THESIS ON NATURAL AND EXACT SCIENCES B94

Structure and Regulation of BDNF Gene

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Dissertation was accepted for the defence of the degree of Doctor of Philosophy in Natural and Exact Sciences on May 21, 2010.

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Defence of the thesis: June 21, 2010 at Tallinn University of Technology

Declaration
Hereby, I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for any academic degree.

This dissertation was supported by European Social Fund.

ISSN 1406-4723
ISBN 978-9985-59-997-6

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BDNF geeni struktuur ja regulatsioon

TAMARA AID-PAVLIDIS

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CONTENTS

INTRODUCTION

Development of the mammalian nervous system occurs through complex genetic mechanisms that control the differentiation and maturation of neurons and glia, and make sure that proper interneuronal connections – synapses – are established in correct time and place. However, not only the genotype is responsible for the development and efficient functioning of the nervous system. By stimulating neuronal activity sensory, cognitive, and motor experiences in postnatal period play a key role in shaping neuronal networks. Synaptic function is being modified throughout life, forming long-lasting memories and alterations in the behavior of the adult organism.

During the last two decades, neuronal activity-regulated genes have received special attention. Genes whose products modulate learning and memory by controlling synapse development, function and plasticity have been also implicated in numerous neurological disorders such as Alzheimer's, Parkinson's, Huntington's disease, schizophrenia, depression, epilepsy, drug addiction and autism spectrum disorders. Brain-derived neurotrophic factor, BDNF, has been one of the most 'popular' genes studied. BDNF mutations and disturbances in the regulation of its expression underlie the above-mentioned neurological disorders as well as obesity and some types of cancer. BDNF gene has been thoroughly studied: its exon-intron organization has been described in several species; numerous transcription factors that regulate its promoters have been discovered; BDNF protein processing and localization has gained a lot of attention. Nevertheless, the data that is appearing in the literature poses more questions than answers. How many promoters does BDNF have exactly and how are they regulated? What other regulatory elements could control BDNF expression? What are the differences between human and rodent BDNF gene structure and regulation and why do they exist? In addition, the exact mechanisms of BDNF transcriptional and translational regulation in pathological conditions remain obscure. Answering these questions could shed light on the mechanisms of many human neurological diseases, and lead to the development of new therapies.

OUTLINE AND AIMS OF THE THESIS

The goal of this study was to gain a deeper understanding of BDNF gene organization and its transcriptional regulation. In the first part of the thesis, I review the literature regarding BDNF role in the nervous system and molecular mechanisms that govern BDNF gene expression. First, BDNF actions in the nervous system and its signaling via TrkB and $p75^{NTR}$ receptors are described. Then, the most recent data on the BDNF gene structure, protein processing and secretion are given. After that, BDNF role in synaptic plasticity and neuronal activity-dependent transcription of the BDNF gene is discussed. Neuronal activity-dependent regulation of BDNF transcription by numerous transcription factors and epigenetic modifications is presented in detail. Further, I discuss the role of BDNF in various neurological diseases, drug addiction, depression, obesity and cancer as well as therapy options involving neurotrophins. And finally, I discuss transgenic mouse models that have been used for studying transcriptional regulatory elements in the BDNF gene.

In the second part of the thesis the results of the presented study are discussed. I provide a detailed description of BDNF gene structure in rodents (Publication I), and propose novel regulators of BDNF transcription based on meta-coexpression conservation analysis of microarray data (Publication II). Finally, I describe transgenic mouse models generated to study transcriptional regulation of human and rodent BDNF gene *in vivo* (Publication III and IV). The results of the presented study expand our understanding of the transcriptional regulation of neuronal genes and brings us one more step further to the future prospects of the new drug design.

ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid-type glutamate receptor
Aβ	β-amyloid peptide
GABA	γ-aminobutyric acid
AD	Alzheimer's disease
APP	amyloid precursor protein
AED	antiepileptic drugs
BACE-1	beta-site APP–cleaving enzyme 1
BBB	blood-brain barrier
BDNF	brain derived neurotrophic factor
CaRE	calcium-responsive element
CaMK	calcium calmodulin kinase
CaRF	calcium-responsive transcription factor
CRE	cAMP response element
CREB	cAMP response element-binding
CNS	central nervous system
CBP	CREB-binding protein
cAMP	cyclic AMP
DAG	diacylglycerol
DRG	dorsal root ganglion
DS	Down's syndrome
GABAA	GABA receptor subtype A
GAD	glutamic acid decarboxylase
HD	Huntington's disease
HFS	high-frequency stimulation
HAT	histone acetyltransferase
HDAC	histone deacetylase

HDMT	histone demethyltransferase
HMT	histone methyltransferase
IE	immediate early
IP3	inositol trisphosphate
kb	kilobase pairs
L-DOPA	L-3,4-dihydroxyphenylalanine
LID	L-DOPA-induced dyskinesia
L-VGCC	L-type voltage-gated calcium channels
L-VSCC	L-type voltage-sensitive calcium channels
LTD	long-term depression
LTP	long-term potentiation
LFS	low-frequency stimulation
MMP	matrix metalloproteinase
mRNA	messenger RNA
mGluR	metabotropic glutamate receptor
Met	methionine
MeCP2	methyl CpG binding protein 2
miRNA	microRNA
MAPK	mitogen-activated protein kinase
MEF2	myocyte enhancer factor 2
NMDA	N-methyl-D-aspartic acid
NMDAR	N-methyl-D-aspartic acid receptor
NGF	nerve growth factor
NS	nervous system
NRSE	neuron restrictive silencer element
NTRK2	neurotrophic tyrosine kinase receptor type 2
NT-3-7	neurotrophin-3-7
NAc	nucleus accumbens
PD	Parkinson’s disease
PNS	peripheral nervous system
PI3K	phophoinositide 3-kinase
PIP2	phosphatidylinositol 4,5-bisphosphate
PLC-γ	phospholipase C-γ
KChIP-3	potassium channel interacting protein-3
PSEN	presenilin
PKA	protein kinase A

PKC	protein kinase C
Pol II	RNA polymerase II
polyQ	polyglutamine
REST/NRSF	RE-1 silencing transcription factor/neuron-restrictive silencer factor
RE	regulatory element
SNP	single nucleotide polymorphism
SN	substantia nigra
SP	synaptic plasticity
SSRI	selective serotonin reuptake inhibitor
tPA	tissue plasminogen activator
TFBS	transcription factor binding site
Trk	tropomyosin receptor kinase
UTR	untranslated region
USF-1/2	upstream stimulating factor-1/2
Val	valine
VTA	ventral tegmental area

ORIGINAL PUBLICATIONS

I Aid, T., Kazantseva, A., Piirsoo, M., Palm, K., Timmusk, T. (2007). Mouse and rat BDNF gene structure and expression revisited. Journal of Neuroscience Research 85, 525535.

II Aid-Pavlidis, T.*, Pavlidis, P.*, Timmusk, T. (2009). Meta-coexpression conservation analysis of microarray data: a 'subset' approach provides insight into brain-derived neurotrophic factor regulation. BMC Genomics, 10:420.

III Koppel, I.*, Aid-Pavlidis, T.*, Jaanson, K., Sepp, M., Pruunsild, P., Palm, K., Timmusk, T. (2009). Tissue-specific and neural activity-regulated expression of human BDNF gene in BAC transgenic mice. BMC Neuroscience, 10:68.

IV Koppel, I.*, Aid-Pavlidis T.*, Jaanson, K., Sepp, M., Palm, K., Timmusk, T. (2010). BAC transgenic mice reveal distal cis-regulatory elements governing BDNF gene expression. Genesis, 48, 214219.

* Equal contribution

1 REVIEW OF THE LITERATURE

1.1 Molecular mechanisms of BDNF actions

Brain-derived neurotrophic factor (BDNF, rarely used synonym – abrineurin) belongs to the family of neurotrophins – secreted growth factors that promote neuronal survival, migration and differentiation in vertebrates (Leibrock *et al.*, 1989; Lewin and Barde, 1996). Neurotrophin family includes structurally related NGF, BDNF, NT-3 and NT-4/5 proteins (Radziejewski *et al.*, 1992). Recently identified NT-6 and NT-7 are present only in fishes (Dethleffsen *et al.*, 2003). Each neurotrophin homodimers bind specifically to their receptor - one of the members of tropomyosin receptor kinase (Trk) family. Activation of Trk receptors by a corresponding neurotrophin leads to transcriptional activation of multiple target genes that control cell growth and survival (Kaplan and Miller, 2000). Also, all neurotrophins bind to a common neurotrophin receptor $p75^{NTR}$ (Barker, 2004, 2007) which is a member of the tumor necrosis factor family. In the presence of Trk receptor, it enhances the specificity of the neurotrophin binding to Trk (Carter *et al.*, 1996). It also signals independently by inducing signaling cascades, some being associated with the induction of apoptosis (e.g. Rac1, JNK), and others (e.g. RhoA) – with cell growth inhibition (Barker, 2004).

BDNF promotes differentiation and survival of peripheral and central neurons and glia. It is expressed at high levels in specific neuronal populations in the central nervous system (CNS) and in the peripheral nervous system (PNS) (Leibrock *et al.*, 1989; McAllister *et al.*, 1997), although some studies have detected BDNF expression also in rodent astrocytes (Condorelli *et al.*, 1994; Zafra *et al.*, 1992), microglia (Elkabes *et al.*, 1996), and oligodendrocytes (Dai *et al.*, 2003). In the CNS, BDNF is highly expressed both in the developing and in the adult brain. Importantly, BDNF expression is markedly upregulated by neuronal activity.

1.1.1 Multiple functions of BDNF

The pro-survival effect of BDNF was for the first time demonstrated in 1982, when it was purified from pig's brain and was shown to promote survival of dorsal root ganglion (DRG) sensory neurons (Barde *et al.*, 1982). Besides neuronal survival,

BDNF fulfills many other tasks during the development of the nervous system. Neuronal proliferation, neuronal migration, axon pathfinding, dendritic growth, synapse formation and maintenance, synaptic competition and pruning, neuronal excitability, both inhibitory and excitatory synaptic transmission, long-term plasticity – these are the processes that BDNF actively participates in (Huang and Reichardt, 2001). It modulates such processes in the adulthood like memory (Alonso *et al.*, 2002; Egan *et al.*, 2003), food intake (Lyons *et al.*, 1999; Kernie *et al.*, 2000), energy balance (Xu *et al.*, 2003), and mood (Hariri *et al.*, 2003; Hashimoto *et al.*, 2004). Moreover, BDNF is produced by activated T cells (Moalem *et al.*, 2000) and has been implicated in T cell–dependent neurogenesis in the adul brain (Ziv *et al.*, 2006). Studies have also shown that BDNF participates in cholesterol biosynthesis. During synapse development it acts via TrkB signaling, inducing gene transcription of cholesterol biosynthesis enzymes in neurons but not in glial cells thus mediating a presynaptic exocytosis of synaptic vesicles (Suzuki *et al.*, 2007).

$BDNF^{-/-}$ knockout mice show severe neuronal deficits and die shortly after birth exhibiting reduced axonal diameters and myelination (Cellerino *et al.*, 1997). Also, reduced neuron numbers have been observed in the cerebellum of $BDNF^{-/-}$ mutants (Schwartz *et al.*, 1997). Mice carrying deletions in the Trk genes show increased numbers of degenerating neurons in the CNS (Minichiello and Klein, 1996; Alcántara *et al.*, 1997). A number of studies have shown that BDNF is essential for differentiation and maintenance of GABAergic (secreting γ-aminobutyric acid) striatal neurons (Mizuno *et al.*, 1994; Ventimiglia *et al.*, 1995). BDNF is also known to have trophic effect on serotoninergic neurons. The levels of serotonin and the density of serotoninergic axons are decreased in $BDNF^{+/-}$ animals (Mamounas *et al.*, 2000). This can explain the fact that $BDNF^{+/-}$ animals, although having a normal lifespan, develop enhanced aggressiveness (Linnarsson *et al.*, 1997; Lyons *et al.*, 1999). Finally, long-term potentiation (LTP), a cellular model of learning and memory, is impaired in $BDNF^{-/-}$ animals (Korte *et al.*, 1995; Patterson *et al.*, 1996).

The early postnatal lethality of $BDNF^{-/-}$ mice had suggested a wider function for this neurotrophin. It had been previously shown that in addition to the brain, BDNF is expressed at high levels in the heart and lung (Timmusk *et al.*, 1993; Maisonpierre *et al.*, 1991). Later, it was demonstrated that in the early postnatal period BDNF is expressed in the endothelial cells of intramyocardial arteries and capillaries of the heart. BDNF deficiency led to the reduction in endothelial cell-cell contacts, endothelial cell apoptosis, intraventricular wall hemorrhage, depressed cardiac contractility and early postnatal death (Donovan *et al.*, 2000). However, little is known about the function of BDNF in the lung.

1.1.2 BDNF-induced signaling via TrkB and p75^{NTR} receptors

Tropomyosin receptor kinase (Trk) was first identified as an oncogene (Martin-Zanca *et al.*, 1986). Only after some years it was found to act as a neurotrophin receptor (Kaplan *et al.*, 1991a,b). Members of the Trk family are highly expressed in neuronas. BDNF-specific receptor TrkB (also known as NTRK2) exists both in full-length form (TrkB.FL) as well as in truncated forms which lack the kinase domain (TrkB.T1 and TrkB.T2). Both truncated versions of TrkB are up-regulated during early postnatal development and predominate over full-length TrkB in the adult brain (Fryer *et al.*, 1996). Truncated TrkB receptors can interfere with BDNF signaling by sequestering BDNF (Biffo *et al.*, 1995) or by forming heterodimers with the full-length TrkB (Eide *et al.*, 1996; Haapasalo *et al.*, 1999). TrkB.T1-deficient mice develop normally but show increased anxiety and morphological abnormalities in the length and complexity of neurites in the basolateral amygdala (Carim-Todd *et al.*, 2009). However, it has been shown that BDNF binding to the truncated TrkBs activates glial calcium signaling in astrocytes (Rose *et al.*, 2003; Ohira *et al.*, 2005) and microglia (Mizoguchi *et al.*, 2009). It has also been reported that full-length TrkB increases proximal dendritic branching, whereas truncated TrkB promotes elongation of distal dendrites, and these actions of the two isoforms inhibit one another (Yacoubian and Lo, 2000). Studies have shown that TrkB signaling system is essential in the adult CNS. Postnatal Cre-mediated deletion of TrkB in forebrain neurons resulted in the reduction in size of the cerebral cortex, likely caused by a decrease in size and the number of neurons and their dendrites (Minichiello *et al.*, 1999; Xu *et al.*, 2000).

Most of the BDNF actions are related to its binding to the full-length TrkB receptor. BDNF binding to TrkB induces receptor dimerization, autophosphorylation, and activation of the intracellular tyrosine kinase domain. This leads to the activation of three main signaling cascades: Ras/MAPK (Ras–mitogen-activated protein kinase), PI3K (phosphoinositide 3-kinase) and PLC-γ (phospholipase C-γ) pathways (Kaplan and Miller, 2000; Minichiello, 2009) and the subsequent activation of immediate-early (IE) target genes such as FOS, EGR1 and EGR2 (Calella *et al.*, 2007). Phosphorylated tyrosine 515 of TrkB binds two complexes of adaptor molecules: Shc/Grb2/SOS and FRS2/SHP-2/Grb2/SOS. Shc phosphorylation by tyrosine 515 (Kavanaugh and Williams, 1994) leads to the activation of Ras/MAPK pathway (Minichiello, 2009). It is possible, that recruitment of different Shc is specific for each Trk and could be a basis for Trk-specific responses to neurotrophins. The same tyrosine residue is able to dock another membrane-anchored adaptor protein, FRS2 (Meakin *et al.*, 1999) and activate Ras/MAPK pathway as well (Kouhara *et al.*, 1997). Ras/MAPK pathway controls such processes as neuronal differentiation and neurite growth. Also, Shc phosphorylation and the formation of Shc/Grb2/SOS complex recruits Gab-1 adaptor protein that mediate activation of PI3K. PI3K activates PKB/AKT kinase, which results in phosphorylation and inactivation of proapoptotic protein BAD from the Bcl-2 family. PI3K pathway thus controls neuronal survival and apoptosis. Phosphoryla-

tion of TrkB tyrosine 816 induces binding of PLC-γ to TrkB and its phosphosylation. The association of PLC-γ with TrkB regulates intracellular Ca^{2+} levels and protein kinase C (PKC) activity via the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2) substrate to diacylglycerol (DAG) and inositol trisphosphate (IP3). This pathway seems to play an important role in neurotrophin-mediated neurotrophin release (Canossa *et al.*, 1997) and in synaptic plasticity (Minichiello, 2009).

Actions of BDNF mediated by $p75^{NTR}$ receptor include myelination (Cosgaya *et al.*, 2002), neuronal migration (Carter *et al.*, 2003), neuronal process retraction (Cahoon-Metzger *et al.*, 2001; Gehler *et al.*, 2004), and neuronal apoptosis (Teng *et al.*, 2005; Troy *et al.*, 2002). Also, the role of BDNF/$p75^{NTR}$ signaling in long-term depression (LTD) has been established in adult animals. Mutant $p75^{NTR}$-null mice do not express LTD, exhibit anxiety-like behavior and have difficulties coping with stress (Woo *et al.*, 2005). It has been hypothesized that acute stress may enhance secretion of BDNF precursor, proBDNF, which facilitates LTD in the adult hippocampus through $p75^{NTR}$ signaling. The proBDNF/$p75^{NTR}$/LTD may serve as a pathway that helps to recover from stress (Greenberg *et al.*, 2009).

1.2 Structure of the BDNF gene

BDNF is the most conserved protein in the neurotrophin family exhibiting high sequence similarity among vertebrates, from human to fish (Maisonpierre *et al.*, 1990; Hallböök *et al.*, 1991). There is no evidence for BDNF orthologs in the genomes of non-vertebrate chordate or invertebrate species. The BDNF gene is comprised of multiple exons and introns that span 52.3 kB on chromosome 2 in mouse, 50.2 kB on chromosome 3 in rat, and 66.8 kB on chromosome 11 in human. For almost ten years, descripion of the BDNF gene had been available only for rat (Timmusk *et al.*, 1993) and BDNF gene structure in other species remained neglected. Recently, however, BDNF organization has been also studied in human (Aoyama *et al.*, 2001; Marini *et al.*, 2004; Liu *et al.*, 2005; Pruunsild *et al.*, 2007), mouse (Liu *et al.*, 2006; Aid *et al.*, 2007), frog (Kidane *et al.*, 2009), zebrafish (Heinrich and Pagtakhan, 2004), and seabass fish (Tognoli *et al.*, 2010). The exon-intron sturcture of the BDNF gene is largely similar in different species (Figure 1). Common features of BDNF gene in different species include: i) expression of a large number of alternatively spliced mRNA transcripts; ii) differential usage of tissue-specific and neuronal activity-regulated promoters; iii) usage of alternative transcription start sites, and polyadenylation signals; iv) presence of in-frame ATG start-codons in one or more exons that could produce pre-proBDNF peptides with alternative N-termini. These features reflect the intricate nature of the regulation of BDNF gene expression. However, the differences in BDNF gene structure among species may reflect the differences in the regulation of BDNF expression and function.

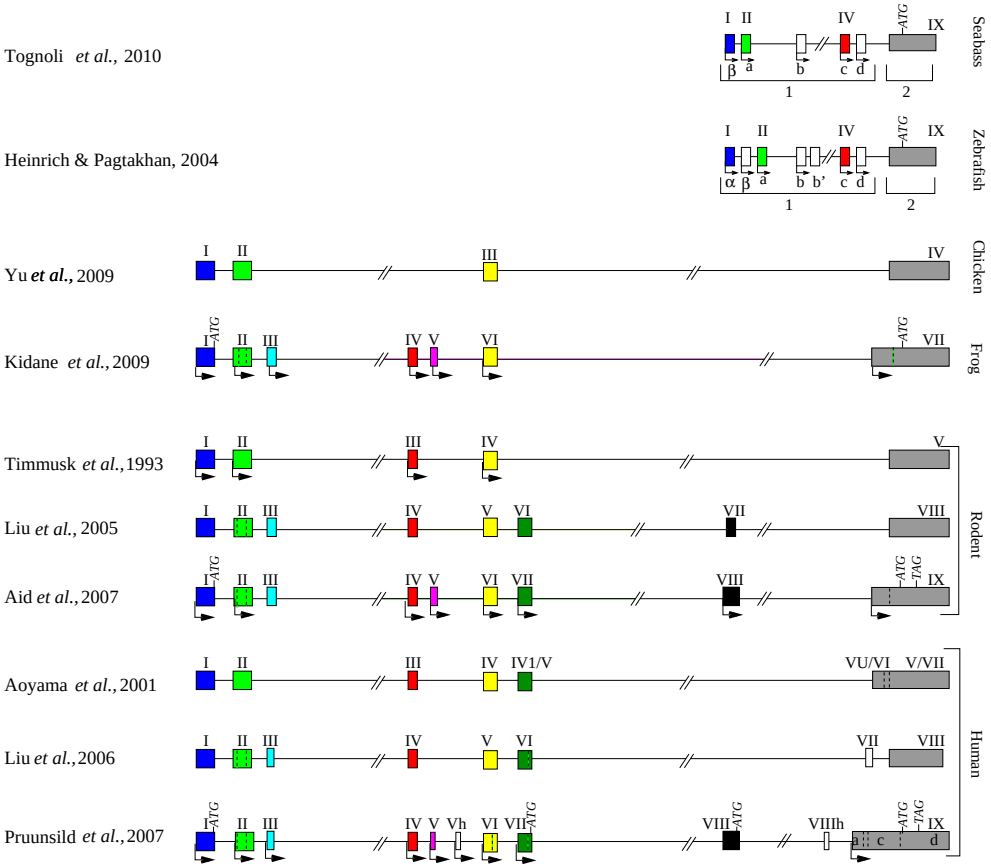

FIGURE 1. Schematic representation of BDNF gene structure in fish, chicken, frog, rodents and human

Exons are shown as boxes and introns are shown as lines. Homologous exons and exons that share some short regions of homology are in the same color. On the top of fish BDNF exons, rodent/human counterparts are shown. Arrows designate promoters that have been discovered by the respective study. In-frame ATG codons are marked in the exons that can be potentially used for translation of N-terminally extended pre-proBDNF peptides. Dotted lines designate alternative splice sites (modified from Pruunsild *et al.* (2007)).

1.2.1 Rodent and human BDNF

The first description of BDNF gene structure was given by Timmusk *et al.* (1993) (Figure 1). Four BDNF promoters were identified in rat, one of each driving the transcription of BDNF mRNAs containing one of the four 5′ noncoding exons (I, II, III, or IV) spliced to the common 3′ exon that contained the coding sequence and the 3′ UTR with two alternative polyadenylation sites (Timmusk *et al.*, 1993). After more than a decade, BDNF gene organization in rat has been updated, mouse BDNF gene structure has been described, and a new numbering has been given to BDNF exons in mouse and rat (Figure 1) (Aid *et al.*, 2007; Liu *et al.*, 2006). The most recent comprehensive study of the rodent BDNF gene organization and its expression profile will be discussed in greater detail in the Results and discussion section, and in Publication I of this dissertation. Shortly, both rat and mouse BDNF genes contain eight 5′ noncoding exons (I-VIII) and one 3′ protein coding exon (IX) (Aid *et al.*, 2007). Eight promoters drive BDNF transcription upstream from 5′ exons and produce ten different transcripts that contain one of 5′ exons alternatively spliced to exon IX (usage of alternative splice donor sites within BDNF exon II leads to three different exon II-containing transcripts) (Aid *et al.*, 2007). In addition, a tripartite transcript variant consisting of exons VII, VIII, and IX has been reported in rodents (Liu *et al.*, 2006). Also, 5′ -extended coding exon (IXA) has been discovered which does not undergo splicing and whose transcription is driven by a separate ninth promoter (Aid *et al.*, 2007). Exon I contains an in-frame ATG start-codon that could potentially add eight aminoacids to the pre-proBDNF N-terminus in case of the translation of exon I-containing BDNF transcript (Aid *et al.*, 2007; Liu *et al.*, 2006).

Human BDNF has a very similar structure, its exons and promoters sharing a high degree of sequence homology in rodents and human (Figure 1). In human, ten 5′ noncoding exons (I-V, Vh, VI-VIII, and VIIIh) have been discovered (Liu *et al.*, 2005; Pruunsild *et al.*, 2007). Nine promoters have been shown to drive BDNF expression giving rise to eighteen alternative transcripts, some of them containing four spliced exons (Pruunsild *et al.*, 2007). Exons I, VII, and VIII contain in-frame ATG start-codons that could be used as translation initiation sites leading to the prepro-BDNF peptides with extended N-termini (Marini *et al.*, 2004; Liu *et al.*, 2005; Pruunsild *et al.*, 2007).

In human, BDNFOS (opposite strand) antisense RNAs are synthesized from the complementary strand of the BDNF gene locus from a single promoter (Liu *et al.*, 2005; Pruunsild *et al.*, 2007). Hundreds of different noncoding RNAs can be generated from the BDNFOS gene as a result of alternative splicing, and each antisense RNA has a region of complementation to BDNF coding exon (Pruunsild *et al.*, 2007). It was shown that these antisense transcripts form double-stranded RNA duplexes with BDNF mRNA *in vivo* in the human brain, and therefore could control human BDNF gene transcription or translation, adding to the complexity of its regulation (Pruunsild *et al.*, 2007).

Since rodent and human BDNF exon nomenclature proposed by Aid *et al.* (2007) and Pruunsild *et al.* (2007) is currently accepted and used by the scientific community, it will be used further in this literature review as well.

1.2.2 BDNF in other species

Zebrafish (*Danio rerio*) BDNF gene is almost as complex as its rodent and human counterparts, spanning about 18 kB. Zebrafish BDNF gene has eight 5′ non-coding exons and six identified promoters (Heinrich and Pagtakhan, 2004). Exon 1α' shares homology with rodent and human exon I, exon 1a - with exon II, exon 1c - with exon IV, and exon 2 is homologous to mammalian BDNF coding exon (Figure 1) (Heinrich and Pagtakhan, 2004; Kidane *et al.*, 2009). Similarly to rodents and humans, the majority of zebrafish BDNF mRNAs contain one 5′ exon spliced to a protein coding 3′ exon. However, a mature BDNF mRNA containing three spliced exons (1b, 1b' and 2) has been reported (Heinrich and Pagtakhan, 2004). Tissue-specific expression pattern of BDNF alternative transcripts has been described in zebrafish (Heinrich, 2003; Heinrich and Pagtakhan, 2004).

Organization of the BDNF gene in seabass (*Dicentrarchus labrax*) is similar to that of zebrafish (Tognoli *et al.*, 2010). In seabass BDNF mRNAs, one of the five alternative 5′ exons (1β, 1a, 1b, 1c or 1d) are spliced to a common 3′ exon (Figure 1). Transcripts containing exons 1b, 1b, and 1d carry in-frame upstream ATG codons, adding amino acids to the alternative prepro-BDNF N-termini (Tognoli *et al.*, 2010).

