileptic mice did not differ between genotypes.

5.3.3 Decreased BDNF signaling in epilepsy

Severe epilepsy may dictate the life of the patient and easing the symptoms might improve the life quality of patients remarkably. Therefore disease–modifying effects of compounds is becoming a new interest in the field of epilepsy. While analyzing the EEG data from epileptic mice, we noticed that decreased BDNF signaling not only reduced epileptogenesis, but also alleviated epilepsy. First, the frequency of interictal spiking activity was considerably lower in epileptic tg mice in comparison to epileptic wt animals. Secondly, tg mice developing epilepsy had spontaneous seizures less frequently than wt littermates suffering from the disease. Thirdly, TK- mice experienced shorter and behaviorally less severe spontaneous seizures than epileptic wt mice did. These results suggest that overexpression of truncated trkB alleviates epileptic symptoms, if the disease develops.

In addition to EEG and behavioral data, the histology of the mice was studied to see whether decreased BDNF signaling had any influence in cellular level in the brain. Markers of cellular damage and plastic network reorganization were studied in the hippocampus after

follow-up in both tg and wt mice. Cell death was assessed by counting hilar neuron numbers and KA treatment was shown to induce cell death in both genotypes. However, no difference between tg and wt mice were seen in the survival of the hilar neurons. Further, we wanted to test whether neuronal subtypes differ in vulnerability in response to KA-induced SE. Neuropeptide Y is a BDNF-associated marker expressed by a subtype of interneurons and therefore we counted the number of neuropeptide Y immunoreactive neurons in hilus. Surprisingly, KA treatment didn't influence the amount of neuropeptide Y positive neurons at chronic state. Although we found a few cases with increased neuropeptide Y positive fiber varicoses, no differences in neuropeptide Y immunoreactive hilar neuron numbers were observed between TK- and wt mice. Lastly, we addressed plastic changes in the hippocampal neuronal network by visualizing sprouting dentate granule cell axons with Timm staining. In both genotypes, KA treatment increased sprouting especially in the septal hippocampus, but no differences were observed between genotypes. In conclusion, decreased BDNF signaling reduced epileptogenesis and alleviated epilepsy, although no protection from epilepsy-associated changes was seen at the cellular level.

Table 1. Epileptogenesis and the severity of developing epilepsy after systemic KA injection.

			Epileptogenesis			Epilepsy		
Genotype of animals	Total number of animals	Number of included survivors	Total number of epileptic animals	Presence of spontaneous seizures in animals (*n*)	Presence of interictal spiking in animals (*n*)	Frequency of spontaneous seizures	Mean duration in seconds [range]	Presence of interictal spiking in recordings
TK- Exp I								
Wt	24	12	33% (4)	25% (3)	33% (4)	1.18 ± 0.5	39.3 [18-64]	61 % (97/159)
Tg	24	16*	31% (5)	6% (1)	31% (5)	0.20	27.5 [15-40]	12 %* (25/199)
TK- Exp II								
Wt	29	13	38% (5)	23% (3)	31% (4)	0.29 ± 0.2	48.7 [28-84]	10 % (18/192)
Tg	26	12	8% (1)	8% (1)	0% (0)	0.15	29.5 [19-40]	0 %* (0/48)
TK+								
Wt	28	17	41% (7)	35 % (6)	35 % (6)	1.27 ± 0.7	47.7 [37-62]	27 % (49/181)
Tg	23	11	27% (3)	27% (3)	27% (3)	0.98 ± 0.3	47.2 [35-57]	52% (31/60)

Abbreviations: Exp = experiment, TK- = tg mice overexpressing truncated trkB, TK+ = tg mice overexpressing full-length trkB, wt = wild type, tg = transgenic, * = $p<0.05$ compared to wild type animals of the same experiment.

5.4 Effect of increased BDNF signaling in epileptogenesis; systemic KA (II)

The effect of increased BDNF signaling to epileptogenesis was studied after discovering the effect of decreased BDNF signaling. As a tool, we used transgenic mice overexpressing full-length trkB receptor. Similar to the previous study, SE, latent phase and the disease state were analyzed separately. Besides the acute response to excitotoxic insult, SE-induced damage was studied 24 and 48 h after induction of SE. The development of spontaneous seizures and interictal spiking were monitored from the 13th week to 18th week after SE. As in the previous study, histology was further studied to see whether TK+ mice had differences in epileptogenesis associated changes in cellular level in comparison to wt littermates.