In frog (*Xenopus laevis*), six alternative 5′ exons (numbered I-VI) in addition to 3′ coding exon (VII) have been described recently by Kidane *et al.* (2009) (Figure 1). Also, a transcript with a 5′ extension of the protein coding exon was found and named VII5′ext (Kidane *et al.*, 2009). Exons I and IV showed sequence homology with their respective counterparts in rodents, human, and zebrafish (corresponding exons 1α' and 1c). Exons II, III, V, and VI did not show appreciable homology with mammalian or zebrafish BDNF exons (Kidane *et al.*, 2009). Frog BDNF exons contain multiple ATG sequences, in-frame (exons I, VI and VII'ext) and out-of-frame (all exons), and, therefore, possible coding regions for alternative N-terminally extended precursors of BDNF. Upstream ORFs in exons I and IV are conserved among mammals, frog and fish, suggesting their functional importance.

In chicken (*Gallus gallus*), three 5′ exons have been described (I-III), each being spliced to the common 3′ exon IV. Exon I, III and IV are highly conserved between chicken and mammals, whereas exon II is unique for chicken (Yu *et al.*, 2009) (Figure 1). Tissue-specific and epigenetic regulation of alternative transcripts has been also described for chicken BDNF (Yu *et al.*, 2009).

1.2.3 The role of alternative 5′ untranslated exons of BDNF

Despite the fact that complex structure of the BDNF gene was discovered more than 15 year ago, the biological meaning of alternative BDNF transcripts had remained enigmatic. The first attempt to address the importance of different BDNF transcripts and the role of different 5′ and 3′ UTR sequences was made by Timmusk *et al.* (1994). In this study, in order to determine the translational status of the alternative BDNF mRNAs, polysomal fraction was isolated from the rat brain and the mRNA composition was analyzed by RNase protection assay, using probes specific for the 5′ exons and the long 3′ UTR of the rat BDNF gene. The results showed that none of the four 5′ exon-specific transcripts was selectively enriched in polysomes suggesting similar translatability. In frog, all 5′ BDNF exons contain multiple out-of-frame ATGs, several of them being conserved in rodents and humans as well. Out-of-frame ATGs in exons I and IV have been shown to markedly decrease translation efficiency of the reporter gene (Kidane *et al.*, 2009), indicating a functional role of untranslated BDNF exons.

To address the issue of BDNF untranslated exons, Pattabiraman *et al.* (2005) investigated the localization of BDNF transcripts in the rat visual cortex during the postnatal development. They reported that BDNF exon IV and VI transcripts (according to the new nomenclature) showed differential intracellular localization: while exon IV transcripts were detected only in neuronal cell bodies (somata), exon VI transcripts were present both in neuronal somata and dendritic processes. Inhibition of visual activity reduced the levels of BDNF mRNA, exon VI transcript almost disappearing from the dendrites (Pattabiraman *et al.*, 2005). Furthermore, epileptogenic seizures were shown to induce differential dendritic localization of BDNF transcripts. After pilocarpine administration, exon II and exon VI transcripts were localized in dendrites, while exons I and IV transcripts displayed somatic localization. In contrast, after kainate administration, only exon VI transcripts were observed in dendrites (Chiaruttini *et al.*, 2008). Another study investigated the subcellular localization of BDNF transcripts in cultured rat hypothalamic neurons (Aliaga *et al.*, 2009). Under basal conditions, BDNF transcripts containing exons I and II were weakly expressed in neuronal somata while the expression of transcripts containing exons IV and VI in somata was strong. In addition, total BDNF mRNA and exon VI mRNA were detected in proximal dendritic processes and in astrocytes. K^+-induced depolarization increased total BDNF mRNA and exon VI mRNA dendritic targeting (Aliaga *et al.*, 2009), while *N*-methyl *D*-aspartate (NMDA) treatment decreased their levels in dendrites. Interestingly, upon NMDA receptor inhibition, all BDNF transcripts were targeted to dendrites (Aliaga *et al.*, 2009). Also, a recent study discovered that rat BDNF coding region contained a constitutively active dendritic targeting signal. This signal is suppressed in exon I and IV mRNAs, which are restricted to the soma and proximal dendrites. This study showed that dendritic targeting of BDNF transcripts was mediated by the RNA-binding protein translin (Chiaruttini *et al.*, 2009).

In long-lasting forms of LTP, local synthesis from pre-existing BDNF mRNA at synapses seems to be crucial for maintaining long-lasting synaptic changes underlying memory formation (Tongiorgi, 2008). Although the majority of proteins are produced in the neuronal soma, some key molecules for plasticity can be delivered in the form of silent mRNAs to the synapses in extra-somatic compartments where they are locally translated. It has been found a long time ago that in cultured hippocampal neurons (Tongiorgi *et al.*, 1997) and also in the hippocampus *in vivo* (Tongiorgi *et al.*, 2004) under basal conditions BDNF mRNA is localized to the proximal dendritic compartment, however, it can be transported to neuronal dendrites in the activity-dependent manner after membrane depolarization or epileptogenic stimuli. Taken together, it is possible to suggest that BDNF alternative transcripts can be important for the regulation of temporal and spatial expression of BDNF and possibly play a role in synaptic transmission and morphology.

1.2.4 The role of long $3'$ UTR in BDNF mRNA

Short (0.35 kb) and long (2.85 kb) BDNF 3UTRs arise from alternative polyadenylation. The primary sequence of BDNF $3'$ UTRs is highly conserved between human, mouse, rat, seabass and zebrafish with a stretch of 39 bp of identical sequence 63 bp downstream from the stop codon (Heinrich and Pagtakhan, 2004; Tognoli *et al.*, 2010). BDNF mRNA species with short and long $3'$ UTRs are equally abundant in the rat cortex (Timmusk *et al.*, 1993). The results of BDNF transgenic studies (Timmusk *et al.*, 1995) showed that not only promoter regions but also $3'$ region downstream of BDNF coding exon are required for the cell-specific and neuronal activity-dependent expression of the rat BDNF gene. Experiments with a transgenic construct containing zebrafish BDNF exon 1c, BDNF $3'$ UTR and a reporter gene showed that BDNF $3'$ UTR was responsible for cell-specific expression of the reporter gene (Heinrich and Pagtakhan, 2004). In the abovementioned study by Timmusk *et al.* (1994), in addition to $5'$ UTRs the translatability of BDNF transcripts with alternative $3'$ UTRs was examined using polysomal fractions from the adult rat brain tissue. It was discovered that transcripts containing long BDNF $3'$ UTR were less abundant in the polysomal fraction than transcripts with short $3'$ UTR suggesting their translational discrimination. Thus, long BDNF $3'$ UTR was suggested to contain negative regulatory elements that repressed translation (Timmusk *et al.*, 1994).

A recent study showed that the production of short $3'$ UTRs as a result of terminating at upstream polyadenylation sites removes microRNA (miRNA) binding sites that repress mRNA translation and suggested a general translational regulatory role for long $3'$ UTRs (Sandberg *et al.*, 2008). MicroRNAs regulate gene expression by interfering with mRNA translation or promoting its degradation. So far, several miRNAs were predicted to bind rodent and human BDNF mRNA. Mir-1 was the first miRNA that was shown to downregulate BDNF expression *in vitro* in HeLa cells

(Lewis *et al.*, 2003). In human BDNF mRNA, mir-1 was predicted to bind 250 bp and 420 bp downstream from the stop-codon in the region of the long 3′ UTR. (Lewis *et al.*, 2003, 2005). In mouse, miR-1 accounts for 45% of all mouse miRNAs found in the heart. It is also expressed in the liver and in the midbrain (Lagos-Quintana *et al.*, 2002). More recently, it was shown that a set of miRNAs differentially expressed in the human prefronatal cortex, including miR-30a-5p and miR-195, repress reporter gene expression linked to BDNF 3′ UTR when overexpressed in HEK292 cells (Mellios *et al.*, 2008). These findings suggest the potential role of miRNA in the regulation of stability and/or translatability of BDNF mRNAs with long 3′ UTR.

The study of An *et al.* (2008) showed that BDNF mRNAs with short and long 3′ UTR are localized in different cellular compartments. The short 3′ UTR mRNAs are restricted to neuronal soma whereas the long 3′ UTR mRNAs are localized in soma as well as in dendrites. In a mouse mutant where the long BDNF 3′ UTR was truncated, dendritic localization of BDNF mRNAs was impaired in the hippocampus despite the normal levels of total BDNF protein. These mice exhibited deficient pruning and enlargement of dendritic spines. Moreover, in this mutant, selective impairment of LTP in dendritic synapses, but not somatic synapses, was observed in CA1 hippocampal neurons lacking dendritic BDNF mRNA (An *et al.*, 2008). These results demonstrate the importance of the long 3′ UTR for BDNF mRNA localization and synaptic functioning in the hippocampus.

1.3 BDNF protein synthesis and secretion

In addition to various BDNF mRNA species, multiple forms of BDNF protein can be secreted by neurons in the brain. BDNF is initially synthesized in the endoplasmic reticulum as a 32-kDa N-glycosylated and glycosulfated precursor protein (pre-proBDNF) (Mowla *et al.*, 2001) which dimerizes after translation (Kolbeck *et al.*, 1994). Thereafter, pre-proBDNF undergoes cleavage to release mature 14-kDa BDNF protein or a minor truncated form of the precursor (28 kDa) (Mowla *et al.*, 2001). First, following the cleavage of the signal peptide, proBDNF is transported to the Golgi for sorting either into constitutive or, preferentially, into regulated secretory vesicles. Then, proBDNF may be converted into mature BDNF intracellularly in the trans-Golgi by the members of subtilisin-kexin family of endoproteases such as furin, or in the immature secretory granules by proprotein convertases (Mowla *et al.*, 1999). ProBDNF form can also be secreted and cleaved extracellularly by serine protease plasmin (Pang *et al.*, 2004) or by selective matrix metalloproteinases (MMPs) (Lee *et al.*, 2001). ProBDNF cleavage by plasmin is accomplished through the activation of plasminogen by tissue plasminogen activator (tPA) - the second secreted protein after BDNF that has been implicated in late-phase LTP and long-term memory (Pang *et al.*, 2004). It was shown that proBDNF is rapidly internalized by perineuronal astrocytes via $p75^{NTR}$–clathrin-mediated internalization in endocytic compartments,

where it undergoes recycling and can be later released by astrocytes (Bergami *et al.*, 2008).

1.3.1 The role of different isoforms of BDNF protein

The diversity of neurotrophin actions in the nervous system might in part be modulated via differential processing of proneurotrophins. After low-frequency stimulation (LFS) that induces LTD in neurons, predominantly proBDNF is secreted (Nagappan *et al.*, 2009). In contrast, when the neurons are subjected to high-frequency stimulation (HFS—a condition that induces LTP), mature BDNF isoforms are dominating. Interestingly, tPA is secreted only under HFS. Thus, both LFS and HFS increase the secretion of proBDNF in the extracellular space, but only high-frequency neuronal activity induces tPA secretion resulting in the extracellular cleavage of proBDNF to produce mature BDNF (Nagappan *et al.*, 2009). Thus, neuronal activity may regulate the balance of BDNF isoforms, allowing BDNF to induce opposite forms of synaptic plasticity.

Past studies have shown that proneurotrophins induce apoptosis in neurons via $p75^{NTR}$ activation in the absence of Trk signaling (Lee *et al.*, 2001). Teng *et al.* (2005) showed that proBDNF, but not mature BDNF, acts via a dual receptor system consisting of $p75^{NTR}$ and transmembrane protein sortilin to mediate cell apoptosis in rodent sympathetic neurons. It was also shown that proBDNF facilitates LTD at hippocampal (Woo *et al.*, 2005) and neuromuscular synapses (Yang *et al.*, 2009a) through the activation of $p75^{NTR}$. There is an indication that proBDNF is expressed at significant levels at early postnatal stages, whereas mature BDNF is the dominant isoform in the adulthood (Yang *et al.*, 2009b). Considering the fact that $p75^{NTR}$ is highly expressed in the postnatal period its levels decreasing during adolescence up to adulthood (Yang *et al.*, 2009b), it can be speculated that spatial and temporal expression of $p75^{NTR}$ and proBDNF are coordinated to achieve proper regulation of synaptic outgrowth and maturation.

1.3.2 BDNF Val66Met polymorphism

A single-nucleotide polymorphism (SNP) in the BDNF gene – G to A substitution – leads to a Val substitution with Met at BDNF codon 66 in the prodomain. This polymorphism is found only in humans, with Met allele frequency in Caucasian populations about 20–30% (Shimizu *et al.*, 2004), and in Asian populations above 40% (Gratacòs *et al.*, 2007). The results of the studies examining the effect of Val66Met polymorphism have been somewhat confusing. Humans heterozygous for Met allele have smaller hippocampal volume (Pezawas *et al.*, 2004), poorer episodic memory and lower hippocampal activation (Hariri *et al.*, 2003; Egan *et al.*, 2003) as compared to Val/Val homozygous individuals. It has been observed, however, that homozygosity for the BDNF Val allele is associated with a greater susceptibility to Alzheimer's

disease (Ventriglia *et al.*, 2002). It has been reported that transgenic $BDNF^{Met/Met}$ mice exhibit anxiety when placed in stressful settings and this condition could not be normalized with antidepressants (Chen *et al.*, 2006). However, human studies have reported an opposite effect of the Val66Met polymorphism: Val/Val genotype was strongly associated with the anxiety personality trait in non-depressed individuals as compared to Val/Met and Met/Met genotypes (Lang *et al.*, 2005). In humans, Val allele is associated with higher BDNF secretion in response to neuronal stimulation compared to the Met allele. It was shown that Val/Val genotype contributed to the substance abuse vulnerability (Tsai, 2007a; Cheng *et al.*, 2005) which was explained by the increased central activity of BDNF.

It has been shown that neuronal activity-regulated secretion of BDNF protein is strongly impaired for $BDNF_{Met}$ isoform (Egan *et al.*, 2003). Retention of $BDNF_{Met}$ has been observed in the Golgi apparatus (del Toro *et al.*, 2006). This effect was suggested to be due to the disrupted binding of $BDNF_{Met}$ to the sorting protein sortilin which directs BDNF to the secretory vesicles (Chen *et al.*, 2005). Curiously, recent human studies suggested that BDNF Met allele, which showed abnormal intracellular trafficking and secretion, had a protective effect on the development of depression (Pezawas *et al.*, 2008). Moreover, there is evidence that epistasis exists between BDNF Met allele and serotonin transporter gene (SLC6A4) in humans (Pezawas *et al.*, 2008; Kaufman *et al.*, 2006). A polymorphism in SLC6A4 promoter region, HTTLPR S allele, is associated with the decreased serotonin transporter mRNA transcription, increased anxiety, risk of depression and increase of amygdala reactivity (Pezawas *et al.*, 2005). It has been speculated that the BDNF Met allele reduces the impact of the HTTLPR S allele on amygdala circuitry, leading to the reduced susceptibility to depression. These observations support the results from the $BDNF^{Met/Met}$ mouse model study (Chen *et al.*, 2006), explaining why anxiety behavior in animals expressing $BDNF_{Met}$ is unresponsive to antidepressant action of serotonin re-uptake inhibitors, which can be viewed as pharmacological analogs of 5-HTTLPR S allele (Pezawas *et al.*, 2008).

1.4 Synaptic plasticity and BDNF

Neurons communicate via special cellular formations – synapses – to propagate environmental signals and to respond back. To propagate the signal, neurons fire at frequencies ranging from 1 Hz (less than once per second) to several hundred Hz. Changes in firing rate induce synaptic modifications that alter the amplitude of the postsynaptic response. Synaptic plasticity (SP) is thus the ability of synapses to change in strength. Short-term SP, which occurs on a timescale of milliseconds to minutes, regulates the activity of neural networks and information processing in the nervous system (Catterall and Few, 2008). Whereas long-term changes at synapses in the hippocampus and cortex underlie learning and memory formation

(Whitlock *et al.*, 2006; Gruart *et al.*, 2006; Rioult-Pedotti *et al.*, 2000). During long-term changes new synapses can be made, old ones destroyed, and existing synapses can be strengthened or weakened. Dysfunctions in the synaptic transmission underlie various human neurological diseases such as depression, Parkinson's disease, epilepsy, and neuropathic pain and play a role in Alzheimer's disease and drug addiction (Malenka and Bear, 2004). This section will further discuss molecular mechanisms of SP, regulation of BDNF gene transcription in response to neuronal activity, and BDNF role in modulating SP and memory formation.

1.4.1 Molecular mechanisms of synaptic plasticity

Molecular mechanisms of SP involve: i) post-translational modifications of the existing synaptic proteins; ii) regulation of gene expression in post-synaptic cells, thus changing the levels of key proteins at the synapse; iii) mRNA targeting to the synapses for local translation; iv) rearrangement of receptor molecules in the post-synaptic membrane, such as delivery of new receptors to the membrane to strengthen synaptic function.

Synaptic potentiation or depression can occur throughout the brain, but long-term potentiation (LTP) and depression (LTD) – cellular models of learning and memory – have been most intensively studied in the hippocampus (Derkach *et al.*, 2007; Lisman *et al.*, 2002; Lüscher *et al.*, 2000). LTP is defined as a prolonged strengthening and LTD – as a prolonged weakening in excitatory synaptic communication. LTP can be induced by multiple paradigms, including high-frequency stimulation (HFS), theta-burst stimulation (TBS), and pairing of pre- and postsynaptic depolarizations (pairing-induced). The TBS protocol is considered to be the most physiological as it resembles hippocampal firing patterns during active exploration and learning in rodents (Otto *et al.*, 1991).

At synapses, communication between neurons is mediated by the release of neurotransmitters from a presynaptic neuron that induces numerous changes in a post-synaptic neuron. In the CNS, neurons receive most of the excitatory synaptic input from glutamatergic neurons and inhibitory input from GABAergic interneurons, except during early development, when the first GABAergic synapses are depolarizing and provide the excitatory drive critical for the subsequent development of glutamatergic synapses (Ben-Ari, 2002). Neurotransmitter binding to its specific receptor in the postsynaptic neuron or post-synaptic membrane depolarization by above-mentioned protocols activates multiple biochemical events, the most significant being rapid and transient rise in intracellular calcium levels. As a result, LTP or LTD can occur, depending on the pattern of synaptic activity and the previous history of the synapse. It is not known exactly which mechanisms are responsible for the LTD induction. A recent study showed that differential metabotropic glutamate receptor (mGlurR) activation, rather than differences in intracellular calcium concentrations,

is crucial for generating LTD versus LTP (Nevian and Sakmann, 2006). The signaling mechanism that has been proposed to underlie LTP involves Ca^{2+} influx through NMDA receptors in response to synaptic activity (Malenka and Bear, 2004) and activation of calcium calmodulin kinase II (CaMKII) (Wayman *et al.*, 2008), Ser/Thr kinases (PKA,PKC, MAP-kinases, etc.) and tyrosine kinases (Src, Fyn, and others) (Smolen *et al.*, 2006). Activity-dependent calcium influx into neurons leads to a number of short-term and long-term alterations, including: i) insertion or removal of synaptic AMPAR (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid-type glutamate receptor), and alterations in its subunit composition and trafficking; ii) posttranslational modifications of synaptic proteins involved in trafficking, cytoskeletal organization and protein synthesis; iii) stimulation of local translation or protein degradation at the synapse; iv) actin reorganization, and modulation of spine morphology (Derkach *et al.*, 2007; Malenka and Bear, 2004). Also, calcium signaling results in the activation of gene expression program in the nucleus, driving the transcription of the genes that promote dendritic growth, synapse development, and neuronal plasticity (Mellström *et al.*, 2008).

1.4.2 Neuronal activity-dependent regulation of BDNF transcription

The first study to propose that neuronal activity regulates gene expression showed that in neuronal cell cultures, membrane depolarization and influx of calcium into cells through L-type voltage-sensitive calcium channels (L-VSCCs) trigger rapid and transient activation of the c-fos proto-oncogene (Greenberg *et al.*, 1986). Further studies discovered hundreds of neural activity-regulated genes. These genes are known to encode i) transcription factors that mediate synaptic activity by inducing target genes which regulate cell survival, dendritic and axonal growth and synaptic development; ii) proteins that that act specifically at synapses to control synaptic development and function (Greer and Greenberg, 2008).

BDNF expression studies in cultured rat embryonic cortical neurons have shown that the route of calcium entry into the cell upon membrane depolarization determines which genes will be induced in the nucleus. Channel properties such as conductance, open time, subcellular localization and association with the key signaling molecules affect the choice of the genes to be induced by calcium influx (Greer and Greenberg, 2008). Numerous studies have demonstrated that BDNF transcription is highly induced following calcium entry through L-VSCCs (Ghosh *et al.*, 1994). L-type VSCCs have slow inactivation rate and high conductance for calcium (Gallin and Greenberg, 1995). They are somatodendritically localized, which enhances calcium signal propagation to the nucleus (Westenbroek *et al.*, 1990; Catterall, 2000). L-VSCCs are associated with protein kinase A anchoring protein (AKAP79/150) that recruits PKA to the channel (Gray *et al.*, 1998), leading to its phosphorylation and activation (Bence-Hanulec *et al.*, 2000). AKAP79/150 recruits calcineurin, which

is required for activation and translocation of transcription factor NFATc4 into the nucleus (Graef *et al.*, 1999) that in turn activates MEF2 family transcription factors (Chin *et al.*, 1998; Mao and Wiedmann, 1999). Also, calmodulin binding to L-type VSCCs activates Ras/MAPK signaling cascade and induces transcription in the nucleus (Dolmetsch *et al.*, 2001) (Figure 2).

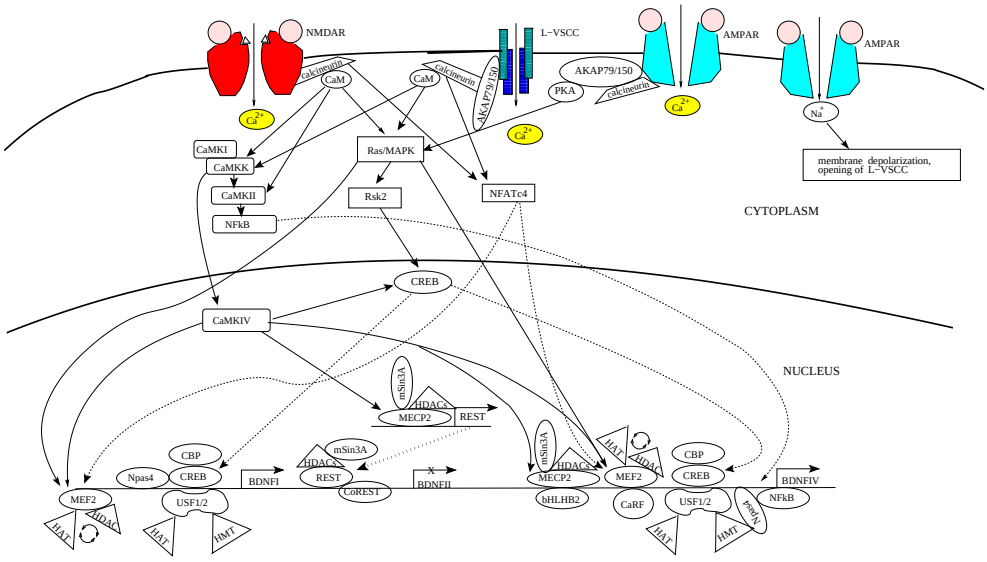

FIGURE 2. Neuronal activity-mediated activation of BDNF transcription
Activation of glutamate receptors (NMDAR or AMPAR) by ligand binding or activation of L-type VSCCs by membrane depolarization causes these ion channels to open and allows calcium influx into the cytoplasm. Direct binding of calcium to receptor-associated calcium sensors such as calmodulin and calcineurin activates Ras/MAPK pathway and calcium/calmodulin-dependent protein kinases. These pathways induce BDNF gene transcription via activation of numerous transcription factors that bind to BDNF promoters. Ligands: triangles - glycine, circles - glutamate. Arrows represent protein activation by direct phosphorylation/dephosphorylation or via intermediates that are not depicted on the diagram. Dotted lines: NFATc4 - translocation to the nucleus; CREB - binding to BDNF promoters. Circle arrows show the exchange of HDAC and HAT, with HDAC leaving from the MEF2 complex and HAT (p300 coactivator) binding to MEF2 upon MEF2 activation. Following calcium influx, MeCP2 is phosphorylated and released from BDNF promoter IV, allowing for its transcriptional activation. MeCP2 derepresses REST/NRSF gene transcription in responce to neuronal activity. As a result, the product of REST/NRSF gene – REST/NRSF protein – is translocated to the nucleus after translation and represses BDNF promoter II (shown with a dashed line).

In addition to L-VSCC, NMDA-type glutamate receptors (NMDAR) are also important mediators of BDNF transcriptional activation (Bhave *et al.*, 1999; Lipsky *et al.*, 2001; Hong *et al.*, 2008). Especially during early brain development, NMDAR have beeb shown to play a role in synaptogenesis and activation of BDNF transcrip-

tion by associating with numerous signal-transducing molecules such as EphB family of receptor tyrosine kinases (Takasu *et al.*, 2002), calmodulin and calcineurin. Also, BDNF is moderately activated by calcium influx via AMPA-type glutamate receptors (AMPAR) or other types of VSCCs (Ghosh *et al.*, 1994). Schematic representation of calcium-mediated induction of BDNF transcription is shown in Figure 2 (Greer and Greenberg, 2008).

1.4.3 Regulatory elements in BDNF promoters

As mentioned above, BDNF gene is regulated by multiple promoters. In neurons, BDNF transcription is activaed by a number of different neurotransmitters, including glutamate analogs (Timmusk *et al.*, 1993; Metsis *et al.*, 1993; Marini *et al.*, 1998), acetylcholine (Knipper *et al.*, 1994), GABA (Marty *et al.*, 1996), serotonin (Zetterström *et al.*, 1999), and dopamine (Küppers and Beyer, 2001; Fang *et al.*, 2003). *In vivo*, environmental stimuli possess specificity in relation to BDNF promoter activation, certain BDNF promoters being activated in distinct brain regions in response to specific stimuli (West, 2008, see []. At least six out of nine BDNF promoters are induced by neuronal activity (Aid *et al.*, 2007), promoters I and IV being the most responsive (Timmusk *et al.*, 1993; Metsis *et al.*, 1993; Timmusk *et al.*, 1995). Transcription from BDNF promoters I, II and IV has been studied extensively and several transcription factors that regulate their activity have been identified.