5.4.1 Increased BDNF signaling during SE

Seizure threshold was determined with three KA doses (25 - 35 mg/kg). Wt mice showed dose-dependent increase in the number and severity of seizures and the duration of SE in comparison of the lowest dose (25mg/kg) and the higher doses (30-35 mg/kg). The two highest doses were considerably similar indicating saturation of the response at the middle dose. Instead, tg mice showed no dose-dependent aggravation in responses; in tg mice, the response to KA appear to be saturated already at the lowest dose. In line with this, more tg that wt mice developed SE at the lowest dose. Moreover, tg mice experienced increased number and severity of seizures and prolonged duration of SE at the lowest dose in comparison to wt mice. At the higher doses, no marked differences between genotypes were observed, although TK+ mice still experienced more seizures during SE. The mortality or the latency to the first seizure did not differ between genotypes in any of the dose groups. These results suggest that increased BDNF signaling may lead to more severe acute excitotoxic insult in the brain.

Acute neuronal damage was assessed 24 and 48 h after initiation of SE so that all studied animals experienced similar SE. 24h after induction of SE, neuronal death was evident in several brain regions, but no difference was seen between genotypes in any brain region studied; the hippocampal subfields, amygdala, cortex, thalamus or caudate-putamen. Instead, 48h after initiation of SE tg mice showed increased acute neuronal damage both in septal hippocampal CA3b and stratum moleculare and temporal hilus, dentate gyrus and CA1 in comparison to wt mice. Increased cell death was also seen in the basal nucleus of amygdala and in the caudate-putamen. On the contrary, exacerbation of neuronal death was not seen in wt mice between 24 and 48 h. Therefore we suggest that in addition to acute excitotoxic insult, increased BDNF signaling aggravates also acute neuronal death.

5.4.2 Increased BDNF signaling during the latent period

During the follow-up period, a higher percent (41%) of wt mice developed spontaneous seizures or interictal spiking activity than in previous studies. Similar to that, 27% of tg mice showed epileptiform activity during the follow-up. Thus, the number of mice developing epilepsy did not differ between genotypes. From epileptic animals, 6 of 7 wt and 3 of 3 tg mice developed clear interictal spiking activity. The presence of interictal spiking in the EEG epochs analyzed did not differ between genotypes, although tg mice showed a mild tendency towards increased interictal spiking.

All epileptic tg mice experienced spontaneous seizures. Likewise, 6 of 7 epileptic wt mice suffered from spontaneous seizures. Thus, increased BDNF signaling in tg mice did not alter the percentage of animals developing epilepsy. Moreover, it did not influence the frequency of the seizures, which was similar in both genotypes. These results suggest that increased BDNF signaling does not affect to the percentage of animals developing epilepsy in transgenic mice after systemic KA injection. Still, we have to keep in mind that these results do not exclude the effect of BDNF in epileptogenesis.

5.4.3 Increased BDNF signaling in epilepsy

We did not observe any changes in epileptogenesis due to increased BDNF signaling in tg mice in comparison to wt animals. Accordingly, both the frequency of spontaneous seizures and the presence of interictal spiking activity were similar in tg and wt mice. Moreover, the spontaneous seizures were similar in behavioral severity and mean duration in both genotypes.

To assess the epilepsy-related changes in cellular level, we performed histology after the long-term follow-up. As previously, we calculated hilar neuron numbers to estimate cellular damage. No differences in hilar neuron numbers were observed between control and KA treated mice, but epileptic tg mice showed decreased neuronal numbers in comparison to non-epileptic mice. Surprisingly, untreated tg mice showed increased neuron numbers in septal hilus in comparison to wt mice, although no differences in neuronal number were observed between genotypes in KA treated mice.

To study whether the increased hilar neuron number in untreated tg mice was due to increased neurogenesis or survival of neurons, we performed TUC-4 immunohistochemistry. TUC-4 is a neuron specific marker expressed by immature neurons when they start migrating, and it has been used as a marker for new born neurons. We observed increased number of TUC-4 positive neurons after KA treatment in the hippocampal hilus of both genotypes. The increase was more pronounced in the hilus of KA treated wt mice in comparison to wt

controls and in the dentate granule cell layer of KA treated tg mice in comparison to tg controls. However, no difference in TUC-4 immunoreactive neuron numbers was observed between genotypes in any treatment group. These results suggest that increased BDNF signaling in tg mice does not affect neurogenesis. Instead, it may increase the cell survival under normal conditions, because naive tg mice show increased hilar cell numbers in comparison to wt mice. Moreover, increased acute cell death in tg mice after SE may be caused by the destruction of the extra neurons survived in the naive hilus of the tg mice. Increased cell death in tg mice might therefore balance the neuronal network after SE.

We further assessed the epileptogenesis-related plastic changes in the hippocampus similar to the previous experiment. KA treated wt mice showed an increase in mossy fiber sprouting in comparison to untreated controls both in the septal and temporal hippocampus. In tg mice, KA treatment did not increase sprouting markedly. However, most epileptic tg mice showed at least modest sprouting in comparison to slight or no sprouting in control animals. However, no difference in sprouting was observed between genotypes in any group. These results further confirm the results of the previous study, that BDNF signaling per se does not influence the sprouting of the dentate granule cell axons.