REs in BDNF promoter I

Calcium influx via L-VSCCs has been shown to induce BDNF expression from promoter I in cultured rat embryonic cortical neurons (Tabuchi *et al.*, 2000). Proximal region of rodent BDNF promoter I contains a cAMP-responsive element (CRE) that overlaps with the binding site of upstream stimulatory factor 1/2 (USF) (Tabuchi *et al.*, 2002). BDNF promoter I CRE is conserved in rat, human and mouse (Figure 3). Both elements are responsible for the activation of BDNF promoter I by neuronal activity through the binding of CREB and USF1/2 transcription factors as was demonstrated in cultured rat cortical neurons (Figure 2) (Tabuchi *et al.*, 2002). CREB activates and binds to its target genes containing CRE in response to the elevation of cellular cAMP/calcium levels (Montminy and Bilezikjian, 1987). It is turned on in the activated brain areas during a wide range of behaviours, including birdsong, cocaine reward, fear conditioning, and spatial learning (Shaywitz and Greenberg, 1999). USF1/2 are expressed in the adult mouse brain (Sirito *et al.*, 1994) and the transcriptional activity of USFs in rat embryonic cortical neurons was shown to be activated by Ca^{2+} influx (Chen *et al.*, 2003b). Interestingly, USF1/2 have been shown to recruit histone methyltransferase (HMT), histone acetyltransferase (HAT), and ATP-dependent nucleosome remodeling complexes to insulator sequences (West *et al.*, 2004; Huang *et al.*, 2007) blocking gene silencing.

Recently, it has been reported that myocyte enhancer factor 2 (MEF2), binds to a distant MEF2 binding site in BDNF promoter I ($\sim$ 6.5 kb upstream from exon I) and regulates its activity (assayed using BDNF promoter-luciferase construct in cultured rat hippocampal neurons) (Flavell *et al.*, 2008). MEF2 family transcription factors are critical for the development and function of musculoskeletal, cardiac, vascular, immune and nervous systems (Potthoff and Olson, 2007). MEF2 suppresses excitatory synapses in a neuronal activity- and calcineurin-dependent manner during hippocampal synapse development (Flavell *et al.*, 2006). Association of MEF2 with class II histone deacetylases (HDACs) results in the suppression of MEF2-dependent genes. In response to increased neuronal activity, calcium/calmodulin-dependent protein kinase (CaMK) phosphorylates HDACs, and HDACs are released from MEF2 (Lu *et al.*, 2000). Once released from the associated repressors, MEF2 is phosphorylated and bound by the p300 coactivator, which possesses HAT activity. MEF2 coactivator relaxes chromatin structure and stimulates MEF2 target gene transcription. Also, calcium influx into neurons via L-VSCCs or NMDAR activates calcium/calmodulin-regulated phosphatase calcineurin, which dephosphorylates nuclear factor of activated T-cells (NFATc4). Activated NFATc4 then translocates to the nucleus where it directly associates with MEF2 (Graef *et al.*, 1999; Vashishta *et al.*, 2009). NFATc4 stimulates MEF2-dependent transcription by facilitating the recruitment of p300 coactivator to MEF2 (Figure 3) (McKinsey *et al.*, 2002). When activated, MEF2 promotes the transcription of the genes that restrict synapse number while strengthening specific synapses and promoting inhibitory synapse development (Flavell *et al.*, 2008).

Mutations in the methyl CpG binding protein 2 (MeCP2) gene are the primary cause of Rett syndrome (RTT) – an X-linked autism spectrum disorder (Amir *et al.*, 1999). MeCP2 has been shown to derepress BDNF promoter IV activity following membrane depolarisation and calcium influx through the L-VSCCs (Chen *et al.*, 2003a; Martinowich *et al.*, 2003) (Figure 2). A recent study of Tian *et al.* (2010) suggested that MeCP2 has a role in regulating BDNF promoter I and IV in cultured rat hippocampal neurons upon NMDA receptor activation (Tian *et al.*, 2010). It was shown that the regulation of BDNF promoters I by MeCP2 is accomplished by MeCP2 binding to CpG sequence in the CRE element of promoter I. Thus, CREB and MeCP2 compete for the CRE site in BDNF promoter I and this competition is probably responsible for a slower activation of BDNF promoter I upon NMDAR stimulation as compared to promoter IV (Tian *et al.*, 2010).

REs in BDNF promoter II

BDNF promoter II contains REST/NRSF binding site (a palindromic $NRSE^{bdnf}$ sequence) (Timmusk *et al.*, 1993) (Figure 3). REST/NRSF, a RE-1 silencing transcription factor/neuron-restrictive silencer factor, was identified as a zinc finger transcrip-

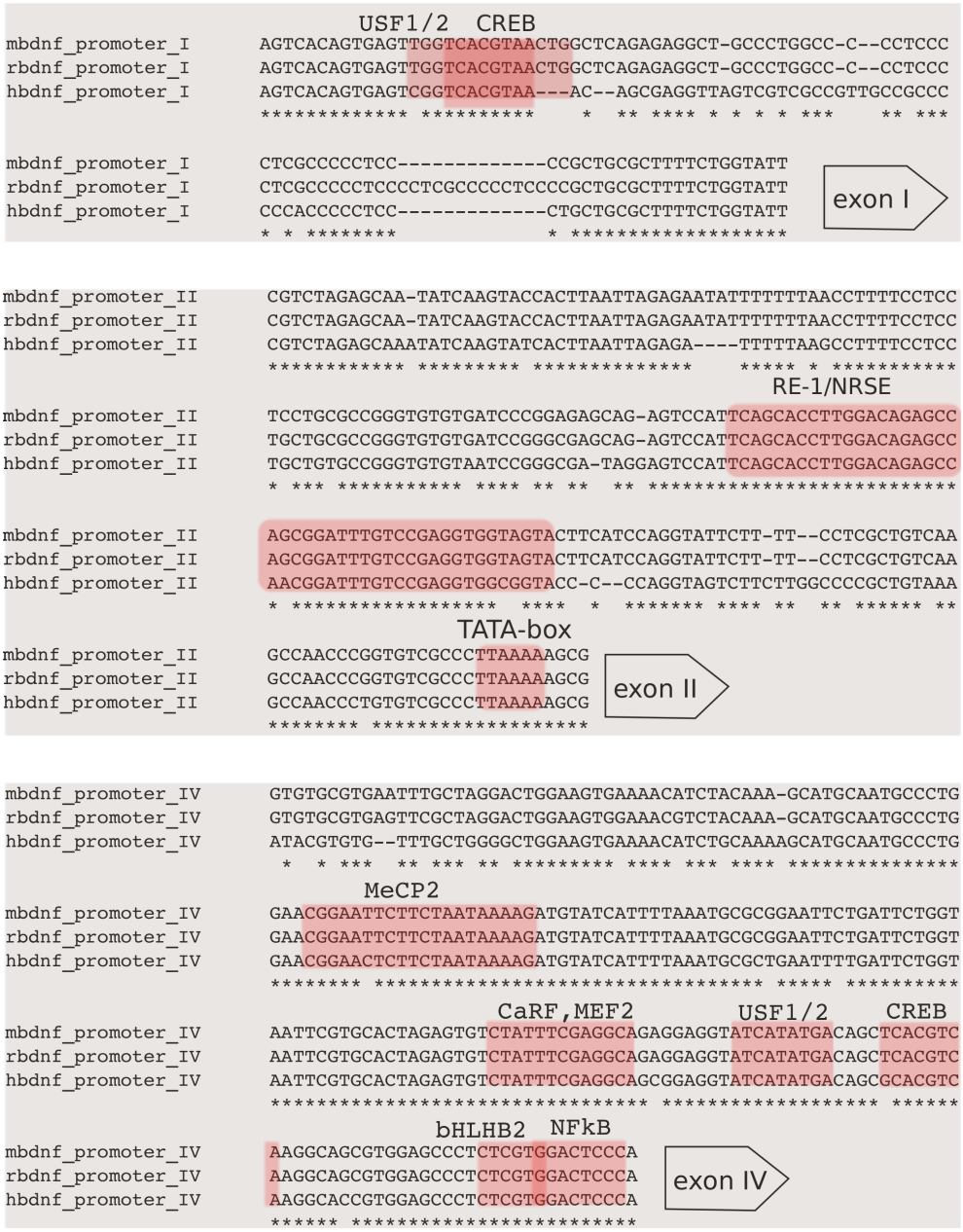

FIGURE 3. Regulatory elements in BDNF promoters I, II and IV
Alignment of the nucleotide sequences of BDNF promoters I, II and IV in mouse, rat and human. Mapped regulatory elements have been shown to activate BDNF transcription. Promoters are shown up to the most 5′ transcription start sites according to Aid *et al.* (2007).

tion factor that recognized a 23 bp *cis*-element, NRSE, which mediated silencing of neuronal genes in non-neuronal cells (Chong *et al.*, 1995; Schoenherr and Anderson, 1995). It was also shown that REST acted as a negative regulator of neuronal gene expression in neurons (Palm *et al.*, 1998; Timmusk *et al.*, 1999). REST/NRSF recruits multiple cofactors including CoREST corepressor, HDAC1, HDAC2, and mSin3A to repress its target genes (Ballas and Mandel, 2005). REST/NRSF was shown to repress basal and neuronal activity-dependent expression of the BDNF gene from promoters II and I *in vitro* and *in vivo* in transgenic mice (Palm *et al.*, 1998; Timmusk *et al.*, 1999). It is involved in the regulation of BDNF gene expression by huntingtin, a protein that is mutated in Huntington's disease. Wild-type huntingtin induces BDNF mRNA and protein expression from BDNF promoter II. This activity of huntingtin is lost when the protein becomes mutated, resulting in a decreased production of BDNF and neuronal cell death (Zuccato *et al.*, 2001). Studies suggest that this effect is due to the loss of function of the wild type huntingtin, which binds to REST and sequesters it in the cytoplasm, derepressing the expression of RE-1 containing genes in the nucleus (Zuccato *et al.*, 2003).

It has been reported that MeCP2 deficiency in human and mouse brain induces the expression of REST and CoREST (Abuhatzira *et al.*, 2007). MeCP2 deficiency in the brain has been shown to decrease an overall expression of BDNF in spite of an observed increase in the activity of promoter IV that is controlled directly by MeCP2 (Chen *et al.*, 2003a; Martinowich *et al.*, 2003). How MeCP2 deficiency caused an overall downregulation of BDNF expression had for a long time remained an enigma. Recently, it has been discovered that MeCP2 binds to and is involved in repression of REST and CoREST promoters despite their unmethylated state (Abuhatzira *et al.*, 2007). MeCP2 depletion is associated with a change in the histone modification profile at REST and CoREST promoters - increase in dimethylation of histone H3 at lysine K4 and decrease dimethylation in histone H3 at lysine K9 – which corresponds to a more active chromatin conformation. Upon neuronal activity, MeCP2 is phosphorylated and released from REST and CoREST promoters, which induces their transcription, translation and subsequent repression of BDNF promoter II (Figure 2). Thus, the elevated levels of REST and CoREST in the brain of RTT patients and MeCP2-deficient mice result in downregulation of BDNF, apparently by their binding to the RE1/NRSE in the BDNF gene (Abuhatzira *et al.*, 2007).

REs in BDNF promoter IV

BDNF promoter IV can be activated by Ca^{2+} influx through either NMDAR or L-VSCC (Tabuchi *et al.*, 2000). Detailed analysis of proximal region of BDNF promoter IV (promoter III according to the old nomenclature) has shown that it contains three distinct Ca^{2+}-responsive elements (CaREs) (Figure 3). In cultured rat embryonic cortical neurons CaRE1 mediated calcium-responsive induction of BDNF

promoter IV expression by recruiting calcium- and neural-selective transcription factor CaRF (Tao *et al.*, 2002; Shieh *et al.*, 1998). CaRF contains consensus phosphorylation sites for a number of kinases including CaMKII, MAPK and PKC (Tao *et al.*, 2002). The second element, CaRE2, is a Ca^{2+}-responsive E-box that binds upstream stimulatory factors 1 and 2 (USF1/2) (Chen *et al.*, 2003b). The third element, cAMP/Ca^{2+}-response element-like element (CaRE3/CRE), proximal to the exon IV transcription start site (Figure 3) is important for the induction of BDNF promoter IV by CREB following membrane depolarization (Tao *et al.*, 1998; Shieh *et al.*, 1998). CREB bound at CRE in promoter IV becomes phosphorylated by calcium-regulated kinase cascades in response to neuronal activity and recruits components of the basal transcriptional machinery to BDNF promoter IV (Lonze and Ginty, 2002; West *et al.*, 2001). Coordinate activity of USF1/2 together with CaRF and CREB is required to regulate BDNF gene expression from promoter IV in Ca^{2+}-dependent manner (Chen *et al.*, 2003b; Tao *et al.*, 2002). Moreover, human BDNF promoter IV was shown to be activated via its CRE element in responce to dopamine binding to D1 class of dopamine receptors in human NT2 cells (Fang *et al.*, 2003). As reported by this study, dopamine binding mediated activation of BDNF transcription via cAMP, PKA, and CREB (Fang *et al.*, 2003).

In frog, transcription from BDNF promoter IV is strongly induced by neuronal activity during black-background adaptation. A sequence that shares high homology with rodent and human CRE along with sequences resembling CaREs in BDNF promoter IV have been found upstream from frog BDNF exon IV transcription initiation site (Kidane *et al.*, 2009). In addition, in the region of CRE, a sequence resembling downstream regulatory element (DRE) was identified and found to be conserved in human and rat BDNF promoter IV (Kidane *et al.*, 2009). DREAM, also termed KChIP-3 (potassium channel interacting protein-3) or calsenilin, binds to the DRE in the promoters of its target genes and represses their transcription in the absence of neuronal activity. It is widely expressed in the brain, and in particular in sensory neurons (Mellström *et al.*, 2008). Upon neurotransmitter release, DREAM binds directly to calcium ions that enter the nucleus, dissociates from the promoters of its target genes, thus relieving transcriptional repression and allowing the transcription of these genes (Carrión *et al.*, 1999). Interestingly, DREAM null mice showed enhanced learning and memory abilities and delayed aging. DREAM functions as a negative regulator of CREB-dependent transcription of BDNF in the hippocampus by binding to unphosphorylated CREB in the absence of neuronal activity and preventing CREB interaction with CBP (CREB binding protein) in a Ca^{2+}- dependent manner (Fontán-Lozano *et al.*, 2009).

In addition to CaREs, MeCP2 binding site (CpG sequences) has been found in the proximal region of the BDNF promoter IV (Figure 3). MeCP2 binds to BDNF promoter IV and represses the expression of the BDNF gene from promoter IV (Chen *et al.*, 2003a; Martinowich *et al.*, 2003). Membrane depolarisation and calcium influx

through L-VSCCs decreases CpG methylation and increases histone H3 H4 acetylation at BDNF promoter IV, thereby facilitating transcription (Chen *et al.*, 2003a; Martinowich *et al.*, 2003). Neuronal activity-dependent induction of the BDNF gene transcription is a consequence of the MeCP2 phosphorylation and the release of a repressor complex containing MeCP2, histone deacetylases HDAC1 and HDAC2, corepressor mSin3A (Martinowich *et al.*, 2003) and probably also Ski, N-CoR (Kokura *et al.*, 2001), and SWI/SNF complex (Harikrishnan *et al.*, 2005). As mentioned above, MeCP2/HDAC regulates BDNF promoter I and IV in cultured hippocampal neurons upon NMDA receptor activation (Tian *et al.*, 2010).

Other transcriptional regulators at BDNF promoter IV include nuclear factor kappa B (NF-κB) (Lipsky *et al.*, 2001; Marini *et al.*, 2004), class B2 basic helix-loop-helix domain containing protein (BHLHB2) (Jiang *et al.*, 2008), neuronal PAS domain protein 4 (NPAS4) (Lin *et al.*, 2008b), and MEF2 (Hong *et al.*, 2008, S.W. Flavell, T.K. Kim, and M.E.G., unpublished data). NF-κB family of transcription factors regulate genes involved in immunologic responses, cell proliferation, growth regulation, and apoptosis. NF-κB was shown to regulate BDNF promoter IV during NMDAR-mediated neuroprotection (Lipsky *et al.*, 2001). BHLHB2 is an immediate-early gene expressed in the hippocampal neurons. It binds BDNF promoter IV between CRE and NF-κB binding sites (Figure 3) in response to neuronal activity upon NMDAR activation and act as a trascriptional repressor of BDNF (Jiang *et al.*, 2008). Npas4 is critical for activity-dependent regulation of GABAergic synapse development. Npas4 expression is rapidly activated by excitatory synaptic activity and turns on a program of gene expression that triggers the formation and/or maintenance of inhibitory synapses on excitatory neurons (Lin *et al.*, 2008b). Initial studies indicate that Npas4 is associated with BDNF promoters I and IV (Figure 2) and regulates BDNF expression during the development of GABAergic synapses (Lin *et al.*, 2008b).

MEF2 has been detected as one of the components of the multifactoral transcriptional activation complex containing CBP, RNA polymerase II (Pol II) and MEF2 that binds to BDNF promoter IV (Hong *et al.*, 2008, S.W. Flavell, T.K. Kim, and M.E.G., unpublished data). Disruption of the ability of CREB to bind BDNF promoter IV in transgenic mice resulted in impaired activity-dependent transcription of BDNF in response to NMDA in cultured cortical neurons or sensory experience-driven synaptic activation in the brain (Hong *et al.*, 2008). The impaired CREB binding to BDNF promoter IV disrupts the binding of CBP, Pol II, and MEF2 to BDNF promoter IV as well. This indicates that the loss of CREB binding to BDNF promoter IV disrupts the multifactor transcriptional activating complex and suggest a new function for CREB in the assembly of transcriptional complexes at its target promoters (Hong *et al.*, 2008). Recent evidence suggests that MeCP2, in addition to functioning as a repressor of gene expression, may work as an activator in the complex with CREB (Chahrour *et al.*, 2008). It is possible that MeCP2, CREB, and MEF2 act together to recruit CBP to BDNF promoter IV once CREB and MeCP2 are phosphorylated at

serine-133 and serine-421, respectively, and MEF2 is dephosphorylated at serine-408 (Greer and Greenberg, 2008).

REs in BDNF promoter VI

Reporter gene assays using BDNF promoter VI sequences (promoter IV according to the old nomenclature) have identified several regulatory elements required for rodent BDNF promoter VI transcriptional activation by the MAPK, CaMKII, and PKA signaling pathways (Takeuchi *et al.*, 2002). Potential C/EBP/β and Sp1 binding sites in BDNF promoter VI were suggested to mediate BDNF activation (West, 2008). In addition to neuronal activity, NGF (Park *et al.*, 2006) and corticosteroid hormones (Hansson *et al.*, 2006) have been shown to regulate BDNF promoter VI. NGF is likely to act through the MAPK pathway to induce transcription (Park *et al.*, 2006). Steroid hormones reduce BDNF exon VI expression possibly by direct binding of a steroid hormone receptor repressor complex to promoter VI. A putative glucocorticoid response element-like sequence has been identified in promoter VI (Funakoshi *et al.*, 1993), but its role in the regulation of BDNF is not yet established. It must be noted, however, that human BDNF promoter VI shares very little sequence similarity with rodent BDNF promoter VI, and the abovementioned elements are not conserved in human BDNF promoter VI.

1.4.4 Activity-dependent epigenetic modifications at BDNF promoters

Generally, histone acetylation, regulated by histone deacetylases (HDACs) and histone acetyltransferases (HATS), is associated with open chromatin and allows for increased transcription. While histone methylations regulated by histone methyltransfereases (HMTs) and histone demethylases (HDMs) are more stable and can be associated either with the repression or activation of transcription in the given locus. Neuronal activity-dependent chromatin remodeling at BDNF promoter I and IV has been shown to regulate BDNF expression *in vitro* and *in vivo*. In cultured rat neurons, membrane depolarization induced histone H3 and H4 acetylation at BDNF promoter IV (Chen *et al.*, 2003a; Martinowich *et al.*, 2003). Also, recent study reported that HDAC1 was released from BDNF promoters I and IV following NMDA receptor activation in cultured rat hippocampal neurons (Tian *et al.*, 2010). *In vivo*, seizures (Tsankova *et al.*, 2004), epilepsy (Huang *et al.*, 2002), antidepressants (Tsankova *et al.*, 2006), and cocaine exposure (Kumar *et al.*, 2005) have been demonstrated to increase acetylation of histones H3 and H4 at BDNF promoters IV and VI and induce BDNF mRNA transcription.

Neuronal activity-dependent regulation of histone methylation at BDNF promoter IV and VI contributes to the transcriptional control of BDNF expression (Chen *et al.*, 2003a; Martinowich *et al.*, 2003; Tsankova *et al.*, 2006). Histone methylation code is more complex than acetylation as it has been associated with either transcriptional ac-

tivation or repression depending on the particular methylated lysine (K). Amino acid residues of histones may be either mono-, di-, or tri- methylated resulting in different effects on gene transcription (Lachner and Jenuwein, 2002). At BDNF promoter IV, membrane depolarization *in vitro* drives dimethylation of histone H3 at K4, which is associated with transcriptional activation (Martinowich *et al.*, 2003), while at the same promoter, a repressive methylation event – dimethylation of histone H3 at K9 – is reduced by neuronal activity (Chen *et al.*, 2003a; Martinowich *et al.*, 2003). *In vivo*, defeat stress in mice induced prolonged downregulation of BDNF promoters IV and VI by strongly increasing the repressive histone H3 K27 dimethylation at these promoters (Tsankova *et al.*, 2006).

Furthermore, epigenetic DNA modifications regulate neuronal activity-dependent activation of the BDNF gene by decreasing CpG methylation of BDNF promoter IV DNA and the release of a MeCP2/HDACs/mSin3A repressor complex (Chen *et al.*, 2003a; Martinowich *et al.*, 2003). Amazing results have been reported by Roth *et al.* (2009a). This study investigated whether early-life maltreatment by caregivers leaves long-lasting epigenetic marks at the BDNF gene in the CNS. Methylation status of BDNF promoter IV and BDNF gene expression was studied throughout the life span of the animals. Also, DNA methylation patterns was studied in the next generation of infants. The results showed that maltreatment in infancy produced persisting changes in methylation of BDNF promoter IV and caused the reduction in BDNF mRNA levels in the adult prefrontal cortex. Furthermore, altered BDNF promoter methylation was observed in offspring of the females that had themselves experienced maltreatment in the childhood. This study presented one more proof that epigenetic regulation of BDNF expression can have a long-lasting effect and can be linked with neuronal plasticity and psychiatric illnesses (Roth *et al.*, 2009a).

1.4.5 The role of BDNF in synaptic plasticity

Neuronal activity strongly increases BDNF expression in hippocampal neurons (Patterson *et al.*, 1992; Dragunow *et al.*, 1993). Activity-dependent regulation of BDNF transcription as well as the localization of BDNF and TrkB at glutamatergic synapses (Drake *et al.*, 1999) suggested that these molecules might modulate synaptic plasticity. First, Zafra *et al.* (1990) showed that depolarization of cultured embyonic rat hippocampal neurons and subsequent elevation of intracellular calcium lead to an increase in BDNF mRNA levels. Then, *in vivo*, BDNF mRNA expression in the hippocampus and cortex was reported to be induced by seizures (Ballarn *et al.*, 1991; Ernfors *et al.*, 1991; Isackson *et al.*, 1991). Later, it was shown that the blockade of visual input results in the down-regulation of BDNF mRNA levels in the rat visual cortex (Castrén *et al.*, 1992). Now it is known that BDNF mRNA expression in the CNS is altered by multiple stimuli such as nerve lesion, antidepressant treatment, stress, drugs, exercise, administration of kainic acid (KA), ischemic insults, hypo-

glycaemic coma, and others (see West, 2008). Also, exercise (Gómez-Pinilla *et al.*, 2002) learning (Hall *et al.*, 2000; Mizuno *et al.*, 2003), and short- or long-term memory formation (Alonso *et al.*, 2002) induce BDNF mRNA in the adult hippocampus.

LTP is compromised at synapses in hippocampal slices prepared from $BDNF^{-/-}$ or $BDNF^{+/-}$ animals (Korte *et al.*, 1995) , but it can be restored when BDNF is added to the slices (Korte *et al.*, 1996; Patterson *et al.*, 1996). Knocking out TrkB in the hippocampus also severely compromises LTP (Minichiello *et al.*, 1999). BDNF also contributes to homeostatic regulation of excitatory synaptic strengths and in the maintenance of the balance in cortical excitation and inhibition (Rutherford *et al.*, 1998). In mice over-expressing BDNF in sympathetic neurons, increased number of synapses was observed, whereas decreased number of synapses was detected in $BDNF^{-/-}$ animals (Causing *et al.*, 1997). The mechanisms of BDNF action during LTP are still unclear. There is good evidence for both pre- and postsynaptic effect of BDNF in modulating LTP (Schuman, 1999).

In mice carrying mutations in TrkB gene fewer synaptic contacts as well as decreased numbers of synaptic vesicles in presynaptic neurons were recorded in the hippocampus (Martnez *et al.*, 1998). Numerous experiments with CNS neurons showed that BDNF was able to rapidly modulate neurotransmission (Schuman, 1999). BDNF can act as a retrograde signal that enhances presynaptic release (hui Zhang and ming Poo, 2002). It was suggested that the stimulation of neurotransmitter release by BDNF involves phosphorylation of synapsins (Jovanovic *et al.*, 2000). BDNF can alter the functional state of neurons within milliseconds (Kafitz *et al.*, 1999). It causes depolarization and elicits action potential in pyramidal cells of the hippocampus or cortex and in the Purkinje cells of the cerebellum. This depolarization results from an increased conductance for sodium ions, and it is as rapid as that induced by the neurotransmitter glutamate. It has been shown that TrkB codistributes and associates with the cation channel TRPC3 (Li *et al.*, 1999). BDNF binding to TrkB leads to a TRPC3-dependent cation influx in CNS neurons through the activation of PLC-γ (Li *et al.*, 1999).

There is strong evidence that activity-dependent regulation of GABAergic synapses is important for the plasticity of the nervous system (Hensch, 2005). BDNF is an important mediator of the development of cortical inhibition induced by neuronal activity (Rutherford *et al.*, 1997). BDNF participates in the control of the number of exitatory/inhibitory synapses to be formed on neurons. The density of inhibitory synapses in the brain regions such as primary sensory cortex, hippocampus and cerebellum is regulated by the level of excitatory synaptic activity and sensory input (Benevento *et al.*, 1995; Seil, 1996; Marty *et al.*, 2000). It is remarkable that mutation of the CRE site at endogenous BDNF promoter IV results in animals that exhibit a reduction in the number of inhibitory synapses formed by cortical neurons in culture, a reduction in spontaneous inhibitory transmission, and a reduction in the level of inhibitory presynaptic markers in the cortex (Hong *et al.*, 2008). However, even though activity-

dependent BDNF promoter IV induction is required for the appropriate development of inhibition in the cortex, it does not appear to affect the survival or differentiation of inhibitory GABAergic neurons (Hong *et al.*, 2008). Interestingly, these findings point to a previously unappreciated role for CREB in regulating inhibitory synapse development (Hong *et al.*, 2008).

It is known that elimination of some synapses and potentiation of other synapses occur in parallel within individual neurons during synapse development (Chen and Regehr, 2000). Activation of MEF2, a transcription factor that regulates BDNF promoters I and IV (see above), might lead to a decrease in the total number of synapses formed onto cells through one group of target genes (e.g. HOMER1A, ARC, KCNA1) and at the same time lead to the strengthening of a separate subset of synapses through another group of target genes (e.g., ADCY8, BDNF) (Flavell *et al.*, 2008). MEF2 may play a positive role in promoting inhibitory synapse development in addition to its effects on excitatory synapse development since MEF2 target genes such as BDNF and gephryn (Giesemann *et al.*, 2003) encode proteins that function at inhibitory synapses (Flavell *et al.*, 2008).

It has been recenlty discovered that transcription factor NPAS4 which is regulated by neuronal activity plays a key role in the formation of GABAergic inhibitory synapses onto excitatory neurons. NPAS4 expression is rapidly activated by excitatory synaptic activity. Visual stimulation results in an increase in NPAS4 mRNA and protein levels specifically in the visual cortex. NPAS4 induction in cultured neurons requires an influx of extracellular Ca^{2+} through L-VSCCs and is partly dependent on the activation of NMDA and AMPA receptors (Lin *et al.*, 2008b). NPAS4 decreases the number of excitatory synapses that form on neurons and presynaptic neurotransmitter release probability which results in a decrease in excitation of a neuron. NPAS4 appears to be a direct target of MEF2 (Lin *et al.*, 2008b). Together with MEF2, it regulates BDNF gene transcription. Therefore, BDNF's role in synapse development might be exerted through activation by MEF2 and NPAS4.

Imbalance in the excitatory-inhibitory synaptic strength in the brain can have significant consequences for the nervous system, leading to mental retardation, neurodevelopmental and autism spectrum disorders (Möhler, 2006; Hensch and Fagiolini, 2005; Rubenstein and Merzenich, 2003). Mutations in the components of activity-regulated transcription can cause various human cognition disorders. For example, mutation of a subtype of the L-VSCC (Ca_v-1.2) is the cause of Timothy syndrome, an autism spectrum disorder with significant cognitive impairment (Splawski *et al.*, 2004). Mutation of the CBP results in Rubinstein-Taybi syndrome (Petrij *et al.*, 1995). Mutation of RSK2 (ribosomal S6 kinase-2 that activates CREB by phosphorylation at serine 133) results in Coffin-Lowry syndrome (Hanauer and Young, 2002). Both syndromes exhibit severe mental retardation. Mutation of MeCP2, as mentioned above, results in Rett syndrome, a developmental disorder characterized by mental retardation and defects in socialization. Since Timothy syndrome and Rett syndrome

are autism spectrum disorders, it is possible that defects in activity-dependent gene transcription might be a cause of autism (Greer and Greenberg, 2008). Taken together, investigation of neuronal activity-regulated gene expression would expand our understanding how environment shapes the nervous system and how impairment in this process may lead to disorders of cognition.

1.5 The role of BDNF in neurological and psychiatric disorders, obesity and cancer. Therapy options involving neurotrophins

1.5.1 Alzheimer's disease

Alzheimer's disease (AD), also called Alzheimer disease, Senile Dementia of the Alzheimer Type (SDAT) or simply Alzheimer's, is the most common cause of dementia in elderly. AD is incurable and degenerative, it is diagnosed in people over 65 years of age although the less-prevalent early-onset AD can occur much earlier. By the year 2010, there were more than 35 million sufferers worldwide (Querfurth and LaFerla, 2010). AD is predicted to affect 1 in 85 people globally by 2050 (Brookmeyer *et al.*, 2007). In the early stages, the most commonly recognized symptom is a short-term memory loss. As the disease advances, symptoms include confusion, irritability and aggression, mood swings, language breakdown, long-term memory loss. Eventually, gradual loss of bodily functions leads to death (Querfurth and LaFerla, 2010).

AD is considered to be a protein misfolding disease (proteopathy) since protein abnormalities have been described in AD and are used to explain its causes. Pathological features of AD include i) extracellular plaques of β-amyloid peptide (Aβ) and dystrophic neurites in neocortex; ii) intracellular neurofibrillary tangles; iii) loss of neurons, synapses, and white matter in the cerebral cortex and certain subcortical regions; iv) inflammation and oxidative brain damage (Querfurth and LaFerla, 2010; Blennow *et al.*, 2006). The 'amyloid hypothesis' suggests that extracellular aggregates of Aβ cause of AD. Aβ peptides consist of 36–43 amino acids and are natural metabolic products that originate from proteolysis of the amyloid precursor protein (APP) (Haass *et al.*, 1993). Monomers of $A\beta_{40}$ are much more prevalent than the aggregation-prone and damaging $A\beta_{42}$ species. APP is sequentially cleaved by beta-site APP–cleaving enzyme 1 (BACE-1 or β-secretase) and γ-secretase - a protein complex containing presenilins, nicastrin, anterior pharynx-defective 1 (APH-1), and presenilin enhancer 2 (PEN-2) (Haass and Selkoe, 2007). Another APP processing pathway that include cleavage by α-secretase is considered to be non-amyloidogenic since it precludes Aβ formation (Lammich *et al.*, 1999). The proteases neprilysin (Kanemitsu *et al.*, 2003) and insulin-degrading enzyme (Qiu *et al.*, 1998) also regulate levels of Aβ in the brain by degrading Aβ monomers and oligomers and overexpression of these enzymes prevents plaque formation (Leissring *et al.*, 2003). An

imbalance between production and clearance of Aβ peptides causes Aβ to accumulate, and this excess may be the initiating cause of AD. Physiologic levels of Aβ may be important for controlling excitatory transmission and preventing neuronal hyperactivity (Kamenetz *et al.*, 2003), however, oligomers of Aβ are toxic to synapses (Walsh *et al.*, 2005). Neuronal activation rapidly increases Aβ secretion at the synapse. Aβ can form voltage-independent cation channels in lipid membranes (Arispe *et al.*, 1993) resulting in calcium uptake and degeneration of neurites (Lin *et al.*, 2001).

Mutations associated with early-onset familial AD (FAD) are dominantly inherited and are found in the APP gene itself (Haass *et al.*, 1994) or in the PSEN1 and PSEN2 genes (Selkoe and Kopan, 2003). Transgenic mice that express a mutant form of the human APP gene develop fibrillar amyloid plaques and Alzheimer's-like brain pathology with spatial learning deficits (Games *et al.*, 1995; Masliah *et al.*, 1996; Hsiao *et al.*, 1996). Also, apolipoprotein E isoform 4 (APOE4) - the major genetic risk factor for AD - leads to excess amyloid buildup in the brain (Polvikoski *et al.*, 1995) as well as synaptic and cytoskeletal alterations in neurons (Masliah *et al.*, 1995). The 'amyloid hypothesis' is further supported by the studies of Down's syndrome (DS) patients and DS animal models. The APP gene is located on the chromosome 21. People with DS have trisomy 21 (three copies of chromosome 21), thus having an extra APP gene copy, and exhibit AD by 40 years of age (Nistor *et al.*, 2007). Recently, amyloid model has been updated. N-APP, an N-terminal fragment of APP that is cleaved from APP by BACE-1 has been shown to trigger neuronal death by binding to a neuronal death receptor 6 (DR6, also known as TNFRSF21) (Nikolaev *et al.*, 2009). In this model, Aβ plays a complementary role, by disturbing synaptic function.

The 'tau hypothesis' suggests that tau protein abnormalities initiate the disease onset. In this model, hyperphosphorylated tau begins to aggregate. Eventually, neurofibrillary tangles are formed inside neurons (Goedert *et al.*, 1991), the microtubules disintegrate collapsing the neuron's transport system (Iqbal and Grundke-Iqbal, 2005). This may result in malfunctions in synaptic transmission and neuronal death (Chun and Johnson, 2007; Khlistunova *et al.*, 2006). Abnormal tau molecules impair cognition (Santacruz *et al.*, 2005; Oddo *et al.*, 2006). Tau mutations do not occur in AD, but increased levels of tau phosphorylated at threonine residues T181 and T231, and total soluble tau in the cerebrospinal fluid correlate with the decline in cognition (Wallin *et al.*, 2006).

The neuronal degeneration in AD is also suggested to be due to the defects in cholesterol metabolism (Bu, 2009). Cholesterol hypothesis ties together the apolipoprotein E (APOE) genetic risk, Aβ production and aggregation, and vasculopathy of AD. A single APOE4 allele increases the risk by a factor of 4, and two APOE4 alleles increase the risk by a factor of 19 (Strittmatter and Roses, 1996). APOE transports cholesterol in the CNS. APOE4 is a pathological chaperone, promoting Aβ deposi-

tion (Golabek *et al.*, 1996) and tau phosphorylation (Wang *et al.*, 1998). It is the least effective of the three apolipoproteins in promoting normal membrane lipid turnover and the uptake of lipoprotein particles. However, trials that were conducted to reduce free cholesterol levels have not been effective in treatment of AD (Querfurth and LaFerla, 2010).

Synaptic impairment and the role of BDNF in Alzheimer's disease

In AD, the number of synapses decreases at the early stage of the disease in the cortex and hippocampus, and the remaining synapses show compensatory increase in size (Scheff *et al.*, 1990, 2007). As the disease advances, synapses are lost, particularly in the dentate gyrus of the hippocampus and in the neocortex (DeKosky and Scheff, 1990; Masliah *et al.*, 1993). In cultured cortical neurons, Aβ interferes with LTP by intervening with PKA/CREB (Vitolo *et al.*, 2002), Ras/ERK and PI3-K/AKT pathways (Tong *et al.*, 2001) and downregulates BDNF expression (Tong *et al.*, 2004). In late-stage AD, levels of neurotrophins (especially BDNF) and their receptors are severely reduced in cholinergic neurons in the basal forebrain (Connor *et al.*, 1997), in hippocampus and several cortical areas (Phillips *et al.*, 1991) . Disturbance in synaptic transmission occurs partially due to the endocytosis of NMDAR (Snyder *et al.*, 2005) and AMPAR (Hsieh *et al.*, 2006) and the impaired release of neurotransmitters. Moreover, high amounts of truncated TrkB receptors have been found in the Aβ plaques (Connor *et al.*, 1996). Murer *et al.* (1999) demonstrated that in AD brains neurons containing neurofibrillary tangles did not contain BDNF, whereas most intensely BDNF labeled neurons were devoid of tangles. PreproBDNF and mature BDNF are also decreased in the cortex and hippocampus of AD patients (Peng *et al.*, 2005; Michalski and Fahnestock, 2003). In rat cortical neurons, it has been shown that $A\beta_{1–40}$ binds to the $p75^{NTR}$ neurotrophin receptor, inducing apoptosis (Yaar *et al.*, 1997; Sotthibundhu *et al.*, 2008). Also, APP has been shown to bind $p75^{NTR}$ and cause apoptosis in neuroblastoma cells (Fombonne *et al.*, 2009). Aβ or hyperphosphorylated tau can potentially interfere with BDNF mRNA localization into dendritic compartments (Tapia-Arancibia *et al.*, 2008).

Finally, CREB phosphorylation has been reported to be impaired in AD patients (Yamamoto-Sasaki *et al.*, 1999). Aβ blocks nuclear translocation of phosphorylated CREB (Arvanitis *et al.*, 2007). In human neuroblastoma cells, it has also been reported that oligomeric $A\beta_{1–42}$ decreases phospho-CREB and BDNF exon IV and VI mRNA (Garzon and Fahnestock, 2007). Aβ has been shown to interfere with synaptic function through binding to several receptors such as amyloid-binding alcohol dehydrogenase (ABAD), receptor for advanced glycation end products (RAGE) and α-7 nicotinic acetylcholine receptors (Arancio *et al.*, 2004; Takuma *et al.*, 2005; Dineley *et al.*, 2001). Following Aβ binding to these receptors, CREB phosphorylation is decreased and LTP is impaired.

Treatment of Alzheimer's disease

Clinical trials of γ-secretase inhibitor, vaccination with Aβ, and monoclonal antibodies against various Aβ epitopes are in progress. The vaccine was found to clear the amyloid plaques in early human trials, but it did not have any significant effect on dementia (Holmes *et al.*, 2008). Moreover, in a phase IIa trial vaccination resulted in encephalitis, and showed no cognitive or survival benefit. A phase II trial of passive immunization resulted in vasogenic cerebral edema in some patients. Phase III trials of two monoclonal antibodies against Aβ are under way (Querfurth and LaFerla, 2010). Trials of small-molecule inhibitors of Aβ (e.g., scylloinositol) and tau oxidation and aggregation inhibitors (e.g., methylene blue) are under way (Querfurth and LaFerla, 2010).

The L-VGCC blocker, MEM 1003, is in a phase III trial, and memantine, an NMDA-receptor blocker, is approved by the Food and Drug Administration. In AD, synthesis of the neurotransmitter acetylcholine is reduced (Geula and Mesulam, 1995). A potential treatment for AD, the cholinesterase inhibitor drug ladostigil, induced BDNF expression as well as APP processing by α-secretase by inducing the PKC and MAPK cascades (Yogev-Falach *et al.*, 2006) and prevented memory deficits in rats (Shoham *et al.*, 2007). However, although cholinesterase inhibitors improve neurotransmission and provide moderate relief in AD, they lose efficacy over time (Raschetti *et al.*, 2007). The use of agonists and modulators of α-7 nicotinic acetylcholine receptors is under investigation (Querfurth and LaFerla, 2010).

Dysfunctional mitochondria in AD release free radicals and cause oxidative stress (Smith and Perry, 1995). Subsequently, increases in membrane permeability to calcium, other ionic imbalances, and impaired glucose transport (Mark *et al.*, 1996) enhance neurodegeneration. However, trials of AD treatment by antioxidants have generally failed (Praticò, 2008). Removal of divalent metals is potentially harmful because they serve as co-factors in multiple essential enzymatic reactions. In a pilot phase II trial, PBT2, a safe compound derived from clioquinol that attenuates metal proteins (Adlard *et al.*, 2008) showed some efficacy.

Glucose intolerance and type 2 diabetes are known to be the risk factors in AD (Craft *et al.*, 1998; Arvanitakis *et al.*, 2004). Resistance to insulin makes neurons energy-deficient and impairs synaptic plasticity. Moreover, high serum glucose levels up-regulate the tau kinase, glycogen synthase kinase 3β (Takashima, 2006) and reduce levels of insulin-degrading enzyme in the brain in AD (Cook *et al.*, 2003). Treatment with thiazolidine drugs (peroxisome-proliferator-activated receptor (PPAR) agonists, which activate insulin-responsive gene transcription) prevented AD-associated changes and cognitive decline in transgenic mice (Pedersen *et al.*, 2006) and had significant effects in subpopulations of patients with AD (Risner *et al.*, 2006).

Finally, neurotrophin therapy is being developed for AD. A phase I study of *ex vivo* gene therapy was reported: genetically modified cells that express NGF have

been transplantated into the brains of AD patients (Tuszynski *et al.*, 2005). BDNF induces rapid *in vitro* dephosphorylation of tau protein in neural cells (Elliott *et al.*, 2005). Exogenous application of BDNF can rescue neurons from death by preventing Aβ and tau-induced neurodegeneration *in vitro* and *in vivo* (Tapia-Arancibia *et al.*, 2008). However, in humans, brain neurotrophin administration induces strong side effects as pain and weight loss (Schulte-Herbrüggen *et al.*, 2007; Weinreb *et al.*, 2007) limiting its usage as a therapeutic molecule. The challenge for future research will be to develop therapeutic strategies aimed at boosting endogenous BDNF or/and TrkB activity, which would prevent dementia (Tapia-Arancibia *et al.*, 2008).

1.5.2 Parkinson's disease

Parkinson's disease (PD) is a progressive neurodegenerative disorder caused by death of dopaminergic neurons in the substantia nigra (SN), a midbrain strucure that provides dopamine input to the striatum (a forebrain structure). In PD, the balance in the inhibitory/excitatory transmission in these brain structures is lost, which leads to disability to control movement. The main symptoms of PD are difficulty in initiating movements, slowness of movements, stiffness, and tremor. Neurons in PD contain Lewy bodies – abnormal aggregates of *alpha*-synuclein associated with other proteins such as ubiquitin (Engelender, 2008), neurofilament protein, and *alpha* B-crystallin. Tau proteins may also be present (Ishizawa *et al.*, 2003).

Reduced BDNF expression in the PD brain has been observed in the substantia nigra pars compacta (SNpc) (Howells *et al.*, 2000), as well as in the striatum (Mogi *et al.*, 1999; Parain *et al.*, 1999). The total number of neurons containing BDNF is significantly reduced (Parain *et al.*, 1999), and the surviving dopaminergic neurons in PD express less BDNF (Howells *et al.*, 2000). Furthermore, almost all dopaminergic neurons containing Lewy bodies are immunoreactive for BDNF, suggesting that endogenous BDNF protein expression is not sufficient for protecting neurons from the degenerative process in PD. TrkB mRNA levels do not show any change per surviving neuron in the SN of the PD brains (Benisty *et al.*, 1998).

Treatment of Parkinson's disease

Treatments that relieve the symptoms and increase dopaminergic transmission in striatum have been helpful at the initial treatment stage, but later on patients experience severe complications such as L-DOPA-induced dyskinesia (LID), motor fluctuations and hallucinations (Fabbrini *et al.*, 2007). Therefore, non-dopaminergic drug development could provide great benefit for PD patients (Hu and Russek, 2008).

Adenovirus (AdV), adeno-associated virus (AAV), herpes simplex virus (HSV) and lentivirus (LV) vectors have recently been used for transferring genes to specific brain regions (Mandel *et al.*, 2008). Phase I clinical trials have been conducted to

deliver glutamic acid decarboxylase (GAD) gene, that catalyses synthesis of GABA, directly into neurons of the human subthalamic nucleus with an adeno-associated virus (AAV) vector (Kaplitt *et al.*, 2007). In patients with PD, activity of the subthalamic nucleus is increased mainly because of the reduced GABAergic input from the globus pallidus. Therefore, increased GABA synthesis could compensate for this deficiency. The 1 year follow-up of this therapy reported that AAV-GAD treatment was safe and well tolerated by patients with advanced PD, reducing thalamic activity. However, GAD gene therapy did not exhibit a strong disease-modifying effect (Kaplitt *et al.*, 2007).

Increasing evidence suggests that programmed cell death (apoptotic-like cell death) is a key cell death mechanism in the selective loss of dopaminergic neurons in PD (Hirsch *et al.*, 2000). Recently, the anti-PD monoamine oxidase-B inhibitor – rasagiline – has been shown to possess neuroprotective activities by regulating the Bcl-2 family of proteins (thus protecting mitochondrial viability) and by inducing BDNF via activating PI3K/Akt pathway (Weinreb *et al.*, 2007; Sagi *et al.*, 2007). Rasagiline showed beneficial effects in PD patients, and confered significant symptomatic improvement (Biglan *et al.*, 2006). The fact that injured dopaminergic neurons respond to exogenous BDNF *in vivo* (Hagg, 1998; Tsukahara *et al.*, 1995) and that TrkB mRNA expression is normal in PD patients, suggests that PD patients could benefit from a rationally designed BDNF therapy (Hu and Russek, 2008).

1.5.3 Huntington's disease

Huntington's disease (HD) is a fatal, dominantly inherited neurodegenerative disorder with onset in midlife. It is characterized by psychiatric, cognitive and motor dysfunctions. HD is caused by an excessive repetition of the CAG trinucleotide in exon 1 of the huntingtin gene, which results in the production of a protein bearing a polyglutamine (polyQ) expanded tract in its N-terminus. This mutation leads to a widespread brain neurodegeneration with specific loss of striatal and cortical neurons (Zuccato and Cattaneo, 2007). It has been suggested that transglutaminases (TGases) play a critical role in the pathogenesis of HD because they cross-link huntingtin and catalyse the formation of aggregates. As TGase activity is increased in HD brain, they represent an attractive therapeutic target in HD (Gentile and Cooper, 2004; Hoffner and Djian, 2005).

Studies showed that BDNF is involved in the development of HD. Striatal neurons in the brain require BDNF for their activity and survival. Most of the BDNF acting in the striatum is of cortical origin (Zuccato and Cattaneo, 2007). Huntingtin mutation in HD reduces the transcriptional activity of BDNF promoters, thus decreasing protein production in the cerebral cortex (Zuccato *et al.*, 2001, 2005). Obstructed anterograde and retrograde vesicle transport of BDNF protein from cortical neurons to striatal neurons has also been observed (Gauthier *et al.*, 2004). Wild-type huntingtin

might act as a facilitator of RE1/NRSE-containing neuronal gene transcription in the nervous system (Zuccato *et al.*, 2003). It retains REST/NRSF in the cytoplasm, thus reducing REST/NRSF binding within BDNF promoter II and allowing BDNF gene transcription (Zuccato *et al.*, 2003). However, mutated huntingtin causes the pathological entry of REST/ NRSF into the nucleus where it can bind to the RE1/NRSE site and lead to BDNF promoter II repression. Also, mutated huntingting downregulates the levels of synapsin-1, cholinergic receptor, and several other genes in the cerebral cortex (Zuccato *et al.*, 2003).

Also, the transcriptional activity of BDNF promoters IV and VI is affected in cell and mouse models of HD (Zuccato *et al.*, 2001, 2005). However, the mechanism of inactivation of BDNF promoters IV and VI in HD is still unknown. Several findings suggested that CBP can be sequestered into mutant huntingtin aggregates (Nucifora *et al.*, 2001; McCampbell *et al.*, 2000). Reduced CREB phosphorylation may also contribute to the reduced transcription from BDNF promoter IV (Gines *et al.*, 2003). Moreover, CREB co-activator TAFII-130 interacts with mutant huntingtin, which might impair BDNF IV transcription (Dunah *et al.*, 2002). It has been reported that heterozygous HD patients carrying BDNF Met allele have a later age of onset compared with homozygous Val/Val BDNF patients (Alberch *et al.*, 2005). It was shown that mutant huntingtin impairs post-Golgi trafficking of $BDNF_{Val}$ but not of $BDNF_{Met}$, impairing regulated secretion of $BDNF_{Val}$ and reducing activity-dependent release of $BDNF_{Val}$ (del Toro *et al.*, 2006). Finally, mutant huntingtin affects TrkB levels in HD (Ginés *et al.*, 2006). This fact has to be taken into account when designing therapeutic strategies based on modulation of BDNF levels.

Treatment of Huntington's disease

In parallel to the development of the lentiviral delivery of BDNF (Bemelmans *et al.*, 1999), cells that express and continuously release BDNF at safe doses have been applied to protect striatal neurons from neurotoxic damage in mouse models of HD (Ryu *et al.*, 2004). Also, small-molecule BDNF analogues with improved pharmacokinetic properties and the ability to penetrate the blood-brain barrier (BBB) are being developed. Cyclic peptides that mimic BDNF three-dimensional structure and bind to TrkB receptor have been designed. Preliminary studies have demonstrated that such BDNF mimetics act as BDNF agonists that promote the survival of cultured sensory neurons, although their proteoliyic stability and their ability to activate TrkB receptors still require analysis (Fletcher and Hughes, 2006).

The usage of antidepressants that affect BDNF levels such as selective serotonin reuptake inhibitors (SSRIs) (Nibuya *et al.*, 1996) and lithium (Chuang, 2004) have been studied in treating HD (Zuccato and Cattaneo, 2007). Serotonin might have protective effects on striatal and cortical neurons by activating cAMP and CREB which leads to BDNF upregulation (Tardito *et al.*, 2006). However, clinical studies failed

to prove SSRIs useful for non-depressed HD patients (Como *et al.*, 1997). Lithium induces the expression of BDNF in cortical neurons (Fukumoto *et al.*, 2001). Also, lithium inhibits GSK-3beta, which is involved in apoptotic cell death, and induces beta-catenin whose overxpression protects cells from mutant huntingtin-induced toxicity (Carmichael *et al.*, 2002). However, lithium did not have consistent effect on motor functions and did not improve survival in mouse model of HD (Wood and Morton, 2003).

Memantine (Marvanová *et al.*, 2001) and riluzole (non-competitive inhibitors of NMDA receptor) (Mizuta *et al.*, 2001; Katoh-Semba *et al.*, 2002) have recently been shown to increase BDNF levels. A 2-year study of 27 HD patients was carried out in order to investigate the effectiveness of memantine in delaying disease progression. The results suggest that memantine treatment may be useful in slowing-down HD progression (Beister *et al.*, 2004). Another trial has found that riluzole causes transient motor improvement in human HD patients (Seppi *et al.*, 2001).

Cystamine is a competitive inhibitor of TGase activity. It limits the aggregation of proteins with an expanded polyQ tract (Igarashi *et al.*, 1998; de Cristofaro *et al.*, 1999). It has been shown to relieve symptoms and improve survival in HD mice (Dedeoglu *et al.*, 2002; Karpuj *et al.*, 2002), limiting the decrease in brain weight, brain volume and neuronal atrophy (Dedeoglu *et al.*, 2002). Recent findings have linked cystamine and its reduced form, cysteamine (a drug approved by the FDA), to BDNF. It was found that cystamine increases the levels of heat-shock DnaJ domain-containing protein 1b (HSJ1B), whose levels are decreased in HD patients. HSJ1B inhibits the polyQ-induced death of striatal neurons and neuronal dysfunction by stimulating BDNF secretion through the formation of clathrin-coated vesicles containing BDNF (Borrell-Pagès *et al.*, 2006). It has been suggested that cystamine increases BDNF secretion from the Golgi, and that this effect is blocked by reducing HSJ1B levels or by overexpressing transglutaminase. Tolerated cystamine doses have been evaluated in HD patients, which encourages using cystamine and cysteamine for treatment HD (Zuccato and Cattaneo, 2007). Finally, inhibiting REST/NRSF, the silencer of BDNF promoter II, could be the target of the therapeutic design in the future (Zuccato and Cattaneo, 2007).

1.5.4 Epilepsy

Epilepsy is a chronic heterogeneous neurological disorder that affects $\sim$50 million people worldwide regardless of age and gender (Bialer and White, 2010). It is characterized by recurrent spontaneous seizures. Epilepsy can be acquired after a brain insult such as trauma, infection, stroke or tumour. The inherited forms of epilepsy can be caused by genetic mutations in ion channel genes (Singh *et al.*, 1998; Biervert *et al.*, 1998) or neurotrasmitter receptor genes (Bertrand, 2002; Baulac *et al.*, 2001), although other genes and environmental factors can modulate phenotypic expression

of epilepsy (age at onset, duration of seizures, responsiveness to antiepileptic drugs) (Gourfinkel-An *et al.*, 2004). However, for many epilepsy cases, genes whose mutations cause the disease are unknown.