5.5 **Effect of altered BDNF signaling in epileptogenesis; intrahippocampal KA**
(unpublished data from C. Heinrich)

To verify the results from the systemic KA model, effects of altered BDNF signaling to the development of epilepsy were tested in a different animal model of epileptogenesis. Epileptogenesis occurs very rapidly after intrahippocampal KA injection, and the main seizure type elicited is focal self-limited seizures (personal communication, C.Heinrich). In this model, development of epilepsy was not significantly altered in TK-mice in comparison to wt animals, although a tendency towards slower development was seen. On the contrary, quantification of the epileptogenesis after intrahippocampal KA injection revealed accelerated development of seizure severity in TK+ mice in comparison to wt mice, and more pronounced, in comparison to TK- mice (see fig.6).

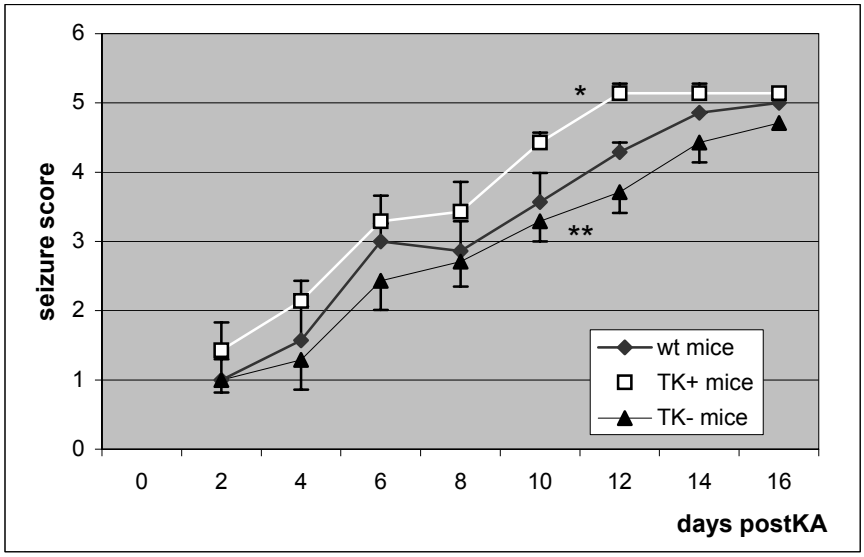

Fig. 6 The development of seizure severity in TK-, wt and TK+ mice after intrahippocampal KA injection.

Moreover, quantification of spontaneous seizures after intrahippocampal KA-injection revealed increased seizure severity in TK+ mice in comparison to wt and TK- mice (see table 2). This was observed as increased number of rapidly generalized seizures, which normally rarely occur in the model, and increased frequency of secondarily generalized hippocampal seizures. Thus, it can be concluded, that overexpression of full-length trkB receptor exacerbates the severity of epilepsy in tg mice, although it could not be detected after systemic KA-induced SE.

Table 2. Classification and numbers of spontaneous seizures after intrahippocampal KA-induced SE

Seizure type	**TK-mice**	**Wt mice**	**TK+ mice**
Rapidly generalized seizure	2	2	4
Slowly generalized seizure	0	0	5
Total number of seizures	2	2	9

5.6 Kainate-induced changes in gene expression

5.6.1 Acute change (IV)

BDNF synthesis is regulated by neuronal activity, and under normal circumstances, BDNF can induce its own synthesis. We have here examined whether BDNF signaling via trkB receptor is involved in the increased BDNF expression during SE. BDNF mRNA expression was measured in the hippocampus of TK- and wt mice 3 h after the induction of SE. A clear increase in the BDNF mRNA was detected in both genotypes in all regions studied: hippocampal CA1, CA3, and dentate granule cell layers. However, the increase was less pronounced in TK- mice, indicating that fl-trkB signaling mediates at least partly the enhancement of BDNF levels also under excitotoxic conditions.

5.6.2 Changes during epileptogenesis (III)

The molecular mechanisms underlying epileptogenesis and BDNF action have remained unclear. We sought to clarify these mechanisms by studying the hippocampal gene expression changes during epileptogenesis in both TK- and TK+ mice and wt littermates. As TK- and TK+ mice have opposite alterations in epileptogenesis, they provide us a perfect tool to identify BDNF-regulated processes and possible mechanisms promoting or preventing epileptogenesis. To minimize the variation caused by the SE, all animals included experienced a statistically similar SE. No differences in SE between genotypes were allowed. The mice were continuously monitored with video-EEG to verify that no spontaneous seizures occurred. Therefore, all mice were concluded to remain in the epileptogenesis phase at the time of sacrifice. Gene expression patterns were studied with microarray and selected genes were confirmed with RT-PCR. It should be noted that gene expression of KA-treated mice was studied relative to control animals of the same genotype. Therefore, the gene expression levels cannot be compared to other genotypes as such.