It is a central idea of epilepsy field that seizures result from imbalance between exitation and inhibittion (Moshé, 2000). The understanding of the molecular mechanisms that underlie epilepsy has come from exploring the mechanisms of antiepileptic drugs (AEDs) action. Voltage-gated sodium channels in the brain are the molecular targets of numerous AEDs. Drugs that enhance GABA receptor subtype A (GABAA)-mediated inhibitory neurotransmission and the $\alpha 2\delta$ potassium chanel ligands gabapentin (GBP) (Neurontin; Pfizer) and pregabalin (Lyrica; Pfizer) are also effective anticonvulsants (Rogawski, 2006). Levetiracetam (Keppra; UCB) and its structural analogue brivaracetam bind to synaptic vesicle glycoprotein 2A (SV2A) that coordinates synaptic vesicle exocytosis and neurotransmitter release (Lynch *et al.*, 2004; Kaminski *et al.*, 2009). Felbamate limits NMDAR, and topiramate attenuates AMPAR and kainate receptor activity (Bialer and White, 2010).

Expression levels of BDNF mRNA and protein increase as a consequence of seizures in several animal models of epilepsy including kindling (repeated small electrical or chemical stimulation to the brain) (Ernfors *et al.*, 1991), kainic acid (Rudge *et al.*, 1998), and pilocarpine treatment (Mud *et al.*, 1996; Poulsen *et al.*, 2004), especially in the hippocampus. Elevated levels of BDNF mRNA (Mathern *et al.*, 1996) and protein (Takahashi *et al.*, 1999) have been detected in hippocampal and temporal lobe tissues of human patients with temporal lobe epilepsy. It was suggested that reduced TrkB activation may inhibit the development of kindling (Kokaia *et al.*, 1995) and that signaling via TrkB promotes epileptogenesis (Binder *et al.*, 1999). Furthermore, compared with wild-type animals, truncated TrkB transgenic mice have less severe seizures with later onset and lower mortality (Lähteinen *et al.*, 2002). Transgenic mice that over-express BDNF have more severe seizures in response to kainic acid and some display spontaneous seizures (Croll *et al.*, 1999). However, chronic intrahippocampal infusion of BDNF inhibits hippocampal kindling, reduces the duration of seizures (Larmet *et al.*, 1995; Reibel *et al.*, 2000b) and decreases TrkB levels by 80% (Knusel *et al.*, 1997). The antiepileptogenic role of BDNF might be due to the up-regulation of neurotransmitter neuropeptide Y (NPY) (Reibel *et al.*, 2000a; Croll *et al.*, 1994). NPY was shown to inhibit hippocampal seizures in model animals (Woldbye *et al.*, 1997). Moreover, NPY has a potent and prolonged presynaptic inhibitory effect on excitatory synaptic transmission in human dentate gyrus (Colmers and Bahh, 2003).

Despite many available AEDs, none is considered to be reliable in curing epilepsy. Also, pharmacoresistant epilepsy forms that cannot be treated by any existing AED represent a challenge for drug design. Also, drugs without side effects are being sought for (Bialer and White, 2010). Carefully designed gene or stem cell therapy to induce neurogenesis and enhance neuroprotection is an attractive strategy

for antiepilepsic therapy. Induction of antinecrotic and anti-apoptotic genes, neurotrophins, and neuropeptides could provide benefits for patients with epilepsy (Vezzani, 2007).

1.5.5 Depression

Depression is a mental illness that affects approximately 17% of the population and is a major cause of disability worldwide (Kozisek *et al.*, 2008). In depressed individuals, decline in hippocampal function and reduced hippocampal cell volume has been observed (Warner-Schmidt and Duman, 2006). Also, chronic stress decreases the neurogenesis of dentate granule cells in the adult hippocampus, and multiple antidepressant drugs increase both cell proliferation and neurogenesis in this brain region (Malberg and Blendy, 2005). Most of the stress models that decrease adult neurogenesis also decrease the expression of BDNF (Duman, 2004). A decrease in the levels of BDNF is seen in the hippocampus and prefrontal cortex of depressed patients (Chen *et al.*, 2001; Karege *et al.*, 2005b). Also, serum levels of BDNF are decreased in depression and antidepressant treatment appears to reverse this reduction (Karege *et al.*, 2005a; Shimizu *et al.*, 2003). CREB mRNA levels and protein phosphorylation are also reduced in postmortem brain samples of depressed patients (Yamada *et al.*, 2003). CREB is considered to mediate BDNF induction by antidepressants (Nestler *et al.*, 2002). Direct infusion of BDNF into the midbrain (Siuciak *et al.*, 1997), hippocampus (Shirayama *et al.*, 2002), or into the lateral ventricles (Hoshaw *et al.*, 2005) induces behavioral responses that mimic antidepressant effects in animal models of depression. In contrast, infusion of BDNF into the ventral tegmental area (VTA) produces a depression-like phenotype and over-expression of dominant negative TrkB in the nucleus accumbens (NAc) produces an antidepressant effect (Eisch *et al.*, 2003). In addition, BDNF Met allele has been associated with the reduced susceptibility to depression (Pezawas *et al.*, 2008). Taken together, BDNF role in depression is complex and brain region specific (Hu and Russek, 2008). Mechanisms of depression seem to involve dysfunction in activity-regulated neuronal networks in amygdala circuitry, where BDNF plays an essential role (Castrén *et al.*, 2007).

Treatment of depression

First antidepressants increased the synaptic concentration of amine neurotransmitters, either by inhibiting their metabolism or blocking neuronal reuptake. However, these two classes of antidepressants are associated with significant side effects and potentially serious adverse reactions. Currently, there are several treatment options for depression, including antidepressants, electroconvulsive therapy (Kozisek *et al.*, 2008), cognitive behavioral therapy, interpersonal psychotherapy, and a combination of nondrug and pharmacologic options. Newer drugs include the selective serotonin reuptake inhibitors, which are now considered the drugs of choice in the treatment

of depression. However, the precise mechanisms underlying antidepressant actions are largely unknown. Many antidepressant drugs acutely increase monoamine levels, but they produce long-term changes that go beyond just enhancing serotonergic or noradrenergic neurotransmission (Duman *et al.*, 1994). Among the longterm targets of antidepressant treatments may be the regulation of neurotrophins such as BDNF (Kozisek *et al.*, 2008). Recently it has been shown that statin therapy that is used clinically to reduce plasma cholesterol levels is associated with a reduced risk of depression. Statins can induce tPA and inhibit plasminogen activator inhibitor-1, the major inhibitor of tPA. It is therefore possible that statins could act through the tPA-plasminogen pathway increasing cleavage of proBDNF to mature BDNF thus achieving an antidepressant effect (Tsai, 2007b).

1.5.6 Drug addiction

Although it is not known with certainty what causes drug addiction, it has been hypothesized that long-term changes that occur within the brain's reward circuitry are important. In particular, adaptations in dopaminergic neurons of the ventral tegmental area (VTA) in the midbrain and in their target neurons in the forebrain striatum structure, nucleus accumbens (NAc), are thought to alter an individual's responses to drug and natural rewards (Russo *et al.*, 2009). These changes lead to drug tolerance, reward dysfunction, escalation of drug intake, and eventually compulsive use. Most classes of addictive substances, when administered chronically, alter structural plasticity throughout the brain's reward circuitry (Nestler, 2001; Koob and Moal, 2005). Opiates have been shown to decrease the number and complexity of dendritic spines in NAc, medial prefrontal cortex, and hippocampus, and to decrease the overall soma size of VTA dopaminergic neurons, with no effect on non-dopaminergic neurons in this brain region (Sklair-Tavron *et al.*, 1996; Nestler, 1997). There is an exception to these findings: morphine has been reported to increase spine number on cortical neurons (Robinson *et al.*, 2002). In contrast to opiates, stimulants such as amphetamine and cocaine have been shown to consistently increase dendritic spines and complexity in NAc medium spiny projection neurons, VTA dopaminergic neurons, and prefrontal cortex pyramidal neurons, with no decrease in structural plasticity (Robinson and Kolb, 1997; Norrholm *et al.*, 2003; Sarti *et al.*, 2007).

Drugs of abuse act by changing gene expression in certain brain regions. For example, acute and chronic exposure to amphetamine and cocaine alters the expression of Fos family and other immediate early genes in the striatum. Fos family proteins are rapidly and transiently induced in striatum following acute administration (Graybiel *et al.*, 1990; Young *et al.*, 1991). However, chronic drug exposure desensitizes the induction of the genes (Hope *et al.*, 1992) and leads to the accumulation of ΔfosB, a truncated splice variant of the FosB gene that appears to mediate enhanced sensitivity and drive for cocaine and other drugs of abuse in animal models

of drug addiction (Nestler, 2001; Peakman *et al.*, 2003; Colby *et al.*, 2003). In contrast, BDNF gene expression is activated chronically but not acutely (McClung and Nestler, 2003). Animal and clinical studies confirm that increased BDNF activity in the brain may be implicated in the pathogenesis of drug addiction. BDNF infusion into rat midbrain enhances the rewarding effects of cocaine while in contrast, cocaine-conditioned place preference was decreased in heterozygous BDNF knockout mice (Hall *et al.*, 2003). Furthermore, in humans plasma BDNF concentrations in methamphetamine users were significantly increased compared with controls (Tsai, 2007a). Similarly to depression, BDNF levels in drug addiction is up-or downregulated depending on the brain region (Russo *et al.*, 2009).

Histone modifications have been shown to differentially regulate gene expression in acute and chronic drug administration. Acute effects of cocaine are associated predominantly with acetylation of H4 at cFos and FosB genes. While chronic cocaine robustly induced levels of H3 acetylation at the FosB and Cdk5 promoters, and BDNF promoter II (Kumar *et al.*, 2005). H3 modifications at BDNF promoter II are very long-lasting and even increase over the course of withdrawal (Kumar *et al.*, 2005). This is interesting considering that the levels of BDNF protein in the striatum increase further during withdrawal (Grimm *et al.*, 2003). The knowledge on the mechanisms of drug-induced changes in the brain should lead to the development of novel therapeutic agents that normalize the plasticity induced by drugs of abuse and thereby reverse the addiction process in humans.

1.5.7 Schizophrenia

Schizophrenia is a severe disorder occurring in up to 1% of the population worldwide (Roth 2009). The risk of developing schizophrenia are increased by several candidate genes that interact with severe obstetric complications (Nicodemus *et al.*, 2008). It is believed that the influence of the environmental factors is exerted through epigenetic mechanisms, such as DNA methylation and histone modifications, which contribute to the regulation of gene activity in the CNS. Candidate genes that might be epigenetically modified in schizophrenia include RELN, DRD2, DRD3, GAD1, MAOA, COMT, BDNF, and others (Roth *et al.*, 2009b).

Studies using neuroimaging in schizophrenia patients indicated altered neural proliferation and migration, delayed myelination, and reduced synapse number in the brain (Heckers, 1997). People diagnosed with schizophrenia usually experience a combination of positive (i.e. hallucinations, delusions, racing thoughts), negative (i.e. apathy, lack of emotion, poor or nonexistant social functioning), and cognitive (disorganized thoughts, difficulty concentrating and/or following instructions, difficulty completing tasks, memory problems) symptoms. The dopamine hypothesis of schizophrenia postulates that hypofunction of the cortical and prefrontal dopamine systems contributes to the negative symptoms and cognitive disorders, and that hy-

peractivity of the subcortical and limbic dopamine systems causes positive symptoms in schizophrenia (Abi-Dargham, 2004). Postmortem studies have shown reduced GABA uptake in the hippocampus and amygdala and in the temporal cortex (Simpson *et al.*, 1989). Decreased glutamate decarboxylase GAD65 and GAD67 mRNA expression has been observed in the prefrontal cortex and other neocortical areas in schizophrenics (Akbarian and Huang, 2006).

There is growing evidence that neurotrophin levels are disrupted in schizophrenia (Shoval and Weizman, 2005). Some post-mortem studies report that BDNF levels are decreased in the hippocampus and increased in the cerebral cortex of patients with schizophrenia (Durany *et al.*, 2001). Increased truncated TrkB expression and decreased BDNF/TrkB signaling in the frontal cortex have been reported in mouse models of schizophrenia (Pillai and Mahadik, 2008). Others report that BDNF and TrkB mRNA levels are decreased in the prefrontal cortex of human patients (Weickert *et al.*, 2005, 2003). Several studies also points to altered plasma neurotrophin levels in patients with schizophrenia (Toyooka *et al.*, 2002). Also, data suggest that Val66Met polymorphism in BDNF may help to distinguish endophenotypes of schizophrenia (Liou *et al.*, 2004). Moreover, BDNF is known to regulate the expression of GAD-related proteins (Arenas *et al.*, 1996).

Treatment of schizophrenia

The prevention of neuropathological processes in schizophrenia is currently a major goal of therapy (Pillai and Mahadik, 2008). All current antipsychotic drugs act primarily through dopamine receptors. The existing drugs are poorly tolerated and have equally low efficiency (Lieberman, 2007). Since schizophrenia is a heterogeneous group of disorders there is a critical need for studies designed to identify other molecular targets for therapeutics. Increasing reports on dysfunctions observed in BDNF/ TrkB signaling in schizophrenia indicate that various components of this system may serve as such molecular targets (Pillai and Mahadik, 2008). All currently known antipsychotics may upregulate BDNF levels when treated for a short period of time, but long-time treatment does not sustain brain BDNF levels (rather down-regulates its expression in the brain). Since for the management of schizophrenia antipsychotics must be used in very-long-term (over 10–50 years), it is important to investigate the possible ways by which BDNF levels can be sustained in the brain (Pillai and Mahadik, 2008). Currently, in addition to conventional antipsychotics, add-on therapy is being searched for that could support BDNF induction in the brain. Erythropoietin (Ehrenreich *et al.*, 2007), cysteamine (Pae *et al.*, 2007) and omega-3 fatty acids (Young and Conquer, 2005) are strong candidates for the add-on therapy of schizophrenia that also modulate BDNF levels.

1.5.8 Obesity

Obesity confers considerable risk for diabetes, cardiovascular disease, stroke and some cancers (McMillan *et al.*, 2006). It was shown that BDNF is important for energy homeostasis in rodents and in humans. Obese phenotypes are found in heterozygous $BDN^{+/-}$ mice (Lyons *et al.*, 1999) and in selective BDNF-hypothalamic knock-down in adult mice (Unger *et al.*, 2007). This mature-onset obesity is associated with hyperphagia, hyperleptinemia, hyperinsulinemia and hyperglycemia. BDNF is believed to act primarily within the hypothalamus to regulate energy intake downstream of the leptin–proopiomelanocortin signaling pathway (Xu *et al.*, 2003). Both peripheral and central administration of BDNF decreases food intake, increases metabolism and leads to weight loss in mice (Xu *et al.*, 2003; Kernie *et al.*, 2000; Pelleymounter *et al.*, 1995). Also, acute intracerebroventricular administration of BDNF impoves hyperinsulinemia and hyperglycemia in diabetic mice (Nakagawa *et al.*, 2002). Moreover, in humans, similar symptoms are associated with the functional loss of one copy of the BDNF gene (Gray *et al.*, 2006) and with a mutation in the BDNF receptor NTRK2 gene (Gray *et al.*, 2007; Yeo *et al.*, 2004). In humans, gene deletions causing haploinsufficiency of the WT1 and PAX6 genes on chromosome 11p13, approximately 4 Mb centromeric to BDNF (11p14.1), result in Wilms' tumor, aniridia, genitourinary anomalies, and mental retardation (WAGR) syndrome (Han *et al.*, 2008). Hyperphagia and obesity have been observed in a subgroup of WAGR syndrome patients. Among persons with the WAGR syndrome, BDNF haploinsufficiency is associated with lower levels of serum BDNF and with childhood-onset obesity. The critical region for the childhood-onset obesity in WAGR syndrome was found to be located within 80 kb of BDNF exon 1 (Han *et al.*, 2008).

A gene therapy strategy for obesity has been proposed by Cao *et al.* (2009). BDNF was delivered to the mouse models of obesity and diabetes by AAV vector carrying two expression cassettes: one constitutively driving BDNF and the other driving a specific microRNA which targeted BDNF (Cao *et al.*, 2009). The microRNA expression was controlled by a promoter responsive to agouti-related protein. As body weight decreased and agouti-related protein was induced, microRNA expression was activated, inhibiting BDNF expression. The results showed that BDNF transfer prevented diet-induces obesity and hyperinsulinemia, hyperleptinemia, hyperglycemia and dyslipidemia that associate with diet-induced obesity (Cao *et al.*, 2009). Also, administration of TrkB agonist resulted in hypophagia and weight loss in mice. However, in Rhesus monkeys the effect was depending on the mode of delivery of the agonist: centrally administered TrkB agonists showed similar anorexigenic effect as in mice, however, peripheral injections increased appetite and body weight (Lin *et al.*, 2008a). Taken together, carefully designed BDNF therapy has a great potential in alleviating obesity and diabetes.

1.5.9 Cancer

Neuroblastoma patients whose tumors have elevated levels of TrkB and BDNF have a poor prognosis (Aoyama *et al.*, 2001; Nakagawara *et al.*, 1994). Also, high expression of TrkB in Wilm's tumor is a poor prognostic marker (Eggert *et al.*, 2001). Neuroblastoma cells that survive repeated exposures to cytotoxic agents express increasing levels of BDNF, suggesting that the BDNF signal transduction pathway contributes to a multidrug-resistant phenotype (Matsumoto *et al.*, 1995). In tumor cells expressing low levels of TrkB receptor the effects of cytotoxic drugs were decreased if cells were in a BDNF-rich environment, and vise versa (Scala *et al.*, 1996). TrkB has been shown to play a key role in metastasis of tumor cells. BDNF stimulates tumor cell disaggregation and increases the ability of TrkB-expressing neuroblastoma tumor cells to invade through the extracellular matrix (Zhang *et al.*, 2008). There are reports on the role of TrkB in the survival of malignant B lymphocytes (D'Onofrio *et al.*, 2000), prostate (Weeraratna *et al.*, 2000), lung (Ricci *et al.*, 2001), breast cancer cells (Cameron and Foster, 2008), and head and neck tumors (Zhu *et al.*, 2007). It has been suggested that tumor cells secreting BDNF are able to induce angiogenesis, therefore, promoting tumor cells invasiveness and survival (Kermani *et al.*, 2005). Most reports have pointed to the importance of the TrkB/PI3K/Akt pathway in mediating invasiveness and resistance of malignant cells to anoikis (anchorage-dependent cell death) (Thiele *et al.*, 2009). Most strategies to treat cancer have been aimed at targeting the Trk tyrosine kinase domain. However, there are currently no reported inhibitors that selectively block TrkA or TrkB kinase activity. Cephalon's CEP-701(lestaurtinib) inhibits Trks, Flt3, and PKC, its phase II studies are in progress (Thiele *et al.*, 2009).

1.5.10 Neuropathic pain and spinal cord injury

Neuropathic pain is a chronic condition that is caused by an injury or dysfunction in the nervous system. There are multiple causes of neuropathic pain such as mechanical nerve injury, metabolic disfunctions (alcoholic neuropathy, Beriberi), viral infections (herpes, AIDS), neurotoxicity, ischaemia, chronic disease (diabetes, malignancies, multiple sclerosis, etc). Neuropathic pain severely affects quality of life. However, present therapies such as non-steroidal anti-inflammatory drugs (NSAIDs), opioids, anticonvulsants, anti-arrhythmics, tricyclic antidepressants and topical agents have modest efficacy in most patients, are palliative rather than curative, and their side effects represent significant limitations (Sah *et al.*, 2003). Neurotrophic factors represent attractive drug candidates, as they have the potential to stop or reverse the pathological changes in the nervous system. BDNF has been implicated as a central pain modulator sensitizing the spinal neurons (Kerr *et al.*, 1999). Therefore, blocking BDNF action could be used in reducing neuropathic pain. In addition to modulating pain, BDNF also participates in the spinal cord repair. BDNF delivery by gene ther-

apy is a promising option for enhancing axonal regeneration in the injured spinal cord (Blesch *et al.*, 2002).

1.5.11 Conclusions

So far, the results of clinical trials using neurotrophins in neurodegenerative diseases both in the PNS and CNS have been disappointing. BDNF therapy has yet unresolved problems: i) targeting BDNF to specific cells in the brain; ii) controlling exogenous BDNF levels; iii) crossing the BBB (Poduslo and Curran, 1996); iv) side effects such as epilepsy, weight gain, downregulation of TrkB by exessive BDNF; v) poor stability (serum half-life of minutes or less) (Poduslo and Curran, 1996; Weinreb *et al.*, 2007). The solution to attenuate neurodegeneration would be administering drugs that selectively modulate endogenous BDNF expression in the brain regions with reduced BDNF levels without flooding the brain with BDNF, or development of efficient BDNF mimetics. Thorough investigation of BDNF expression regulation must be carried out in order to design successful therapy strategies in the future.

1.6 Transgenic mouse models for studying BDNF expression

Various BDNF transgenic constructs have been used to study the regulation of rodent BDNF gene expression in transgenic animals. Plasmid-based constructs in which BDNF proximal promoter sequences were fused to the chloramphenicol acetyl transferase (CAT) reporter gene were used to investigate tissue-specific, axotomy-, and neuronal activity-induced transcription of the rat BDNF gene in transgenic mice (Timmusk *et al.*, 1995). However, using these transgenes had certain shortcomings: they failed to recapitulate BDNF expression in the dentate granule cells, granule cells of the cerebellum and in the heart, as well as displayed relatively high reporter activity in the striatum where endogenous rat BDNF levels were very low (Timmusk *et al.*, 1994). These findings suggested that given transgenic constructs lacked important regulatory elements responsible for a proper spatial expression of BDNF. Even though multiple regulatory elements have been identified in the BDNF gene, not all of them have been tested *in vivo*. To date, the role of NRSE in the regulation of BDNF promoter I and II (Timmusk *et al.*, 1999) and the role of CRE in activation of BDNF promoter IV (Hong *et al.*, 2008) has been established in transgenic mice. However, the effects of many other regulatory elements in the BDNF gene have not been yet described *in vivo*. Furthermore, data on human BDNF regulation is still missing.

A recent study reported BDNF-EGFP transgenic mice with 145 kb YAC construct carrying human BDNF genomic fragment (spanning from 45 kb upstream of exon I to 33 kb downstream from coding exon), where human BDNF coding sequence was partially replaced with the EGFP reporter gene (Guillemot *et al.*, 2007). These transgenic mice failed to fully recapitulate endogenous BDNF expression, indicating

that the 145-kb BDNF fragment did not contain all necessary regulatory elements for its proper spatial expression. Also, BDNF regulatory region has been discovered 850 kb upstream of the human (Gray *et al.*, 2006) and mouse (Sha *et al.*, 2007) BDNF genes that causes obesity, cognitive impairment and hyperactivity when disrupted. The critical region for childhood-onset obesity in the WAGR syndrome was found to be located within 80 kb of BDNF exon 1 (Han *et al.*, 2008). These data proves that large transgenic constructs should be used for identifying new proximal an distant regulatory elements in the BDNF gene.

Alternative vectors for transgenesis need to be used when the gene is too large to be accommodated in a conventional plasmid-based vector. Large insert clones can be produced with the P1 bacteriophage (P1) (Sternberg, 1990), P1 artificial chromosome (PAC) (Ioannou *et al.*, 1994), bacterial artificial chromosome (BAC) (Shizuya *et al.*, 1992), or yeast artificial chromosome (YAC) (Burke *et al.*, 1987) cloning systems. The choice of the cloning vector depends on the size of the gene to be expressed and the distance of the regulatory elements within the gene locus. P1 clones typically contain genomic inserts up to 100 kb (Ioannou *et al.*, 1994), BACs up to 300 kb (Shizuya *et al.*, 1992), and YAC vectors up to 2 Mb (Burke *et al.*, 1987). The preparation of YAC DNA, however, is more difficult, with DNA being susceptible to shearing during *in vitro* manipulations. Because of their stability and the fact that they have been used to map and sequence human and mouse genomes, BACs currently represent the most completely characterized and commercially available source of large genomic fragments. Transgenic mice generated with modified BACs have proven valuable for studying *cis*-elements that act at a distance to regulate tissue-specific and developmental patterns of gene expression (Antoch *et al.*, 1997; John *et al.*, 2001). Because BACs are more likely to include all the necessary regulatory elements (i.e., locus control regions and enhancers) to obtain a dose-dependent and integration-site independent transgene expression (Yang *et al.*, 1997; Heintz, 2001), modified BACs are the most advantageous constructs to be used to generate transgenic mice (Yang and Gong, 2005).

2 AIMS OF THE THESIS

The aim of this doctoral research was to gain insight into the structure and transcriptional regulation of the BDNF gene. In pursuing this goal the following steps were taken:

1. Comprehensive analysis of mouse and rat BDNF gene structure and expression pattern in various brain regions and peripheral organs. Analysis of the regulation of rodent BDNF transcription by neuronal activity and by chromatin remodeling drugs.

2. Microarray meta-coexpression analysis of BDNF co-expression conservation and a search for conserved transcription factor binding sites among co-expressed genes.

3. Generating BAC transgenic mouse models to dissect tissue-specific and neural activity-regulated expression of the human BDNF gene in transgenic mice and to discover novel proximal and distal regulatory *cis*-elements in the rodent BDNF gene *in vivo*.

3 MATERIALS AND METHODS

Detailed description of materials and methods is provided in the publications of this thesis. Briefly, the following methods were used in the presented study:

3.1 Gene sequence analysis

Mouse and rat BDNF gene structure *in silico* analysis was performed using NCBI and UCSC genomic, mRNA and EST databases (Publications I-IV).

3.2 RNA isolation, cDNA synthesis, RT-PCR

Publications I, III, IV

3.3 5' RACE analyses of transcription initiation sites

To determine the transcription start sites of BDNF transcripts, 5' rapid amplification of cDNA ends (RACE) and nested PCR was performed. RACE products were sequenced and aligned to mouse and rat genomic sequences (Publication I).

3.4 Cell culture and animal experiments

Rat glioma C6 and mouse neuroblastoma Neuro2A cells were treated with 5-Aza-2'-deoxycytidine (5-AzadC) or with trichostatin A (TSA) to analyze the effects of DNA methylation and histone acetylation on BDNF transcription (Publication I). Adult male Sprague-Dawley rats were injected with the kainic acid as previously described (Metsis *et al.*, 1993). Animals were sacrificed 1, 3, 6, 12, and 24 hr posttreatment (Publication I). Kainic acid or phosphate-buffered saline was administered intraperitoneally to adult transgenic mice. Only animals with induced tonic-clonic seizures were selected for analysis (Publications III and IV). All animal experiments were performed according to the norms of the local Ethical Committee of Animal Experimentation.

3.5 Microarray datasets and data filtering

Homo sapiens, *Mus musculus* and *Rattus norvegicus* microarray datasets were downloaded from Gene Expression Omnibus (GEO). Affymetrix GeneChips experiments were selected that comprised a minimum of 16 samples. Datasets which conained BDNF Detection call = Absent in more than 30% of the samples were excluded from the analysis. Samples in each dataset were split into subsets and/or sub-subsets according to the experimental conditions (i.e. normal tissue, disease tissue, control, treatment, disease progression, age, etc) (Publication II).