5.6.3 Candidate epileptogenesis-related genes (III)

All genes were annotated with Gene Ontology keywords and divided into functional classes based on annotations. The genes showing at least 25% ($0.8 < x > 1.25$) change in gene expression in comparison of KA-treated and control mice were defined as candidate epileptogenesis-related genes. The study of functional classes represented among those genes revealed several intriguing classes. Firstly, genes coding for cytoskeletal proteins (14.8% vs. 4%) and intermediate filaments (7.4% vs. 1%) were enriched among candidate epileptogenesis-related genes. Furthermore, extracellular matrix components (7.4% vs. 2.3%)

were overrepresented among genes showing altered regulation in response to epileptogenesis. Secondly, several functional classes associated with cell growth and regulation of developmental processes were regulated by epileptogenesis. Among those, both genes involved in neurogenesis and cell differentiation were highly represented among candidate epileptogenesis-related genes.

5.6.4 SE-induced changes in expression of functionally related genes (III)

To study the changes in functional networks, clustering analysis of the annotated gene expression data was used. The clustering analysis revealed several functional gene classes that showed SE-induced changes in gene expression. From those, genes involved in programmed cell death, DNA repair, and DNA recombination are tightly linked to epileptogenesis-related damage in the brain. Furthermore, genes encoding for transcription and transport-linked proteins showed altered expression in response to epileptogenesis. Moreover, a group of cyclin-dependent kinase (cdk)-inhibitors was regulated in response to SE. However, most functional groups did not show analogous changes in gene expression, suggesting that these processes may not be crucial in epileptogenesis.

5.6.5 BDNF modifies changes in expression of functionally related genes after SE (III)

BDNF-signaling appeared to modify the regulation of several functional classes during epileptogenesis. Firstly, it had a surprising influence to cytoskeleton; in clustering analysis, both microtubule- and actin-binding proteins were overrepresented in an area indicating downregulation in TK- mice. No change in gene expression of transport-linked genes was observed either in wt or TK+ mice. Secondly, TK-mice showed also a slight to moderate decrease in gene expression involved in DNA repair and DNA recombination. Thirdly, increased BDNF-signaling in TK+ mice led to a slight to moderate decrease in expression of genes regulating cell death and initiation of programmed cell death, while TK- and wt mice showed a slight decrease or no alteration in gene expression. Although increased BDNF-signaling showed less influence to gene expression during epileptogenesis, these results well support the role of BDNF as a survival factor.

BDNF-regulated altered gene expression during epileptogenesis was confirmed by real time PCR. The intriguing influence to cytoskeleton was confirmed with kinesin family member 3A (Kif3A), a microtubule-binding protein showing downregulation in TK- mice. Kif3A is further involved in axonal growth, and therefore the downregulation of other growth-related gene, growth-associated gene 43 (GAP-43), in TK- mice was chosen for confirmation. The third confirmed candidate epileptogenesis-related gene was tissue inhibitor

of metalloproteinase 3 (TIMP3), an extracellular matrix protein regulating growth and the turnover of extracellular matrix. The real time PCR verified that the expression of Kif3A in TK- mice was reduced to 0.6x in comparison to controls without changes in other genotypes. Similarly, the expression of GAP-43 in TK- mice was 0.7 fold in comparison to controls, whereas TK+ mice showed a tendency to increased expression of GAP-43 (4.3x expression). Lastly, the expression level of TIMP3 in TK- mice was reduced to 0.3 fold from the control levels without changes in other genotypes. These results suggest decreased growth in TK- mice showing reduced epileptogenesis. They further confirm the reduced expression of transport-linked proteins and extracellular matrix components in association with decreased BDNF-signaling and epileptogenesis and suggest that those processes may play a notable role in the development of epilepsy.

Table 3. BDNF-related changes in functional processes during epileptogenesis

Functional processes (from Gene Ontology)	Enriched in clustering analysis	Epilepsy-associated change in TK- mice	Epilepsy-associated change in Wt mice	Epilepsy-associated change in TK+ mice	Enriched in candidate epileptogenesis-related genes	Expression change confirmed in TK- mice
Cytoskeletal protein binding	X	↓	↔	↔	X	X
Actin binding	X	↓	↔	↔		
Microtubule binding	X	↓	↔	↔		X
Extracellular matrix					X	X
Intermediate filaments					X	
Death	X	↔	↔	↓		
Apoptosis regulator	X	↔	↔	↓		
Neurogenesis					X	
DNA repair	X	↓	↔	↔		
DNA recombination	X	↓	↔	↔		
Transcription	X	↔	↓	↔		