3.6 Differential expression analysis

Kruskal-Wallis test was used to measure differential expression of BDNF across subsets in each dataset. The false discovery rate approach (FDR) was applied at the 0.05 level as it is described by Benjamini and Hochberg (1995) (Publication II).

3.7 Co-expression conservation analysis

Standard Pearson correlation coefficient (PCC) was calculated across samples for each subset separately following a resampling bootstrap approach. A threshold value of r = 0.6 was used to retrieve a list of probe sets that were co-expressed with the BDNF probe set. Genes whose co-expression with BDNF in three or more subsets was found to be conserved between human, mouse, and rat constituted an input list for the g:Profiler (Publication II).

3.8 Motif discovery

Combinations of over-represented transcription factor binding sites (TFBS) in the conserved correlated genes were searched for using DiRE (Gotea and Ovcharenko, 2008) and CONFAC (Karanam and Moreno, 2004) tools (Publication II).

3.9 Generation of BAC transgenic mice

BAC clones containing human or rat BDNF locus were modified using Red/ET homologous recombination in *E. coli* (Publications III and IV). Modified BACs were tested for the absence of rearrangements using EcoRV restriction analysis and pulsed field gel electrophoresis. Integrity of the reporter gene was confirmed by BAC sequencing. BAC DNA was purified and transfected into COS-7 cells using DEAE-dextran. To assay for the reporter activity, EGFP expression was visualized using fluorescence microscopy and lacZ reporter activity was tested using β-galactosidase

assay. BAC DNA was purified for microinjection, separated in the low-melt agarose gel using pulsed field electrophoresis, and purified from agarose. Transgenic mice were generated by injection of BAC DNA into CBA×C57Bl/6 mouse pronuclei in the Karolinska Center for Transgene Technologies (Sweden) (Publications III and IV).

3.10 Genotyping

Founder mice carrying the BAC transgene were identified by PCR analysis of genomic DNA. Transgene copy number was analyzed by slot-blot hybridization of genomic DNA (Publications III and IV).

3.11 Ribonuclease protection assay

Publications III and IV

3.12 *In situ* hybridization

Publications III and IV

3.13 Quantitative real-time PCR

Publications III and IV

4 RESULTS AND DISCUSSION

4.1 Mouse and rat BDNF gene structure and expression revisited

More than fifteen years ago BDNF gene structure and the expression pattern of its alternative transcripts was described for the first time in rat (Timmusk *et al.*, 1993). The interest towards this molecule has been growing since then. The involvement of BDNF in important physiological processes and numerous neurological disorders has stimulated research. This is proved by the fact that in the last years BDNF gene organization has been studied also in human (Aoyama *et al.*, 2001; Marini *et al.*, 2004; Liu *et al.*, 2005; Pruunsild *et al.*, 2007), zebrafish (Heinrich and Pagtakhan, 2004), seabass fish (Tognoli *et al.*, 2010), and frog (Kidane *et al.*, 2009). However, the structural organization of the rodent BDNF gene has not been revised since four BDNF 5′ noncoding exons spliced to a common 3′ coding exon and four promoters were first discovered in the rat BDNF gene. The numeration of BDNF exons proposed by Timmusk and colleagues has been used by the scientific community untill recently. However, the results of this doctoral research showed that mouse and rat BDNF gene structure and expression is much more complex than has been accepted before.

Back in 1993, the rat BDNF gene structure was determined using such methods as screening cDNA libraries by hybridization with BDNF coding sequence probe to explore BDNF exon-intron structure, and RNase protection assay to map transcription initiation sites for alternative BDNF mRNAs. Southern blot analysis and *in situ* hybridization were used to detect BDNF mRNA expression pattern. At that time, full rat genome sequence was not yet available. Taking advantage of more up-to-date molecular biology techniques, bioinformatics tools and the availability of mouse and rat full genome sequences, we have revised rat BDNF gene structure and described mouse BDNF gene that has not been studied until now. We identified new BDNF exons and promoters and showed that novel BDNF promoters exhibit tissue-specific and neural activity-dependent regulation. According to our data, mouse and rat BDNF gene structure is identical, having at least eight 5′ noncoding exons (exons I–VIII). In each BDNF transcript, one 5′ exon is spliced to the protein coding exon. In addition, we identified a novel BDNF transcript that contains the 5′ extended protein coding exon (exon IXA). We suggest a new numbering system for mouse and rat BDNF exons. With regard to the old nomenclature (Timmusk *et al.*, 1993), former exon III corre-

sponds to exon IV, exon IV is now exon VI, and the coding exon previously called exon V is now exon IX.

All 5′ exons are controlled by distinct promoters as evidenced by RACE analysis as well as the expression analysis data. We have mapped transcription initiation sites for the novel exons and, using RT-PCR, showed that BDNF alternative transcripts are differentially expressed in various brain parts and non-neural tissues. Nevertheless, exons that are closely located in the genome are expressed in a similar manner: exons I, II, and III (cluster I) have brain-enriched expression patterns and exons IV, V, and VI (cluster II) are widely expressed in nonneural tissues as well as in the brain. This observation suggests that different tissue-specific regulatory mechanisms might exert transcriptional control over these two promoter clusters. Proximal regions of BDNF promoters I and IV contain binding sites for upstream stimulatory factor 1/2 (USF1/2) (Tabuchi *et al.*, 2002; Chen *et al.*, 2003b). Currently, USF1/2 is considered to control calcium-dependent expression of BDNF promoters in concert with other calcium-responsive transcription factors upon neuronal activation. However, USF1/2 have been also shown to recruit histone methyltransferase activity, histone acetyltransferase, and ATP-dependent nucleosome remodeling complexes to insulator sequences blocking gene silencing (West *et al.*, 2004; Huang *et al.*, 2007). It has been shown that basal tissue-specific expression of α-spectrin gene in erythroid cells is regulated by USF1/2 binding within α-spectrin exon 1' that functions as insulator with a barrier-element activity (Gallagher *et al.*, 2009). Barrier elements mark the boundary between euchromatin and heterochromatin. Although they do not directly affect the level of expression of a given gene, they are thought to be responsible for preventing the spread of heterochromatin into the gene, maintaining the open chromatin structure for optimal expression (Grewal and Moazed, 2003). Therefore, it can be hypothesized that two BDNF promoter clusters are separated by a region of heterochromatin, and USF1/2 binding relieves this silencing in a tissue-specific manner, probably by recruiting different barrier protein complexes (Oki *et al.*, 2004) at BDNF I and IV promoters. This means that USF1/2 might have more than one role in BDNF expression: not only it can be activity-dependent regulator of BDNF expression in response to elevated calcium levels, but also regulate BDNF basal tissue-specific transcription.

It has been established earlier in various *in vitro* and *in vivo* models that rat BDNF expression is regulated by neuronal activity through calcium-mediated pathways (Greer and Greenberg, 2008). BDNF exon I and exon IV transcripts (exons I and III according to Timmusk and colleagues (Timmusk *et al.*, 1993)) had previously been characterized as the most highly induced BDNF mRNAs, and BDNF II transcript being more moderately activated in response to kainate treatment of the rat brain (Metsis *et al.*, 1993; Timmusk *et al.*, 1995). Later, several calcium-responsive elements and transcription factors that regulate these promoters had been characterized (Timmusk *et al.*, 1999; Tabuchi *et al.*, 2002; Tao *et al.*, 2002; Chen *et al.*, 2003b;

Shieh *et al.*, 1998). Here we report that novel rat BDNF promoters are as well differentially regulated by neuronal activity in the rat hippocampus. We show that BDNF promoters V, VII, VIII, and IXA are upregulated upon kainate receptor activation by kainic acid and that the induction magnitude is comparable to that of BDNF promoters I, II and IV. Future characterization of the regulatory sequences and transcription factors mediating regulation of novel BDNF transcripts in different disease models is important for understanding BDNF gene regulation and its role in pathological conditions.

The role of histone modifications and epigenetic DNA modifications in the activity of BDNF promoters has been investigated by several studies. Neuronal activity-dependent activation of BDNF gene is mediated by decreased CpG methylation of BDNF promoter IV DNA and the release of a repressor complex containing methylcytosine binding protein MeCP2, histone deacetylases HDAC1 and HDAC2, and corepressor mSin3A (Martinowich *et al.*, 2003). In cultured neurons, depolarization induced histone H3 and H4 acetylation at BDNF promoter IV (Chen *et al.*, 2003a; Martinowich *et al.*, 2003). *In vivo*, seizures (Tsankova *et al.*, 2004), epilepsy (Huang *et al.*, 2002), antidepressants (Tsankova *et al.*, 2006), and cocaine exposure (Kumar *et al.*, 2005) have been demonstrated to increase acetylation of H3 and H4 at BDNF promoters IV and VI, upregulating BDNF mRNA levels. In addition, REST/NRSF which recruits multiple cofactors including HDAC1, HDAC2, and mSin3A (for review see (Ballas and Mandel, 2005) to repress its target genes, negatively regulates BDNF gene expression by binding to NRSE/RE1 element in BDNF promoter II (Palm *et al.*, 1998; Timmusk *et al.*, 1999; Ballas and Mandel, 2005). We showed that the DNA demethylating agent 5-AzadC evoked robust activation of BDNF gene expression in C6 rat glioma cells and more moderate activation in Neuro2A mouse neuroblastoma cells in a promoter-specific manner. Induction of exon I, III, IV, V, VIII, and IXA mRNAs was observed in C6 cells, whereas only exon I and exon III mRNA levels increased in Neuro2A cells. Furthermore, in C6 cells but not in Neuro2A cells, inhibition of histone deacetylation by TSA up-regulated the levels of BDNF exon III, exon VII, and exon IX transcripts. The results presented in this study suggest the contribution of histone modifications and DNA methylation at BDNF promoters to the regulation of BDNF gene expression in the cells of neural and glial origin under basal conditions. It is remarkable, that BDNF promoters responded to the 5-AzadC and TSA treatment differently in neural and nonneural cell lines. It could be hypothesized that in neural cells, epigenetic silencing of BDNF promoters by histone modification can be relieved only following neuronal activation and calcium influx. However, in nonneural cells the regulatory mechanisms of BDNF expression can be different.

Human BDNF gene structure and expression had been also studied by our group (Pruunsild *et al.*, 2007) in parallel with the rodent BDNF. The results showed that human and rodent BDNF gene structure was largely similar. Homology of human and

rodent BDNF 5′ exons ranges from 95 - 45%, being 95% for exon I, 93% for exon II, 62% for exon III, 91% for exon IV, 79% for exon V, 86% for exon VI, 45% for exon VII and 84% for exon VIII. The expression pattern of human BDNF resembles rodent in the way that cluster I exons (I-III) seem to be brain-specific, while cluster II exons (IV-VI) are also expressed in non-neural tissues (Pruunsild *et al.*, 2007). However, several differences have been discovered between organisms: i) human BDNF has two human-specific exons (Vh and VIIIh) that have not been detected in rodents; ii) unlike rodent exon VIII, human exon VIII is not driven by a separate promoter, but is always spliced together with exon V; iii) more complex splicing has been observed in human BDNF – several alternative transcripts contain three to four exons spliced together; iv) human exon VII splice donor site contains GG nucleotides instead of the conventional GU sequence; v) human exon IX, which encodes the BDNF protein and 3′ UTR, is subjected to alternative internal splicing and/or transcription initiation upstream of exon IX that leads to the generation of the transcripts containing variants of exon IX (IXbd and IXabd) that have not been found in rodents; vi) human transcripts containing exons I, VII, and VIII could potentially lead to alternative human prepro-BDNF proteins with longer N-termini since they contain upstream in-frame translation initiation codons (in rodents only exon I contains in-frame ATG). Finally, we report that, in contrast with the human BDNF locus (Liu *et al.*, 2005; Pruunsild *et al.*, 2007), mouse and rat BDNF loci do not contain BDNFOS gene (BDNF opposite strand) which encodes BDNF antisense RNAs. Interestingly, BDNFOS ESTs are also not available for chimpanzee and rhesus monkey although highly homologous sequences are present in the genomes of these animals. This suggests that antiBDNF could have evolved during primate/hominid evolution, as was proposed also by Liu *et al.* (2005). This finding demonstrates that the regulation of BDNF gene expression by antisense-BDNF RNAs is a human-specific phenomenon and proves one more time that the regulation of rodent and human BDNF genes might differ substantially.

4.2 Meta-coexpression conservation analysis of microarray data provides insight into brain-derived neurotrophic factor regulation

Developments in microarray technologies and bioinformatics allow scientists nowadays to utilize genome-wide gene expression data to investigate gene regulatory mechanisms using system biology approaches. Publicly available microarray data contains human genome-wide gene expression profiles in health and disease and under many other conditions, thus being a valuable source of information in human gene expression studies. Little is known about the regulation of human BDNF gene expression *in vivo*. The regulation of BDNF expression is complex due to its multiple activity-dependent and tissue-specific promoters. Thus, analysis of BDNF gene co-expression with other genes under various experimental conditions using microarray data could provide insight into the regulation of this complex gene.

Meta-coexpression analysis uses multiple experiments (datasets) to make more reliable predictions about gene co-expression than could be made using only a single data set. Meta-coexpression analysis postulates that co-regulated genes display similar expression patterns across various conditions. Several studies have successfully applied meta-analysis approach to get insight into various biological processes. For instance, microarray meta-analysis of aging and cellular senescence led to the observation that the expression pattern of cellular senescence was similar to that of aging in mice but not in humans (Wennmalm *et al.*, 2005). Data from a variety of laboratories was integrated to identify a common transcriptional host response to pathogens (Jenner and Young, 2005). Also, meta-coexpression studies have displayed their efficiency to predict functional relationships between genes (Wolfe *et al.*, 2005). However, co-expression alone does not necessarily imply co-regulation. Thus, analysis of evolutionary conservation of co-expression coupled with the search for over-represented motifs in the promoters of co-expressed genes is a powerful criterion to select the genes that are co-regulated from a set of co-expressed genes (Causton *et al.*, 2003; Stuart *et al.*, 2003).

It is a common practice in meta-coexpression studies to assess co-expression by calculating the gene pair correlations after merging the datasets (Stuart *et al.*, 2003) or by confirming the re-occurrence of significant correlations across datasets (Lee *et al.*, 2004). However, it has been shown recently that genes can reveal differential co-expression patterns across subsets in the same dataset (e.g. gene pairs that are correlated in normal tissue might not be correlated in cancerous tissue or might be even anti-correlated) (Choi *et al.*, 2005). We performed meta-coexpression conservation analysis of 80 publicly available microarray datasets using a novel 'subset' approach to discover genes whose expression correlates with BDNF in mouse, rat and human. We divided datasets into subsets with biologically meaningful sample content (e.g. tissue, gender or disease state subsets), analyzed co-expression with BDNF across samples separately in each subset and confirmed the links across subsets. Then, we analyzed conservation in co-expression between human, mouse and rat, and sought for conserved transcription factor binding sites (TFBSs) in BDNF and BDNF-correlated genes. We found a total of 84 genes whose co-expression with BDNF was conserved in all three organisms. Analysis of the list of 84 conserved BDNF-correlated genes using g:Profiler showed significantly low p-values for all the genes and revealed that these genes fall under Gene Ontology (GO) categories related to BDNF actions. Statistically significant GO categories of genes included: i) MYC- associated zinc finger protein (MAZ) targets (44 genes, $p = 1.82 \times 10^{-5}$); ii) signal transduction (36 genes, $p = 3.51 \times 10^{-6}$); iii) nervous system development (17 genes, $p = 5.27 \times 10^{-8}$); iv) Kruppel-box protein homolog (KROX) targets (18 genes, $p = 1.21 \times 10^{-4}$); v) transmembrane receptor protein tyrosine kinase pathway (7 genes, $p = 3.56 \times 10^{-6}$); vi) dendrite localization (5 genes, $p = 1.82 \times 10^{-5}$). According to the GO database, conserved BDNF-correlated gene products partic-

ipate in axonogenesis (BAIAP2), dendrite development (DBN1), synaptic plasticity and synaptic transmission (DBN1, KCND2, MBP, NPTX1, NR4A2 and SNCA), regeneration (GAS6, PLAUR), regulation of apoptosis (XIAP (known as BIRC4), KLF10, NEFL, PLAGL1, PRKCE, SCG2, SNCA, and TBX3), skeletal muscle development (MYH9, PPP3CA, and TBX3) and angiogenesis (ANGPT1, BAIAP2, CYR61, MYH9, SCG2, SERPINE1 and TBX3). Out of 84, 24 BDNF-correlated genes are related to cancer and 14 are involved in neurological disorders. According to the literature, more than 20 out of 84 conserved correlated genes have been reported to have functional interaction (direct or via other proteins) or co-regulation with BDNF. IGFBP5 (Hausman *et al.*, 2006), NR4A2, RGS4 (Schmidt-Kastner *et al.*, 2006) and DUSP1 (Kwon *et al.*, 2004) have been previously reported to be co-expressed with human or rodent BDNF. Other gene products, such as FGFR1 (Soto *et al.*, 2006) and SNCA (Kohno *et al.*, 2004) are known to regulate BDNF expression. Proprotein convertase PCSK1 is implied in processing of pro BDNF (Marcinkiewicz *et al.*, 1998). PTPRF tyrosine phosphatase receptor associates with NTRK2 and modulates neurotrophic signaling pathways (Yang *et al.*, 2006). Thyroid hormone receptor alpha (THRA) induces expression of BDNF receptor NTRK2 (Pastor *et al.*, 1994). Expression of such BDNF-correlated genes like EGR1 (Pollak *et al.*, 2005), MBP (Djalali *et al.*, 2005), NEFL (Kitagawa *et al.*, 2005), NPTX1 (Ring *et al.*, 2006), NTRK2, SERPINE1 (yan Sun *et al.*, 2006), SCG2 (Fujita *et al.*, 1999), SNCA (von Bohlen und Halbach *et al.*, 2005) and TCF4 (also known as ITF2) (Carter, 2007) is known to be regulated by BDNF signaling. CCND2, DUSP1, DUSP6, EGR1 and RGS4 gene expression is altered in cortical GABA neurons in the absence of BDNF (Glorioso *et al.*, 2006). SCG2 protein is found in neuroendocrine vesicles and is cleaved by PCSK1 - protease that cleaves pro-BDNF (Laslop *et al.*, 1998). BDNF and NTRK2 signaling affect SNCA gene expression and alpha-synuclein deposition in substantia nigra (I *et al.*, 2005). ATF3 gene is regulated by EGR1 (Bottone *et al.*, 2005), which expression is activated by BDNF (Pollak *et al.*, 2005).

We applied DiRE (Gotea and Ovcharenko, 2008) and CONFAC (Karanam and Moreno, 2004) motif-discovery tools to search for statistically over-represented TFBSs among conserved BDNF-correlated genes. Using DiRE, we discovered two regulatory regions at the human BDNF locus that were enriched in TFBSs. The first regulatory region spans 218 bp and is located 622 bp upstream of human BDNF exon I transcription start site. The second putative regulatory region is 1625 bp long and located 2915 bp downstream of the BDNF stop codon. Significant over-representation of binding sites for WT1, KROX, ZNF219, NF-κB, SOX, CREB, OCT, MYOD and MEF2 transcription factors was reported by DiRE in BDNF and BDNF-correlated genes. CONFAC results overlapped with DiRE results and suggested additional novel regulatory elements in human BDNF promoters and exons I- IX and in BDNF $3'$ UTR, which were highly conserved among mammals and over-represented in the BDNF-correlated genes. It is remarkable, that the TFBSs discovered in the BDNF

gene are highly conserved: most of the TFBSs are 100% conserved in mammals from human to armadillo, and some are conserved in vertebrates from human to fish.

As detected by g:Profiler, 44 out of 84 conserved correlated genes identified in this study including BDNF carry MAZ transcription factor binding sites. Motif discovery analysis revealed putative binding sites for MAZ in BDNF promoter Vh and in exons III and IV, suggesting that cluster II of BDNF promoters can be regulated by MAZ. MAZ is a transcriptional regulator of muscle-specific genes in skeletal and cardiac myocytes (Himeda *et al.*, 2008). BDNF mRNA expression in the heart is driven by promoters IV, Vh and VI (Pruunsild *et al.*, 2007). Therefore, it is possible, that MAZ can drive tissue-specific expression of these promoters in the heart.

Our analysis revealed that Wilms' tumor suppressor 1 (WT1) transcription factor binding sites are overrepresented in the BDNF-correlated genes. WT1 binding sites were detected in BDNF promoter I, in IRS2 (insulin receptor substrate 2), EGR1, BAIAP2 (insulin receptor substrate p53) and PURA promoters and in 19 other genes. WT1 acts as an oncogene in Wilms' tumor (or nephroblastoma), gliomas (Hashiba *et al.*, 2007) and various other human cancers (Yang *et al.*, 2007). WT1 regulates the expression of several factors from the insulin-like growth factor signaling pathway (Werner *et al.*, 1993). Gene deletions causing haploinsufficiency of the WT1 and PAX6 genes on chromosome 11p13, approximately 4 Mb centromeric to BDNF, result in the Wilms' tumor, aniridia, genitourinary anomalies, and mental retardation (WAGR) syndrome. The critical region for childhood-onset obesity associated with the WAGR syndrome was shown to be located within 80 kb of BDNF exon 1 (Han *et al.*, 2008). Also, high expression of TrkB in Wilm's tumor is a poor prognostic marker (Eggert *et al.*, 2001). Moreover, WT1 might have a role in neurodegeneration observed in Alzheimer's disease brain (Lovell *et al.*, 2003). We propose that WT1 may control BDNF promoter I as well as regulate IRS2, BAIAP2 and other correlated gene expression.

KROX family (EGR1-EGR4) transcription factors' binding sites were abundant in the promoters of BDNF and BDNF-correlated genes. KROX binding motif was detected in BDNF promoter V and EGR2 binding site was found in BDNF promoter IV. Also, correlation of EGR1 gene expression with BDNF was conserved in human, mouse and rat. EGR1 is involved in the maintenance of long-term potentiation (LTP) and is required for the consolidation of long-term memory (Jones *et al.*, 2001), whereas EGR2 is necessary for Schwann cell differentiation and myelination (Nagarajan *et al.*, 2001; Ghislain and Charnay, 2006). Since BDNF plays a significant role in these processes, it would be intriguing to study the regulation of BDNF by EGR factors.

BDNF promoters II-V and exons II, IV and IX contain highly conserved potential BRN2 (POU3F2) binding sequences. BRN2 is driving expression of the EGR2 gene - an important factor for controlling myelination in Schwann cells (Nagarajan *et al.*, 2001; Ghislain and Charnay, 2006). BRN2 also activates the promoter of the Notch

ligand Delta, regulating neurogenesis (Castro *et al.*, 2006). Considering a prominent role of BDNF in myelination and neurogenesis, BRN2 might fulfill its tasks in part by regulating BDNF gene expression.

Several transcription factors that were identified in our study as potential regulators of human BDNF gene have been previously shown to regulate rodent BDNF transcription *in vitro* and *in vivo*. CREB, USF1/2 (Tabuchi *et al.*, 2002) and MEF2 (Flavell *et al.*, 2008) have been shown to regulate rat BDNF promoter I. USF1/2 (Chen *et al.*, 2003b), CREB (Tao *et al.*, 1998; Shieh *et al.*, 1998), MEF2 (Hong *et al.*, 2008), CaRF (Tao *et al.*, 2002; Shieh *et al.*, 1998) and MeCP2 (Chen *et al.*, 2003a; Martinowich *et al.*, 2003) have been shown to regulate rat BDNF promoter IV upon calcium influx into neurons. Rat BDNF promoter II has also shown induction by neuronal activity, though to a lesser extent compared to the promoters I and IV (Aid *et al.*, 2007; Metsis *et al.*, 1993). REST/NRSF was established to bind to the palindromic $NRSE^{bdnf}$ in BDNF promoter II (Timmusk *et al.*, 1993) and to repress basal and activity-dependent expression of the BDNF gene from promoters II and I *in vitro* and *in vivo* in trasngenic mice (Palm *et al.*, 1998; Timmusk *et al.*, 1999). However, calcium responsive elements have not been yet studied in BDNF promoter II. It was believed that its induction is regulated by the elements located in the promoter I. Our analysis of human BDNF detected CREB and USF binding sites in BDNF promoter I, USF and MEF2 binding sites in the promoter II, and USF, MEF2 and CREB binding sites in the promoter IV. We suggest that MEF2 and USF elements might contribute to BDNF promoter II induction by neuronal activity. In addition, we have detected conserved TCF4 (also known as ITF2) binding sequences in BDNF promoter IV, and in exon I. It has been shown that calcium-sensor protein calmodulin can inhibit TCF4 factor activity (Saarikettu *et al.*, 2004). Preliminary experimental evidence (Sepp and Timmusk, unpublished data) suggests that TCF4 transcription factor is involved in the regulation of BDNF transcription. TCF4 might play in concert with CREB, MEF2 and other transcription factors to modulate BDNF levels following neuronal activity.

The results of BDNF transgenic studies (Timmusk *et al.*, 1995) showed that not only promoter regions but also 3′ UTR region downstream of the coding exon is required for cell-specific and activity-dependent expression of the rat BDNF gene. The primary sequence of BDNF 3′ UTRs is highly conserved between human, mouse, rat and zebrafish (Heinrich and Pagtakhan, 2004). It was shown that zebrafish BDNF 3′ UTR was responsible for the cell-specific expression of the reporter gene (Heinrich and Pagtakhan, 2004). We discovered that human BDNF 3′ UTR sequence contains highly conserved potential binding sites for TCF4 (ITF2), BRN2 (POU3F2), NF-κB and MEF2.

In summary, the support of our bioinformatics findings by experimental evidence reported in the literature strongly suggests that the potential regulatory elements discovered in the human BDNF locus using microarray data analysis may be involved in the regulation of BDNF expression.

4.3 BAC transcgenic mice reveal regulatory regions in the rat and human BDNF locus

To date, NRSE role in the regulation of BDNF promoters I and II (Timmusk *et al.*, 1999) and the role of CRE in activation of BDNF promoter IV (Hong *et al.*, 2008) has been established in transgenic mice. However, the effects of many other regulatory elements in BDNF gene have not been yet described *in vivo*. Furthermore, data on human BDNF regulation is still insufficient. Disturbances in BDNF gene expression have been implicated in a variety of human neurological disorders. Although the regulation of the rodent BDNF gene has been extensively investigated in transgenic animals, for the human BDNF gene *in vivo* studies have been largely limited to postmortem analysis.

Various BDNF transgenic constructs have been used to study the regulation of rodent BDNF gene expression *in vivo* in transgenic animals. Plasmid-based constructs in which BDNF proximal I, II, IV and VI promoter sequences (according to the new nomenclature) were fused to the chloramphenicol acetyl transferase (CAT) reporter gene were used to investigate tissue-specific, axotomy-, and neuronal activity-induced expression of rat BDNF promoters in transgenic mice (Timmusk *et al.*, 1995). However, these transgenes failed to recapitulate BDNF expression in the dentate granule cells, granule cells of cerebellum and in the heart, as well as displayed high reporter activity in the striatum where endogenous BDNF levels were relatively low (Timmusk *et al.*, 1994). These findings suggested that given transgenic constructs lacked certain regulatory elements responsible for the tissue-specific expression of BDNF.