6 DISCUSSION

The aim of this thesis work has been to clarify the role and mechanism of BDNF signaling in different phases of epileptogenesis. First, BDNF signaling promoted both the severity of SE and the acute cellular damage induced by SE. Second, we revealed the protecting role of truncated trkB overexpression in epileptogenesis after systemic KA injection and provided data about the time schedule of epileptogenesis in mice via long-term follow-up after SE. Accordingly, epileptogenesis progressed faster in TK+ mice after intrahippocampal KA injection in comparison to wt controls and more markedly, in comparison to TK- mice, supporting the exacerbating role of BDNF signaling in epileptogenesis. Third, decreased BDNF-signaling modified the outcome of epilepsy by alleviating the severity of the developing disease in comparison to wild type littermates. Fourth, altered expression of several gene groups linked to neuronal reorganization was discovered during epileptogenesis phase, suggesting that BDNF-mediated formation of new synaptic contacts after SE may promote epileptogenesis. Fifth, trkB-mediated BDNF signaling during SE was shown to increase the synthesis of BDNF in mice, therefore enhancing the signaling and probably augmenting the synaptic changes induced by seizure activity.

6.1 Methodological considerations

Truncated trkB has been thought to be unable to convey signaling of its own, and therefore mice overexpressing T1 were supposed to mimic purely decreased fl-trkB activation. Surprisingly, Rose and coworkers (2003) recently suggested that truncated trkB has a fl-trkB-independent function in glial Ca^{2+} homeostasis, and it may therefore regulate neuronal excitability and seizure susceptibility both in the normal situation and during or after excitotoxic insults. Although intriguing, astrocytic function does not explain our results. The expression of the T1 in transgenic mice was directed to neurons with neuron-specific thy-1 promoter, and therefore the changes seen in transgenic mice most likely were due to neuronal changes in the BDNF signaling.

The differential genetic background of animal strains is now a significant variable in animal studies, because strains may have very different traits both behaviorally and at the molecular level. This is especially important when using the KA-model of TLE, because the response to KA-induced acute excitotoxicity varies between mouse strains (Ferraro et al., 1995; Schauwecker & Steward 1997; Royle et al., 1999; Schauwecker et al., 2000). In our case, the transgenic mice are hybrids of BALBc and DBA mouse lines that respond differently to KA;

BALB is resistant to the brain damage, DBA shows more vulnerability (Ferraro et al., 1995; Schauwecker & Steward 1997; Royle et al., 1999). As a result, hybrid mice show resistance to kainate induced cellular death in the brain, and may therefore be less suitable to study transgene effects to cell death. The transgenic mice were compared to wt littermates with similar genetic background, which is important to avoid false interpretations of the results. Therefore the results suggesting increased acute neuronal damage after SE in TK+ mice were quite surprising.

As a model for the study, we chose KA-induced status epilepticus. It is well-defined and relatively simple protocol that mimics human TLE in several features including similar histopathological changes as TLE patients have (Mathern 1999; Ben-Ari and Cossart 2000). Because EEG follow-up was performed with cortical electrodes, only generalized seizures could be measured. However, intraperitoneal KA injections affect to the whole brain and therefore most seizures probably are secondarily generalized and can be detected (Mathern 1999; Ben-Ari and Cossart 2000). It should still be considered, although unlikely, observing no seizure activity in cortical EEG recordings cannot exclude all partial seizures. Even so, the long overall EEG recording time greatly increases the possibility to find all epileptic animals, although the first seizures might be of focal origin. However, the non-continuous follow-up and long non-recorded period after SE recordings prevented us from analyzing the temporal evolution of epileptogenesis. This was later done in collaboration with Prof. Antoine Depaulis group that uses intrahippocampal KA-injection, a model with short latency period, as a model for epileptogenesis. They also recorded hippocampal EEG, allowing them to detect focal hippocampal seizures and analyze the seizure generalization from the hippocampus to the cortex (Heinrich et al., 2003).

Microarrays have become a popular research tool. Microarray technologies are a powerful method to search for differences in gene expression between healthy and diseased tissue, or in identification of gene expression altered in response to pharmacological manipulations (Ginsberg et al., 2000; Mirnics et al., 2000; Landgrebe et al., 2002; Sokolov et al., 2003; Lukasiuk and Pitkänen 2004) The possibility to scan thousands of genes in one experiment is tempting, and even more; may help to identify wholly new genes. However, even with the best planning and implementation, microarray experiments require extreme caution in data analysis and interpretation. Since the technique is relatively new, no standardized instructions are available making it difficult to analyze the data. Differing methods further make it difficult to compare results, especially regarding single genes. Therefore, especially the changes in the expression of single genes should always be verified with an independent method to prevent false positives. Likewise, negative findings in single

genes may not be exclusive for the expression of the given gene. For these reasons, results concerning changes in single genes should be considered with caution. An alternative method to analyze the data is to search for altered functional gene groups, where we have been active (Törönen et al., 1999; Kontkanen et al., 2002; Kaski et al., 2003). It is more reliable and has more power than searching for single genes. Furthermore, it reveals pivotal information about functional networks and processes underlying the circumstances. Therefore, microarray techniques offer strong possibilities in identifying biological processes involved, but it requires careful planning, implementation, data analysis, and confirmation of results.