The most serious limitation of conventional transgenic constructs carrying up to 20 kb genomic DNA is the positional effects, that is when expression of the transgene is influenced by its integration site. The position effects may reveal itself in different ways, including lack of transgene expression, ectopic transgene expression (unintended sites of expression), mosaic expression (only a subset of cells express the transgene), and extinction (diminishing transgene expression in successive generations) (Yang and Gong, 2005). The most important cause for the position effects is the lack of important regulatory elements in the genomic fragment. Bacterial artificial chromosome (BACs) clones currently represent the most completely characterized source of large genomic fragments. BACs are more likely to include all the necessary regulatory elements for an integration-site independent transgene expression (Yang *et al.*, 1997; Heintz, 2001). Thus, modified BACs are the most advantageous constructs to be used to generate transgenic mice (Yang and Gong, 2005).

A recent study reported generation of human BDNF-EGFP transgenic mice using a 145 kb YAC clone (spanning from 45 kb upstream of BDNF exon I to 33 kb downstream of coding exon), where hBDNF coding sequence was partially replaced with EGFP reporter gene (Guillemot *et al.*, 2007). Three out of five transgenic founder

lines obtained in that study expressed transgenic mRNA in the nervous system and one showed relatively weak expression in the heart. Also, BDNF regulatory locus has been discovered 850 kb upstream of the human (Gray *et al.*, 2006) and mouse (Sha *et al.*, 2007) BDNF genes that is responsible for obesity, cognitive impairment and hyperactivity. Finally, the critical region for childhood-onset obesity in the WAGR syndrome was shown to be located within 80 kb of BDNF exon 1 (Han *et al.*, 2008). These data proves that distant regulatory elements in the BDNF gene are essential for its proper expression.

We have generated and analysed BAC transgenic mice carrying 207 kb of the rat BDNF locus or 168 kb of the human BDNF locus. Rat BDNF-BAC clone (rBDNF-BAC) encompassing the genomic region from 13 kb upstream of BDNF exon I to 144 kb downstream of BDNF coding exon was modified to replace the BDNF protein coding sequence of exon IX with the lacZ reporter gene. In the human BDNF-BAC (hBDNF-BAC) transgenic construct spanning from 84 kb upstream of exon I to 17 kb downstream of exon IX, enhanced green fluorescent protein (EGFP) was inserted into the C-terminus of BDNF generating BDNF-EGFP fusion reporter gene.

Only one founder line was obtained using rBDNF-lacZ-BAC construct. We demonstrate that rBDNF-lacZ-BAC transgene recapitulates endogenous BDNF expression in the brain, heart and lung, indicating that regulatory elements governing BDNF mRNA expression in these tissues are located within the genomic region from 13 kb upstream of rat BDNF exon I to 144 kb downstream of rat BDNF coding exon. However, rBDNF-lacZ-BAC transgene expression, unlike the expression of endogenous BDNF mRNA, was not detected in the thymus, liver, kidney and skeletal muscle. In the claustrum and hypothalamus, rBDNF-lacZ mRNA expression levels were relatively lower than endogenous BDNF mRNA levels. Also, in the granular cell layer of the olfactory bulb, caudate putamen, and nucleus accumbens, high levels of rBDNF-lacZ mRNA were detected, whereas endogenous mouse BDNF mRNA was not expressed.

Out of three analyzed founder lines carrying hBDNF-EGFP-BAC transgene, one line (C3) largely recapitulated human BDNF mRNA expression throughout the brain as well as in the thymus, lung, skeletal muscle and testis. Founder line E1 mimicked human BDNF mRNA expression in some brain regions, and also in thymus, lung and kidney. Founder line E4 expressed transgene only in the thymus and testis. These results show integration site-dependent expression of the transgene and suggest that given BAC constructs do not contain necessary insulator elements to protect the transgene from the influence of the genomic regions flanking the transgene integration site.

Human BDNF-EGFP-BAC failed to drive EGFP reporter gene expression in the heart. Since expression of rBDNF-lacZ mRNA was detected in the heart of transgenic mice, this suggested that distant heart-specific regulatory elements could be potentially located in the $3'$ of the gene, from 17-144 kb downstream of BDNF cod-

ing exon. The results of Guillemot and colleagues (Guillemot *et al.*, 2007), narrow this region down to 17-33 kb downstream from BDNF coding exon, since their transgene spanning from 45 kb upstream of BDNF exon I to 33 kb downstream of coding exon also drove the expression of BDNF-EGFP mRNA in the heart in one of the founder lines. However, this hypothesis should be treated with caution, since BAC and YAC transgenes demonstrate position effect, therefore, multiple founder lines must be analysed before comparing different transgenis models.

Neither hBDNF-EGFP-BAC nor rBDNF-lacZ-BAC could direct transgene expression to the granule cells of dentate gyrus in the hippocampus suggesting that BDNF expression in this brain region is controlled by distant regulatory elements located further than 84 kb upstream of BDNF exon I or 144 kb downstream of BDNF coding exon. It is noteworthy, that granule cells in the dentage gyrus (together with olfactory bulb granule cells) are two major cell populations that undergo neurogenesis in the adulthood (reviewed in Balu and Lucki, 2009). Therefore, it is important to determine the regulatory regions that drive BDNF exrpession in these cells. Interestingly, both rat and human BAC transgenes exhibited particularly high expression in the testis of transgenic mice. One possible explanation is that the integration sites of these transgenes belong to the euchromatin regions transcriptionaly active during spermatogenesis.

Unfortunately, we could not detect EGFP and lacZ reporter proteins in the brains of the transgenic mice neither with fluorescence microscopy/X-gal staining assay nor with Western blot analysis. This could be explained with low levels of the reporter proteins as transgenic mRNA levels were about tenfold lower than endogenous BDNF mRNA.

Neuronal activity-induced promoter-specific expression of the transgene mRNA in the rBDNF-lacZ-BAC mice mimicked the induction of the respective promoters of endogenous BDNF in the adult cerebral cortex and hippocampus. Also, we showed that kainic acid differentially induced alternative hBDNF-EGFP transcripts in the cortex and hippocampus. The induction pattern of human BDNF transcripts upon neuronal activation was consistent with the induction pattern of respective BDNF mRNAs in mouse and rat: pronounced induction of human promoters I, IV and IX as well as moderate induction of promoters II and III was observed. This is the first report on human BDNF promoter induction by neuronal activity *in vivo* in transgenic animals. Several calcium-responsive elements have been previously identified in the rat BDNF gene (Timmusk *et al.*, 1999; Tabuchi *et al.*, 2002; Tao *et al.*, 2002; Chen *et al.*, 2003b; Shieh *et al.*, 1998), however, human BDNF gene regulation by neuronal activity has not been studied yet. Since in our mouse model transgene activation mimics that of the endogenous BDNF, this model can be used to study the regulation of human BDNF gene by neuronal activity *in vivo*.

In summary, our mouse models represent useful tools for further studying of proximal and distal regulatory elements in the rat and human BDNF gene.

CONCLUSIONS

1. Mouse and rat BDNF gene structure is similar, the gene being comprised of eight 5′ noncoding exons (exons I–VIII). In each BDNF transcript, one 5′ exon is spliced to the protein coding exon IX. Furthermore, alternative BDNF transcript containing 5′ extended protein coding exon (exon IXA) has been identified. All 5′ exons are controlled by distinct promoters that exhibit tissue-specific and neuronal activity-dependent regulation *in vivo*. Also, chromatin remodeling drugs differentially affect the activity of BDNF promoters in neural and non-neural cell lines.

2. Several structural and functional differences exist between rodent and human BDNF gene. The most prominent difference is that mouse and rat BDNF loci do not encode antisense-BDNF RNAs (BDNFOS gene). This suggests that the regulation of rodent and human BDNF might differ substantially.

3. Meta-coexpression conservation analysis of microarray data proposed novel regulatory elements in the human BDNF gene. We hypothesize that transcription factors MAZ, EGR, WT1, TCF4 (ITF2), MYOD, MEF2, BRN2 (POU3F2) and several others might control the expression of BDNF as well as BDNF-correlated genes.

4. BAC transgenic mice carrying 207 kb of the rat BDNF locus or 168 kb of the human BDNF locus were generated and characterized as they represent a useful tool for studying rat and human BDNF regulation *in vivo*. The transgenes largely recapitulated the expression of endogenous BDNF mRNA in the brain and peripheral tissues. Also, neuronal activity-dependent regulation of the transgene transcription was similar to the endogenous BDNF in the adult cerebral cortex and hippocampus.

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ACKNOWLEDGMENTS

This work was carried out at the Department of Gene Technology, Tallinn University of Technology, Estonia. I would like to express my gratitude to many excellent people I was lucky to work with. First of all, I would like to thank my supervisors, Kaia Palm and Tõnis Timmusk. Kaia - for her enthusiasm and believe in our work, for her passion in science. I am endlessly thankful to Tõnis, whose thoroughness and support made this thesis a reality.

I am grateful to professor Erkki Truve and Andres Veske for giving me the opportunity to work and defend my thesis at Tallinn University of Technology. I thank professor Madis Metsis for reviewing the manuscript of this thesis. Also, I want to thank Annely Rätsep and Tiina Varatalu, who was my administrative support grupp.

Many thanks to my colleague and co-author, Indrek Koppel, who led the transgenic mice project to the final stage and contributed most to the writing of the Publication III and IV manuscripts. I would like to thank my other lab-mates: Anna Kazantseva, Priit Pruunsild, Mari Sepp, Marko Piirsoo, Richard Tamme, Karin Tamm, Anne Kaljas, Kaur Jaanson ja Epp Väli. Working with these people was a pleasure indeed, and I hope we will stay connected by scientific and friendship bonds in the future. Also, my special thanks are referred to Jüri Reimand, who made valuable comments on our manuscript on meta-coexpression analysis of microarray data. I want to express my admiration to Igor Kuzmitshov for his amazing talent to see formatting mistakes in the text.

I want to officially thank my husband, Pavlos Pavlidis, who motivated me to pursue the doctoral degree, and provided me with countenance and technical support for the writing of this thesis. Working with him on the microarray data analysis was a lot of fun, and I hope we will continue to generate discoveries (and children). Also, my Mom and my sister were of a great support during all the years of studies. Finally, I am grateful to my children, Alexandra and Constantinos, who were simultaneously a challenge and a motivation for me.

PUBLICATION I

Mouse and rat BDNF gene structure and expression revisited.
Aid, T., Kazantseva, A., Piirsoo, M., Palm, K., Timmusk, T.
Journal of Neuroscience Research (2007), **85** 525-535

Journal of Neuroscience Research 85:525–535 (2007)

Mouse and Rat BDNF Gene Structure and Expression Revisited

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Brain-derived neurotrophic factor (BDNF) has important functions in the development of the nervous system and in brain plasticity-related processes such as memory, learning, and drug addiction. Despite the fact that the function and regulation of rodent BDNF gene expression have received close attention during the last decade, knowledge of the structural organization of mouse and rat BDNF gene has remained incomplete. We have identified and characterized several mouse and rat BDNF transcripts containing novel 5′ untranslated exons and introduced a new numbering system for mouse and rat BDNF exons. According to our results both mouse and rat BDNF gene consist of eight 5′ untranslated exons and one protein coding 3′ exon. Transcription of the gene results in BDNF transcripts containing one of the eight 5′ exons spliced to the protein coding exon and in a transcript containing only 5′ extended protein coding exon. We also report the distinct tissue-specific expression profiles of each of the mouse and rat 5′ exon-specific transcripts in different brain regions and nonneural tissues. In addition, we show that kainic acid-induced seizures that lead to changes in cellular Ca^{2+} levels as well as inhibition of DNA methylation and histone deacetylation contribute to the differential regulation of the expression of BDNF transcripts. Finally, we confirm that mouse and rat BDNF gene loci do not encode antisense mRNA transcripts, suggesting that mechanisms of regulation for rodent and human BDNF genes differ substantially.

Key words: BDNF; exon; promoter; DNA methylation; histone deacetylation; calcium; kainic acid

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family consisting of small secreted proteins that play important roles in the development of the nervous system in vertebrates (for recent reviews see Bibel and Barde, 2000; Binder and Scharfman, 2004; Chao et al., 2006). BDNF supports the survival and differentiation of specific populations of embryonic neurons in vivo, and growing evidence indicates that BDNF is also involved in several functions in adulthood, including neuronal homeostasis and brain plasticity-related processes such as memory, learning (Tyler et al., 2002; Yamada et al., 2002), and drug addiction (Bolanos and Nestler, 2004). Alterations in BDNF expression in specific neuron subpopulations contribute to various pathologies, including depression, epilepsy, and Alzheimer's, Huntington's, and Parkinson's diseases (Bibel and Barde, 2000; Murer et al., 2001; Binder and Scharfman, 2004; Castren, 2004; Cattaneo et al., 2005; Russo-Neustadt and Chen, 2005).

In addition to BDNF, the neurotrophin family includes nerve growth factor, neurotrophin-3, and neurotrophin-4/5 (Binder, 2004). All neurotrophins bind to $p75^{NGFR}$ receptor but selectively interact with their individual high-affinity protein kinase receptors of the *trk* (tropomyosin-related kinase) family (Kaplan and Miller, 2000; Chao, 2003; Teng and Hempstead, 2004). BDNF mediates its biological effects via TrkB and $p75^{NGFR}$ receptors. Binding of mature BDNF protein to TrkB and $p75^{NGFR}$ promotes cell survival, neurite outgrowth, synaptic transmission, plasticity, and cell migration (Dechant and Barde, 2002). Uncleaved precursor BDNF protein (pro-BDNF) has altered binding characteristics and distinct biological activity in comparison with mature BDNF protein (Lee et al., 2001; Teng et al., 2005).

In mouse and rat, BDNF mRNA is expressed throughout development and differentially in adult tissues (Ernfors et al., 1990; Hofer et al., 1990; Hohn et al., 1990). In the brain, BDNF mRNA and protein expression becomes detectable during embryonic development, reaching the highest levels by days 10–14 postnatally and decreasing thereafter. In the adult animal, BDNF is expressed throughout the brain, with the highest levels in the neurons of hippocampus (Ernfors et al., 1990; Hofer et al., 1990; Kawamoto et al., 1996; Conner et al., 1997; Yan et al., 1997). Neuronal BDNF expression is affected by many stimuli, such as γ-aminobutyric acid (GABA)-ergic and glutamatergic neurotransmission and membrane depolarization through calcium-mediated pathways (Zafra et al., 1990, 1991; Ghosh et al., 1994; Shieh and Ghosh,

Sequence data from this article have been deposited in the GenBank Data libraries under Accession Nos. EF125669-EF125690.

Contract grant sponsor: Wellcome Trust; Contract grant number: 067952; Contract grant sponsor: Estonian Ministry of Education and Research; Contract grant number: 0222602 (to T.T.).

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Received 25 April 2006; Revised 16 August 2006; Accepted 12 October 2006

Published online 5 December 2006 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.21139

1999; West et al., 2001). BDNF gene expression is controlled by multiple activity-dependent and tissue-specific promoters. Four BDNF promoters have been previously identified in rat (Metsis et al., 1993; Timmusk et al., 1993; Timmusk et al., 1995), each driving the transcription of BDNF mRNAs containing one of the four 5′ noncoding exons (I, II, III, or IV) spliced to the common 3′ coding exon. Several transcription factors contributing to the regulation of BDNF promoters have been characterized. Among these factors are cAMP-responsive element binding protein (CREB; Shieh et al., 1998; Tao et al., 1998; Tabuchi et al., 2002) and upstream stimulatory factors 1/2 (USF1/2; Tabuchi et al., 2002; Chen et al., 2003b), which regulate BDNF promoters I and III. In addition, calcium-responsive transcription factor (CaRF) has been found to mediate BDNF transcription through binding to BDNF promoter III upon neuronal activation (Tao et al., 2002). Chromatin remodeling by DNA methylation and histone deacetylation also plays an important role in cell-specific and activity-dependent regulation of BDNF gene by recruiting global repressors such as REST/NRSF to promoter II (Palm et al., 1998; Timmusk et al., 1999; Zuccato et al., 2003) and MeCP2 to promoter III (Chen et al., 2003a; Martinowich et al., 2003).

Unraveling the regulation of BDNF gene expression is important for understanding its contribution to nervous system function and pathology. Provided that BDNF actions are most frequently modeled in rodents, detailed knowledge of the structural organization of rodent BDNF genes would be imperative. We undertook this study to specify the structure and expression of BDNF gene in mouse and rat. We show that rodent BDNF gene structure and expression are more complex than initially characterized (Timmusk et al., 1993) and that novel, as yet unidentified regulatory sequences may contribute to cell-specific and activity-dependent regulation of rodent BDNF expression.

MATERIALS AND METHODS

DNA and Amino Acid Sequence Analysis

Mouse and rat BDNF gene structure in silico analysis was performed using genomic, mRNA and EST databases (http://www.ncbi.nlm.nih.gov and http://genome. ucsc.edu). Alignment tools available at http://www.ncbi. nlm.nih.gov as well as software provided by the BIIT group at the University of Tartu, Estonia, were used for homology searches and analysis. AntiHunter software (available at http://bio.ifom-firc.it/ANTIHUNTER/) was used to search for opposite-strand transcripts in mouse and rat BDNF genomic region.

RNA Isolation, cDNA Synthesis, RT-PCR

Total RNA from developing and adult mouse and rat total brain and brain regions and nonneural tissues was purified by RNAwiz (Ambion, Austin, TX) as recommended by the manufacturer. DNase treatment of total RNA was perfomed by using a Turbo DNA-Free Kit (Ambion) according to the manufacturer's instructions. Five micrograms of total RNA from different tissues was used for first-strand synthesis using oligo(dT) and SuperScript III First-Strand synthesis system (Invitrogen, Carlsbad, CA). To analyze expression of BDNF transcripts, reverse primer specific for 3′ BDNF coding exon and forward primers specific for 5′ noncoding exons were used. To identify homologues of human antisense BDNF exons in mouse and rat, primers were designed corresponding to mouse and rat BDNF genomic regions that showed significant homology with human exons. Total RNA was normalized to the expression of ubiquitously expressed HPRT gene. All primers used in the study are listed below, where m designates mouse, r, rat; h, human; for, forward; rev, reverse; and Arabic numbers BDNF exons as follows: mrBDNFI, GTGTGACCTGAGCAGTGGGCAAAGGA; mrBDNFII, GGAAGTGGAAGAAACCGTCTAGAGCA; mBDNFIII, GCTTTCTATCATCCCTCCCCGAGAGT; rBDNFIII, CCTTTCTATTTTCCCTCCCCGAGAGT; mrBDNFIV, CTCTGCCTAGATCAAATGGAGCTTC; mrBDNFV, CTCTGTGTAGTTTCATTGTGTGTTC; mBDNFVI, GCTGGCTGTCGCACGGTTCCCATT; rBDNFVI, GCTGGCTGTCGCACGGTCCCCATT; mrBDNFVII, CCTGAAAGGGTCTGCGGAACTCCA; mrBDNFVIII, GTGTGTGTCTCTGCGCCTCAGTGGA; mBDNFIXA, CCCAAAGCTGCTAAAGCGGGAGGAAG; rBDNFIXA, CCAGAGCTGCTAAAGTGGGAGGAAG; hmrHPRT for, GATGATGAACCAGGTTATGAC; hmrHPRTrev, GTCCTTTTCACCAGCAAGCTTG; and mrBDNFrev, GAAGTGTACAAGTCCGCGTCCTTA.

To analyze expression of mouse and rat exons I–IV-, exon VI-, and exon IXA-specific transcripts, cDNA was amplified in a total volume of 25 μl with 35 cycles of PCR using HotFire polymerase system (Solis BioDyne, Estonia). An annealing temperature of 60°C was used for all primer combinations. Because of relatively low expression levels of BNDF mRNAs containing exons V, VII, and VIII, a more robust HotStartTaq Master Mix kit (Qiagen, Chatsworth, CA) was used for cDNA amplification for 40–45 PCR cycles. All RT-PCR reactions were performed in triplicate. PCR products were resolved in 1.2% agarose gel and visualized by staining with ethidium bromide. PCR fragments were subsequently excised from the gel, cloned by using pCRII-TOPO cloning system (Invitrogen), and subjected to sequence analysis.

5′ RACE Analyses of Transcription Initiation Sites

To determine the transcription start sites of novel BDNF transcripts, 5′ rapid amplification of cDNA ends (RACE) was performed by using the GeneRacer kit (Invitrogen) according to the manufacturer's instructions. PCR amplification was performed with a HotStartTaq Master Mix kit (Qiagen) and GeneRacer 5′ forward primer and reverse primers specific for exons III, V, VII, VIII, and IXA. Then, nested PCR was performed to increase the specificity and sensitivity of RACE by using GeneRacer 5′ nested primer and nested primers specific for exons III, V, VII, VIII, and IXA. RACE products were analyzed in a 2% gel and cloned into the pCRII-Topo vector (Invitrogen) for sequence analysis. Primers used for RACE analysis are listed below: rBDNFIIIRACE, TCAATGAAGCATCCAGCCCGGCA; rBDNFIIINested, CGGAACTCTCGGGGAGGGAAAATA; rBDNFVRACE, GAACACACAATGAAACTACACAGAG; rBDNFVIIRACE, CTAAAGAGGTGCGCTGGATGGACAGAG; rBDNFVIINested, GGACCTGGAGTTCCGCAGACCCTTT; rBDNF-

VIIIRACE, CCATTTTCAGCAATCGTTTGTTCAGC; rBDNFVIIINested, GAGACACACACCACAGCCTTTCTC; rBDNFIXARACE, GAGTAAACGGTTTCTAAGCAAGTG; and rBDNFIXANested, CTTCCTCCCACTTTAGCAGCTCTG.

Cell Culture and Animal Experiments

Rat glioma C6 and mouse neuroblastoma Neuro2A cells were plated 16 hr before treatment in DMEM (Invitrogen) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin). Trichostatin A (TSA) and 5-aza-2′-deoxycytidine (5AzadC) were purchased from Sigma-Aldrich (St. Louis, MO). Neuro-2A and C6 cells were treated for 48 hr with 5AzadC (1 μM) or with TSA (333 nM) to analyze the effects of 5AzadC and TSA on the expression of BDNF.

Adult male Sprague-Dawley rats were injected with the glutamate analog kainic acid as previously described (Metsis et al., 1993). Animals were sacrificed 1, 3, 6, 12, and 24 hr posttreatment. Total RNA from hippocampi was extracted by using RNAwiz RNA Isolation Reagent (Ambion) according to the manufacturer's recommendations. All animal experiments were performed according to the norms of the local Ethical Committee of Animal Experimentation.

RESULTS

New Nomenclature for Mouse and Rat BDNF Gene

BDNF gene is transcribed from multiple promoters located upstream of distinct 5′ noncoding exons to produce a heterogeneous population of BDNF mRNAs. Although this conserved feature of BDNF has been described for several species, including human (Liu et al., 2005), mouse (Hayes et al., 1997), rat (Timmusk et al., 1993), and zebrafish (Heinrich and Pagtakhan, 2004), detailed analyses of rodent BDNF gene structure have not been performed. In rat, four 5′ noncoding exons (I–IV) that are spliced to the common 3′ coding exon (Fig. 1A) have previously been identified (Timmusk et al., 1993). For mouse, only homologues of rat BDNF exons I and II have been reported (Hayes et al., 1997). In silico analysis of mouse and rat BDNF gene structure performed in the present study showed that BDNF exons III and IV are present and expressed in mouse as well. Moreover, a number of EST and mRNA sequences aligned to the locations of potential novel BDNF exons and the respective sequences turned out to be highly conserved in rat and mouse genome. Furthermore, analysis of BDNF 5′ RACE products from human hippocampal RNA revealed additional novel exons (Kazantseva et al., unpublished), the sequences of which were also conserved in mouse and rat genomes. Identification of rodent BDNF transcripts containing novel exons by RT-PCR and subsequent cloning and sequencing confirmed the bioinformatic analyses data. Together our results show that both rat and mouse BDNF gene contains eight 5′ noncoding exons and one 3′ protein coding exon. All exon–intron junctions display conventional splice-donor and -acceptor sites. A new nomenclature was assigned to mouse and rat BDNF exons (Fig. 1B). In both mouse and rat genomes, the locations of novel BDNF exons are as following: exon III (corresponding to rat exon Ia described by Bishop et al., 1994) is located 0.6 kb downstream of previously described exon II, exon V is 0.25 kb downstream of exon IV (exon IV is the former exon III according to Timmusk et al., 1993), exon VII is located 0.6 kb downstream of exon VI (exon VI corresponds to exon IV in Timmusk et al. 1993), exon VIII is 13.5 kb upstream of the protein coding exon, and exon IXA is a 5′ extended variant of the protein coding exon (Fig. 1B). Homology of human and rodent BDNF 5′ exons ranges from 95% to 45%, reaching 95% for exon I, 93% for exon II, 62% for exon III, 91% for exon IV, 86% for exon VI (corresponds to exon V in human according to Liu et al., 2005), and 45% for exon VII (corresponds to exon VIA in human according to Liu et al., 2005). All exons that have been defined in human (Liu et al., 2005) are also expressed in mouse and rat, except for human exons VIIB and VIII. Rodent exons V, VIII, and IXA have not been previously described in human (Liu et al., 2005), but according to our data these exons are expressed in human as well (Kazantseva et al., unpublished). Rat BDNF gene has been suggested to undergo cryptic splicing within exon II (Timmusk et al., 1995). In agreement with the recently updated version of GenBank's submission (AY057907), our results show that usage of alternative splice donor sites (A, B, and C in Fig. 1B) within BDNF exon II leads to three different exon II transcript variants in both in mouse and rat.

Expression Analysis of Mouse and Rat BDNF Transcripts

Rat BDNF transcripts containing exons I, II, IV (former III), and VI (former IV) and their tissue-specific expression profiles have previously been described (Timmusk et al., 1993), whereas there are no data on the expression of the novel rat BDNF exons V, VII, VIII, and IXA, and only limited data are available on the expression patterns of rat exon III (Bishop et al., 1994). Furthermore, although promoter regions upstream of mouse BDNF exons I and II have been described (Hayes et al., 1997), no data are available for the expression of mouse BDNF transcripts containing exons I–IXA. In the present work, RT-PCR analysis of the expression profiles of all BDNF transcripts was carried out in developing and adult brain as well as in peripheral tissues of mouse and rat (Fig. 2).