6.2 BDNF in SE

BDNF has been suggested to play a role in SE, because it causes hyperexcitability and increases seizure severity in response to KA (Scharfman 1997; Croll et al., 1999; Scharfman et al., 1999). Our results agree with the previous findings and extend them both in response to the role of trkB receptor variants and in terms of the more thorough quantitation of the SE. The increased severity of SE in TK+ mice overexpressing fl-trkB implies that first, increased BDNF signaling promotes acute excitotoxic insult, and second, epilepsy-related BDNF signaling is mediated via fl-trkB receptor. The finding that BDNF induces its own synthesis during SE via fl-trkB further highlights the role of trkB in epileptogenesis. Furthermore, we detected lowered seizure threshold in TK+ mice, possibly reflecting increased excitatory synaptic transmission and/or reduced inhibition in the hippocampus. Therefore, excess BDNF appears to overexcite the neuronal network and facilitate acute excitotoxic insults in the brain. These results are in line with the findings that BDNF enhances excitatory synaptic transmission and is a significant mediator of neuronal activity-induced morphological changes in neuronal connectivity (Kang and Schuman 1995; Poo 2001; Tyler and Pozzo-Miller 2003). The proposed overexcitation of the neurons may further be linked to the exacerbated acute neuronal damage observed in TK+ mice two days after induction of SE, as it is known that overexcitation may cause cellular damage in the brain. Similar exacerbation of KA-induced neuronal damage has been observed also before (Rudge et al., 1998). In conclusion, increased BDNF signaling exacerbates SE and acute SE-induced cell death, thereby possibly promoting the later development of epilepsy.

6.3 BDNF in epileptogenesis

Previously, there has been no direct data concerning BDNF in the development of epilepsy besides the acute phase. To elucidate the role of BDNF, we followed the influence of

both increased and decreased BDNF signaling in epileptogenesis in tg mice. Our data reveals that decreased BDNF signaling by overexpression of T1 in neurons reduced epileptogenesis after systemic KA-induced SE, and produced a trend towards slower progression of epileptogenesis after intrahippocampal KA injection. Accordingly, TK+ mice showed faster progression of epileptogenesis after intrahippocampal KA injection, although we did not detect changes in epileptogenesis after systemic KA. These results are in line with previous studies suggesting that decreased BDNF signaling delay the development of kindling (Kokaia et al., 1995; Binder et al., 1999b). On the other hand, long-term BDNF infusion to the brain has been shown to delay the development of kindling in rats (Larmet et al., 1995; Osehobo et al., 1999; Reibel et al., 2000). These seemingly conflicting results probably are due to down-regulation of fl-trkB receptor after long-term exposure to BDNF, which leads to reduced BDNF signaling (Carter et al., 1995; Frank et al., 1996; Knusel et al., 1997; Binder et al., 2001). These results indicate that BDNF can modify the development of epilepsy via trkB receptor mediated signaling. Therefore, it can be concluded that BDNF is one of the key players not only in seizures, but also in the development of epileptogenesis-related changes in the brain.

6.4 BDNF in epilepsy

Based on available literature, the effect of BDNF on epilepsy had not been studied before. Our results are unique in demonstrating the disease-modifying effect of BDNF signaling; overexpression of truncated trkB can decrease both the frequency and severity of spontaneous seizures in comparison to wt littermates. Furthermore, the presence of interictal spiking activity was decreased. In contrast, increased BDNF signaling did not show disease-modifying effects after systemic KA. However, epileptogenesis did progress faster in TK+ mice after intrahippocampal injection of KA, and those mice experienced more rapidly generalized seizures in comparison to wt and TK- mice. The difference in results between i.p.and intrahippocampal KA may be explained by different recording systems, since with cortical EEG measurement we were not able to analyze the generalization of seizures. On the other hand, these two epileptogenesis models differ both in the duration of latency period and histological changes, which may explain partial divergence between models. The disease-modifying effects of BDNF signaling are intriguing, since spontaneous seizures are considered as an important contributor in the progressive neuronal death occurring in the epileptic brain. Alleviating the seizures might therefore delay or prevent the neuronal degradation and related neurological problems (Pitkänen and Sutula 2002; Pitkänen et al.,

2002a; Sperling 2004). Simultaneously, relieved symptoms would improve the quality of life of epilepsy patients

6.5 Possible mechanisms of BDNF in epileptogenesis

6.5.1 Regulation of neuronal survival

The function of BDNF as a survival factor has led to the hypothesis that it might protect neurons during excitotoxic insults. In line with that, we found epileptogenesis-related changes in the gene expression of functional gene groups associated with cell death, neurogenesis and neuronal differentiation in mice with altered BDNF signaling. TK- mice showed decreased expression of genes linked with neurogenesis and neuronal differentiation, which may imply reduced compensatory mechanisms for neuronal damage. Decrease in compensation of neuronal damage might further associate with reduced neuronal network reorganization and therefore might prevent epileptogenesis. On the other hand, TK+ mice showed a tendency towards increased neuronal survival at two weeks after SE. This alteration is in line with the hypothesis that BDNF may promote survival or neurogenesis in the hilus. Interestingly, it might enhance the reorganization of neuronal network and therefore promote epileptogenesis. Surprisingly, mutation in the trkB Shc-binding site mediating cell survival signals does not alter development of kindling, suggesting that other mechanisms may be responsible for BDNF-mediated plastic changes in the adult brain (He et al., 2002). In contrast to increased cell survival during epileptogenesis, increased neuronal death was observed in TK+ mice shortly after SE. This suggests that BDNF is not capable of protecting neurons during acute excitotoxic insult, but it exacerbates the acute neuronal death in the hippocampus. In line with this, exogenous BDNF was shown to enhance neuronal death after SE (Rudge et al., 1998). Therefore, BDNF may not support neuronal survival during SE but may play a role in neuronal survival during epileptogenesis, and increased neurogenesis and neuronal survival may not be as advantageous in epileptogenesis as thought before.

6.5.2 Regulation of neurite growth, synapse formation, and connectivity

BDNF is strongly associated with neurite growth and branching both *in vivo* and *in vitro* (Danzer et al., 2002; Tolwani et al., 2002) and might therefore participate in sprouting of mossy fibers and the branching of granule cell dendrites in the epileptic hippocampus. These changes require alterations in neuronal cytoskeleton and indeed, both microtubule- and actin binding proteins as well as intermediate filaments showed reduced mRNA expression in TK- mice during epileptogenesis. Furthermore, several growth-related protein mRNAs were

downregulated in TK- mice during epileptogenesis, although no change was observed in wt or TK+ mice. These changes propose reduced neurite growth in TK- mice, which may be associated with reduced network reorganization during epileptogenesis. This may in turn, partly cause decreased epileptogenesis observed in TK- mouse line. In addition to the regulation of neurite growth, BDNF promotes formation, maturation, and maintenance of spines and synapses, which is seen during epileptogenesis (for review, see Vicario-Abejon et al., 2002). Similar to neurite growth, microtubule-and actin binding proteins are needed in synapse modification. Moreover, BDNF-mediated remodeling of neuronal connections, possibly via mechanism similar to late LTP, may enhance hippocampal excitability and seizure spread. As reported earlier, BDNF-mediates LTP mainly via PLCγ pathway. In fact, this signaling is greatly enhanced in TK+ mice, although the basal levels of PLCγ protein are decreased in comparison to control (Koponen et al., 2004). This might partly explain the increased seizure severity and lowered seizure threshold in TK+ mice during SE. In conclusion, this evidence suggests that BDNF signaling may play a significant role in the reorganization of the connectivity of the neuronal network in the epileptic hippocampus and may in part lead to the formation of excitable neuronal network that may further promote epileptogenesis.

6.5.3 Effects outside neurons

In addition to neuronal effects, BDNF signaling may be involved in changes in the glial cells and extracellular matrix. Our gene expression studies revealed an increased proportion of extracellular matrix proteins among candidate epileptogenesis-related genes, and those genes had decreased expression in TK- mice during epileptogenesis. Previously, several extracellular matrix components, such as proteinases and their inhibitors, were shown to be required for axonal and dendritic growth and synapse formation (Brew et al., 2000; Baker et al., 2002). For instance, absence of tissue plasminogen activator prevents mossy fiber sprouting after KA induced SE, and the participation of extracellular matrix components in epileptogenesis has been proposed by microarray studies before (Wu et al., 2000; Lukasiuk et al., 2003; Lukasiuk and Pitkänen 2004) Therefore, decreased expression of extracellular matrix components agrees with other gene expression changes in TK- mice suggesting that reduced growth, repair or network reorganization might be associated with decreased epileptogenesis. Moreover, extracellular matrix proteinases, such as plasmin and matrix-metalloproteinases, are suggested to cleave proBDNF to mature form and the absence of extracellular matrix proteinases may facilitate neuronal death via p75 receptor (Seidah et al.,

1996; Lee et al., 2001). Altogether, BDNF signaling may regulate the epileptogenic processes also via the extracellular milieu.

6.5.4 Histology

BDNF signaling has been suggested to influence histopathological changes occurring in the brain during epileptogenesis. However, we did not detect changes in mossy fiber sprouting, a marker for neuronal plasticity, in either transgenic mouse line in comparison to wt littermates. There were no differences either in control, KA-treated non-epileptic, or KA-treated epileptic mice hippocampus. Consistent with our cellular findings, no association between BDNF and mossy fiber sprouting has been observed in other studies (Elmer et al., 1997; Qiao et al., 1997; Routbort et al., 1997; Bender et al., 1998; Qiao et al., 2001). Although BNDF and mossy fiber sprouting may not be associated, a strong increase in BDNF and trkB activation is seen in dentate granule cell somas and mossy fibers after kindling (Binder et al., 1999a; Danzer et al., 2002; He et al., 2002). Therefore, BDNF signaling may play a role in the formation of synaptic contacts in the mossy fibers, although it may not directly regulate sprouting of axons.

Similarly, no changes in the delayed neuronal damage were detected in either transgenic mouse line in comparison to wt mice. However, acute neuronal death was increased in TK+ mice two days after SE in comparison to wt littermates experiencing similar SE. These results are contradictory to the proposed role of BDNF as a survival factor, but similar results have been obtained earlier (Rudge et al., 1998). Moreover, increased BDNF signaling did not enhance neurogenesis, but it may support the survival of new born neurons in normal conditions. These results support the previous findings proposing that BDNF is required for the survival of new born neurons, but it may not promote neurogenesis *per se* (Linnarsson et al., 2000). Altogether, BDNF may play an important role in exacerbating SE and acute neuronal damage, but it may not be able to affect histological changes later during epileptogenesis.

7 Summary

The purpose of this work has been to clarify the role and mechanisms of BDNF signaling via trkB receptor in all development phases of epilepsy. The main findings can be summarized as follows:

First, BDNF signaling exacerbated the severity of SE and lowered the seizure threshold to KA-induced seizures. Furthermore, increased BDNF signaling led to more severe acute neuronal damage after SE in comparison to wt animals experiencing similar SE. This may be linked to increased neuronal activity during SE, since severity and duration of SE may determine the extent of subsequent cellular death in the brain.

Secondly, decreased BDNF signaling reduced the development of epilepsy, and increased BDNF signaling accelerated epileptogenesis. These effects may be linked to plasticity-related mechanism mediating BDNF-dependent changes in the neuronal connectivity; seizure activity may acutely enhance excitatory synaptic connections, and later on induce BDNF-mediated formation and stabilization of new synaptic contacts. On the other hand, effects to neurogenesis, neurite growth, and other processes may be involved. Therefore, increased BDNF during SE may regulate the later formation of excitatory circuits, promoting seizure generation and seizure spread in the brain.

Thirdly, reduced BDNF signaling alleviated the severity of epilepsy, if it did develop. Both the frequency and severity of seizures, as well as the presence of interictal spiking activity were reduced in comparison to wt mice. Accordingly, increased BDNF led to faster development of epilepsy and faster generalization of spontaneous seizures. These results suggest that modifying of BDNF signaling might be helpful in modifying or even preventing the development of epilepsy also in patients.

Fourthly, BDNF signaling causes several changes in intracellular signaling during epileptogenesis. Genes coding for intermediate filaments and extracellular matrix proteins as well as other genes linked to growth, differentiation and neurogenesis were identified as candidate epileptogenesis-related genes in mice with altered BDNF signaling. Moreover, cytoskeletal genes regulating growth cone and synapse motility as well as genes involved in growth, DNA repair, and DNA replication showed reduced expression when BDNF signaling was decreased. Increased BDNF signaling, on the other hand, tended to increase neuronal

survival in epileptogenesis phase. Furthermore, BDNF signaling enhanced the synthesis of BDNF during SE, therefore promoting BDNF-mediated effects. Altogether, several different mechanisms may be involved in BDNF mediated signaling and neuronal repair and regrowth during epileptogenesis may not be as beneficial as thought previously.

Fifthly, the influence of altered BDNF signaling to histological changes was assessed. No effect to the plastic mossy fiber sprouting or delayed neuronal death was observed in either genotype, nor was neurogenesis altered in TK+ mice. However, acute neuronal damage was exacerbated in TK+ mice. Therefore, BDNF signaling may regulate acute neuronal damage, but may not cause major histological changes during epilepsy.

In conclusion, exaggerated BDNF signaling is harmful throughout epileptogenesis. It is mediated mainly via fl-trkB receptor and may be regulated by truncated T1-trkB. BDNF signaling involved in epileptogenesis is probably mediated via plasticity-related mechanisms, enhancing hyperexcitability and altering connectivity, although other pathways may also convey significant effects. Therefore, modification of BDNF signaling might be used to alleviate epilepsy and even to prevent epileptogenesis in the future. However, this requires more research and new means to better recognize patients at risk of developing epilepsy.

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