Expression of rat exon I BDNF mRNA, which was previously described as a brain-specific transcript (Timmusk et al., 1993), was also observed at low levels in several nonneural tissues, including testis, lung, thymus, liver, and spleen (Fig. 2B). Expression of mouse exon I transcripts was detected in addition to brain only in thymus (Fig. 2A). In adult mouse and rat brain, BDNF exon I mRNAs were expressed in all regions studied, with the lowest levels in cerebellum. In developing mouse and rat brain, low levels of BDNF exon I transcripts were expressed at embryonic (E) days 13 and 15, the expression

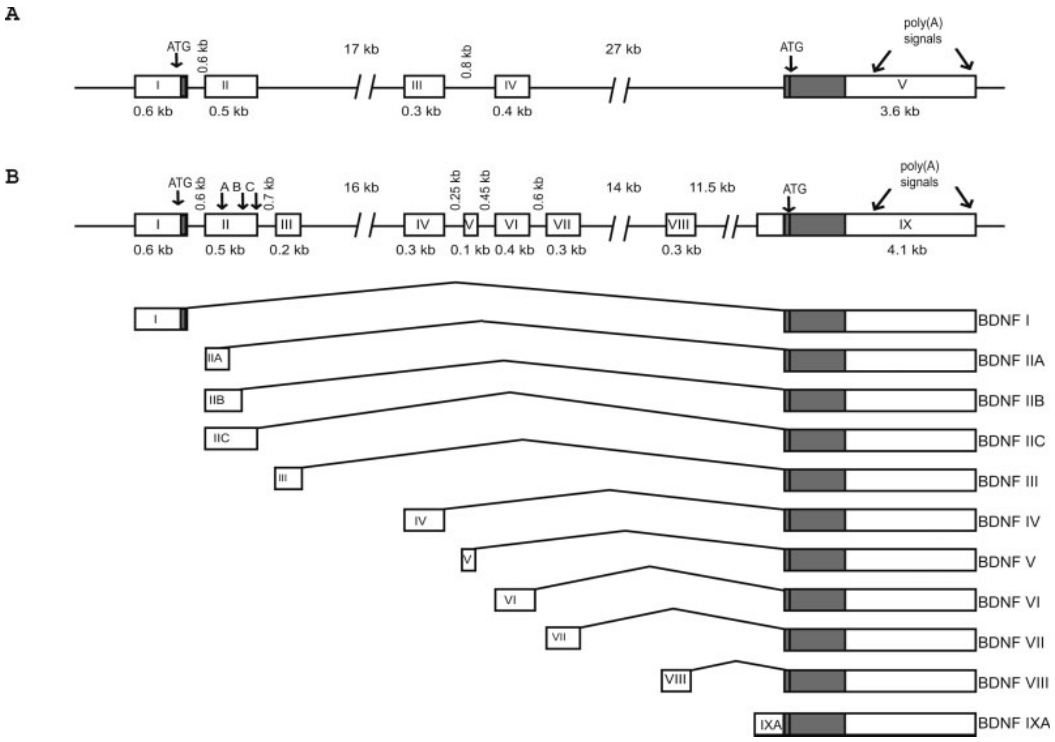

Fig. 1. Exon/intron structure and alternative transcripts of mouse and rat BDNF genes. **A:** Rat BDNF gene structure as described by Timmusk et al. (1993). Exons are shown as boxes and introns are shown as lines. **B:** The new arrangement of exons and introns of mouse and rat BDNF genes as determined by analyzing genomic and mRNA sequence data using bioinformatics, 5′ RACE, and RT-PCR. The schematic representation of BDNF transcripts in relation to the gene is shown below the gene structure. Protein coding regions are shown as solid boxes and untranslated regions are shown as open boxes. Each of the eight 5′ untranslated exons is spliced to the common 3′ protein coding exon IX. In addition, transcription can be initiated in the intron before the protein coding exon, which results in IXA transcripts containing 5′ extended coding exon. Each transcription unit may use one of the two alternative polyadenylation signals in the 3′ exon (arrows). For exon II, three different transcript variants, IIA, IIB, and IIC, are generated as a result of using alternative splice-donor sites in exon II (arrows marked A, B, and C).

levels peaked at postnatal (P) day 1 in mouse and embryonic day 21 in rat and decreased slightly during postnatal development (Fig. 2A,B). BDNF exon II mRNA splice variants A, B, and C revealed differential expression patterns in brain both in mouse and in rat, and their expression was not detected in peripheral tissues. In cerebellum, exon IIA transcript was the most abundant; in hippocampus, all three exon II splice variants were expressed at similar levels. Overall brain-specific expression pattern of mouse and rat novel BDNF exon III transcripts resembled that of BDNF exon II (Fig. 2A,B). In murine nonneural tissues, low levels of exon III transcripts were detected only in spleen and kidney, and, in rat, exon III transcripts were detected in thymus. BDNF exon IV and exon VI mRNAs (formerly exons III and IV, Timmusk et al., 1993) were observed at significant levels in developing mouse and rat brain already at E13, the earliest developmental stage studied. Both in mouse and in rat, BDNF exon IV and exon VI mRNA levels increased gradually during embryonic and postnatal development and decreased slightly in adult brain. In adult brain, exon IV and exon VI transcripts were detected in all analyzed brain regions both in mouse and in rat. Exon IV and exon VI transcripts exhibited wide patterns of expression in mouse and rat nonneural tissues, with the highest levels in heart and lung (Fig. 2A,B).

In both mouse and rat, BDNF novel exons V, VII, and VIII, were expressed at relatively low levels during brain development, broadly in adult peripheral tissues, and differentially in adult brain regions (Fig. 2A,B). In spite of the fact that mouse BDNF mRNA containing exons VII and VIII in the same transcript has been submitted to NCBI GenBank (AY231132), we failed to detect similar mRNAs in any of mouse or rat tissue studied.

Expression of the novel BDNF exon IXA transcripts was detected in rodent brain during embryonic

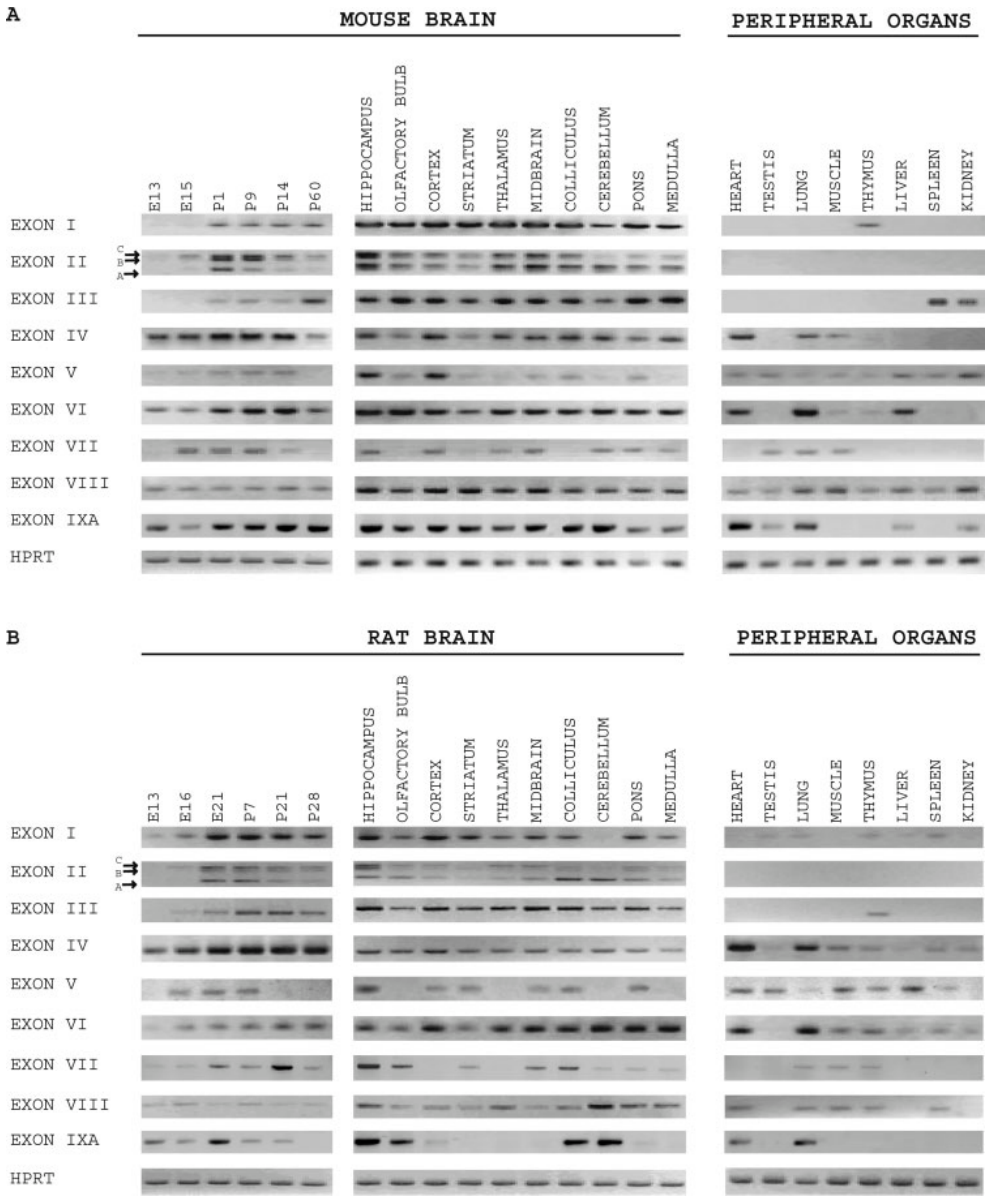

Fig. 2. Expression analysis of mouse and rat BDNF mRNAs. Semiquantitive RT-PCR analysis of tissue-specific expression of mouse (**A**) and rat (**B**) BDNF transcripts and control HPRT mRNA was performed in developing and adult brain and in peripheral organs. E, embryonic day; P, postnatal day.

development as well as in adulthood. In mouse adult brain, exon IXA-containing transcripts were expressed at similar levels in all brain regions (Fig. 2A), whereas, in rat adult brain, exon IXA expression was detected at high levels in hippocampus, olfactory bulb, colliculus, and cerebellum and at lower levels in cortex and pons

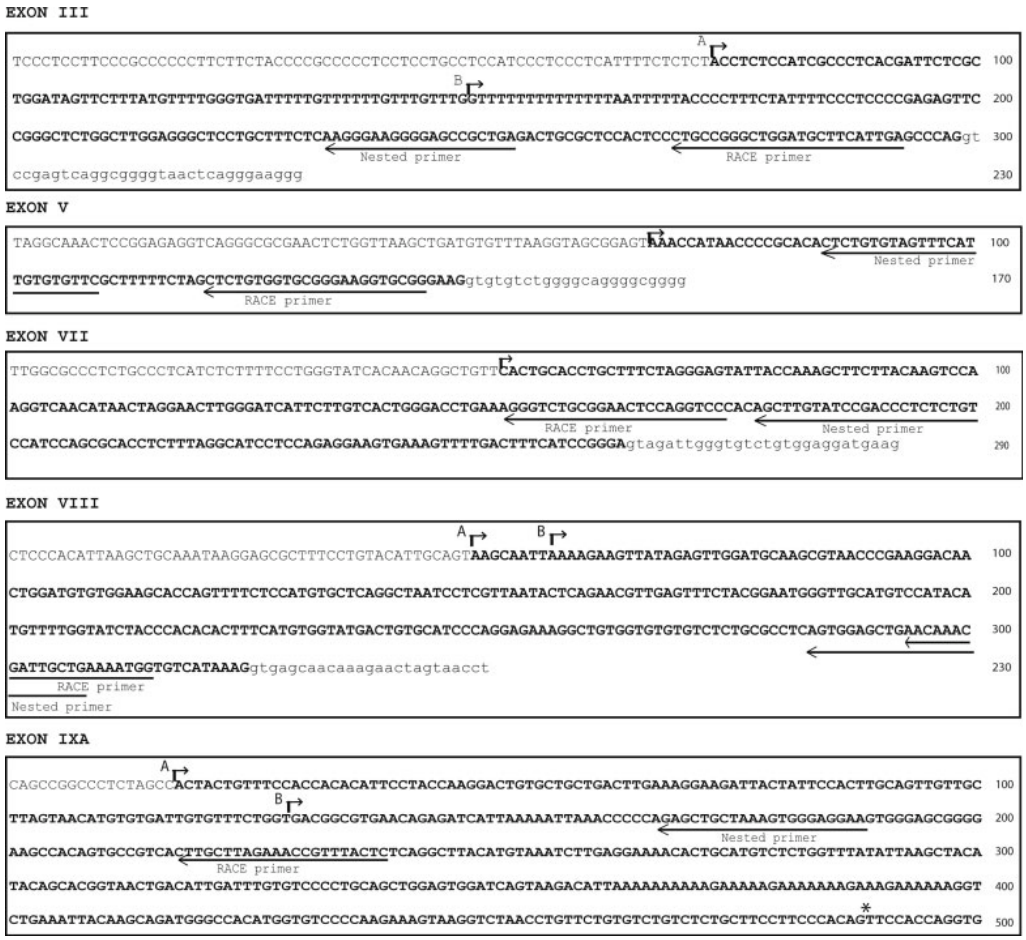

Fig. 3. Identification of the transcription start sites for BDNF new exon III, V, VII, VIII, and IXA mRNAs in rat. 5′ Rapid amplification of cDNA ends (5′ RACE) was performed to determine the transcription start sites for novel BDNF transcripts. Major transcription initiation sites (short arrows) are located at 152 bp and 230 bp for exon III (arrows marked A and B), at 81 bp for exon V and at 277 bp and 286 bp for exon VIII (arrows marked A and B) upstream of the 3′ end of the respective exon and at 476 bp and 363 bp upstream of the major splice acceptor site of the coding exon (asterisk) for exon IXA. For exon VII, the 5′end of the longest EST is shown as a putative transcription initiation site, because 5′ RACE did not result in any specific products as a result of very low levels of exon VII transcripts. Exon sequences are in boldface; intron sequences are in lowercase letters. The locations of primers that were used in 5′ RACE are indicated with long arrows.

(Fig. 2B). In rodent nonneural tissues, relatively high levels of exon IXA transcripts were observed in heart and lung.

Identification of the Transcription Start Sites for BDNF New Exons III, V, VII, and VIII in Rat

The transcription initiation sites for rat BDNF exons I, II, IV, and VI have been determined earlier (Timmusk et al., 1993). To identify the transcription start sites for novel BDNF transcripts, 5′ rapid amplification of cDNA ends (5′ RACE) from rat hippocampal RNA was performed by using antisense primers specific for exons III, V, VII, VIII, and IXA. Sequencing analysis of different RACE clones showed that major transcription initiation sites are located at 152 bp and 230 bp for exon III, at 81 bp for exon V, at 277 bp and 286 bp for exon VIII upstream of the 3′ end of the respective exon, and at 476 bp and 363 bp for exon IXA upstream of the major splice site of this exon (Fig. 3). None of the identified 5′ exons contains upstream open reading frames, so the usage of these 5′UTRs will apparently not affect amino acid composition

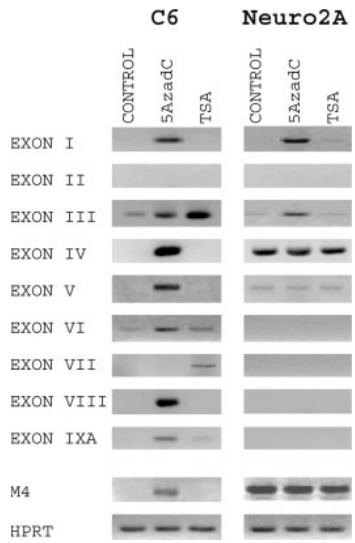

Fig. 4. Differential regulation of BDNF gene expression by DNA methylation and histone deacetylation. The role of DNA methylation in transcriptional activity of BDNF promoters was investigated by treating rat C6 glioma and mouse Neuro2A neuroblastoma cells with 1 μM 5-Aza-deoxycytidine (5AzadC) for 48 hr. The effects of inhibition of histone deacetylation was studied by treating Neuro2A and C6 cells with 300 nM trichostatin A (TSA) for 48 hr. Muscarinic acetylcholine receptor M4 gene and constitutive hypoxanthine-phosphoribosyltransferase (HPRT) gene were used as reference genes.

of the protein product. Because of the very low expression levels, we failed to map the transcription start site for rodent BDNF exon VII. However, 5′ RACE analysis of rodent exon VII homologue in human showed that transcription initiation site for this exon is located at 285 bp upstream of its 3′ end (Kazantseva et al., unpublished). These data strongly suggest that, similarly to BDNF exon I, II, IV, and VI mRNAs (Timmusk et al., 1993), novel exon III, V, VII, VIII, and IXA mRNAs are also transcribed from separate promoters.

Differential Regulation of BDNF Expression by DNA Methylation and Histone Deacetylation

Provided that methylation of the cytosine residues in the CpG dinucleotides in genome and posttranslational modifications of histones in the nucleosome establishes epigenetic codes for gene regulation in different tissues, including nervous system (Hsieh and Gage, 2004; Ballas and Mandel, 2005), we investigated the potential role of chromatin structure on transcriptional activity of BDNF promoters. By treating rat C6 glioma cells and mouse Neuro2A neuroblastoma cells with the DNA methyltransferase inhibitor 5AzadC or with the histone deacetylase (HDAC) inhibitor TSA for 48 hr, we examined the role of DNA methylation and histone acetylation, respectively, in the regulation of BDNF gene expression.

We observed robust activation of the expression of BDNF exon I and IV as well as novel exon V, VIII, and IXA transcripts in rat C6 glioma cells after 5AzadC treatment (Fig. 4). Expression of exons III and VI in C6 cells was moderately induced by inhibition of DNA methylation. Expression of BDNF exon I and exon III transcripts in Neuro2A cells was significantly induced following 5AzadC treatment, whereas there was no change in the levels of other BDNF mRNAs (Fig. 4). In Neuro2A cells, TSA treatment failed to relieve repression of any of the BDNF promoters. However, in C6 cells, inhibition of histone deacetylation by TSA increased the levels of BDNF exon III, exon VII, and exon IXA transcripts. Muscarinic acetylcholine receptor gene M4 was used as a reference because its expression has been shown to be regulated by 5AzadC in various cell lines, other than C6 and Neuro2A, in a cell-type-specific manner (Lunyak et al., 2002; Wood et al., 2003). Our findings suggest that DNA methylation and histone deacetylation could play a role in silencing of BDNF gene in a promoter- and cell-specific manner both in C6 and Neuro2A cells.

Activity-Dependent Regulation of Rat BDNF Exon-Specific mRNAs in the Hippocampus by Kainic Acid-Induced Seizures

Glutamate analogue kainic acid induces a rise in intracellular Ca^{2+} levels and differential activation of four previously characterized BDNF promoters in the hippocampus and cerebral cortex of adult rat brain (Timmusk et al., 1993). We examined whether expression of the BDNF mRNAs containing novel 5′ exons is regulated by kainic acid 1, 3, 6, 12, and 24 hr after drug administration. The results revealed differential regulation patterns for BDNF transcripts. BDNF exon I and IV transcripts (exons I and III according to Timmusk et al., 1993) have previously been characterized as the most highly induced BDNF mRNAs in response to kainic acid treatment. It was remarkable that in our experiments not only were these BDNF transcripts induced by kainate but also the levels of novel exon V, VII, VIII, and IXA mRNAs were up-regulated peaking at 3–6 hr posttreatment and rapidly down-regulated to basal levels thereafter (Fig. 5). The levels of exon IV transcripts remained elevated at 3–24 hr posttreatment. BDNF transcripts with exon IIA, IIB, and IIC exhibited differential expression profiles in response to kainite treatment. The levels of exon IIC transcripts were markedly elevated at 3 hr, peaked at 6 hr, and decreased at 12–24 hr after kainate treatment. Expression levels of exon 2A and exon 2B transcripts increased moderately at 3 hr, dropped at 6 hr, and reached basal levels at 24 hr posttreatment (Fig. 5). In contrast, the expression levels of BDNF exon III and exon VI mRNAs did not change at any time point studied (Fig. 5). These results agree with the previous reports on the transcript-specific regulation of rat BDNF mRNAs in response to kainate-induced seizures (Timmusk et al., 1993; Sathanoori et al.,

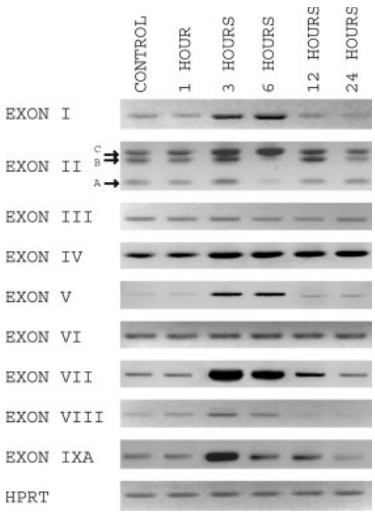

Fig. 5. Activity-dependent regulation of BDNF exon-specific mRNAs in rat hippocampus by kainic acid-induced seizures. The effect of kainate-induced seizures on the expression of different BDNF transcripts in the hippocampus of adult rat brain was examined. Adult rats were injected subcutaneously with kainic acid (8 mg per kg body weight) and sacrificed 1, 3, 6, 12, and 24 hr posttreatment. Total RNA was extracted, and semiquantitative RT-PCR was performed. Untreated rat hippocampus RNA was used as a control.

2004) and provide the first evidence that the novel BDNF mRNAs are differentially regulated by kainic acid. Our data strongly suggest that as yet unexplored regulatory elements within BDNF gene contribute to the activity-dependent regulation of BDNF mRNA expression.

Antisense-BDNF Transcripts Are Not Expressed in Mouse and Rat

It was shown recently (Liu et al., 2005) that protein noncoding antisense transcripts are expressed from human BDNF gene locus. Analyses of mouse and rat BDNF gene loci with the AntiHunter software tool (Lavorgna et al., 2004) did not reveal any BDNF antisense transcripts from EST databases. Moreover, alignment of human antisense BDNF exons sequences with mouse and rat EST databases at NCBI did not reveal any rodent ESTs homologous to human antisense BDNF transcripts. Failure to find antisense ESTs transcribed from mouse and rat BDNF gene loci could be explained by the fact that, although EST databases are growing rapidly, they are still undersampling the full mammalian transcriptome. Therefore, we aligned the sequences of human antisense BDNF exons with the respective regions of mouse and rat BDNF genomic sequence. Interestingly, sequences with significant homology to human antisense exons, though present in chimpanzee genome, were missing from mouse and rat genomes. RT-PCR analysis with mouse- and rat-specific primers annealing to the very short regions of homology with human antisense transcripts failed to detect expression of antisense BDNF transcripts in mouse and rat tissues. Therefore, we concluded that antisense BDNF transcripts are human- or primate-specific, as was proposed earlier by Liu and colleagues (2005).

DISCUSSION

Since the purification of BDNF protein, definitive evidence has emerged for its central role in mammalian brain development, physiology, and pathology. However, the structural organization of rodent BDNF gene has not been revisited since four 5′ exons were first discovered and nomenclature of exons established for rat BDNF gene (Timmusk et al., 1993). This numeration of BDNF exons is currently widely used by the scientific community. In the present work, we show, however, that mouse and rat BDNF gene structure is much more complex than was accepted before. According to our data, mouse and rat BDNF genes consist of a common 3′ exon that encodes the pro-BDNF protein and at least eight 5′ noncoding exons (exons I–VIII). In each BDNF transcript, one 5′ exon is spliced to the protein coding exon. All 5′ exons are controlled by distinct promoters as evidenced by our RACE analysis of the 5′ ends of these exons, as well as expression analysis data. In addition, we identified a novel BDNF transcript both in mouse and in rat that contains only exon IXA, the 5′ extended protein coding exon. Here we suggest a new numbering system for mouse and rat BDNF exons. With regard to the old nomenclature (Timmusk et al., 1993), former exon III corresponds to exon IV, previous exon IV is now exon VI, and the coding exon previously called exon V is now exon IX.

Pro-BDNF, a 32-kDa precursor, undergoes cleavage to release mature 14-kDa BDNF protein as well as a minor truncated form of the precursor (28 kDa). Secreted pro-BDNF activates a heteromeric receptor complex of p75 and sortilin to initiate cell death (Teng et al., 2005) and binds to p75 in hippocampal neurons to enhance long-term depression (Woo et al., 2005). Studies suggest that proneurotrophins account for a significant amount of the total neurotrophins secreted extracellularly, particularly in CNS neurons (Farhadi et al., 2000; Mowla et al., 2001). In mouse, rat, and human, exon I transcripts contain an in-frame AUG that can serve as an alternative translation initiation codon, extending the prepro- region of BDNF by eight amino acids (Timmusk et al., 1993). It can be hypothesized that additional amino acids in the N-terminus of prepro-BDNF can affect the intracellular trafficking of BDNF and play a role in pro-BDNF secretion. In human, BDNF 5′ exons VIB and VII (according to Liu et al., 2005) can contribute to alternative BDNF protein isoforms, because exon VIB can add 15 amino acids to the N-terminus of prepro-BDNF, and exon VII can undergo alternative in-frame splicing leading to the mature BDNF protein isoform that lacks 48 amino acids internally (Liu et al., 2005). None of the novel rodent BDNF exons in-

cludes an in-frame ATG, predicting that for these transcripts translation is initiated from the BDNF coding exon.

BDNF is the most abundant and widely distributed neurotrophin in the mammalian CNS. In addition to refining expression patterns of BDNF transcripts that have been identified earlier, results of this study also show that mouse and rat BDNF novel exons III, V, VII, VIII, and IXA are differentially expressed in adult brain and in peripheral tissues. In general, exons that are closely located in the genome are expressed in a similar manner: exons I, II, and III have brain-enriched expression patterns and exons IV, V, and VI are widely expressed also in nonneural tissues. However, 5′ RACE analysis of transcription initiation sites of rat and mouse BDNF new exons and in silico analysis of the regions upstream of these exons (data not shown) suggest that their expression is driven by distinct novel tissue-specific and development- and activity-regulated promoters.

It has been established earlier by using different cellular and animal models that BDNF gene is regulated by neural activity through calcium-mediated pathways (Shieh and Ghosh, 1999; West et al., 2001; Mellstrom et al., 2004) and that BDNF transcripts containing exons I, II, and IV are differentially regulated. BDNF exon I and exon IV transcripts (exons I and III according to Timmusk et al., 1993) have previously been characterized as the most highly induced BDNF mRNAs in response to kainate treatment and KCl-mediated membrane depolarization in embryonic cortical neuron cultures (Tao et al., 1998). Several calcium-responsive elements and transcription factors binding to these elements have been characterized in the promoter regions upstream of these exons (Timmusk et al., 1999; Tabuchi et al., 2002; Tao et al., 2002; Chen et al., 2003b). Here we show that BDNF exon V, exon VII, exon VIII, and exon IXA transcripts are also regulated by kainic acid and that the induction magnitude is comparable to that of BDNF exon I and IV transcripts. In light of our findings, it is attractive to speculate that differential regulation of nine BDNF exon mRNAs would become apparent in different neurodegenerative diseases in which BDNF levels are altered (Phillips et al., 1991; Mogi et al., 1999; Parain et al., 1999; Zuccato et al., 2001). Also, differential regulation of BDNF mRNAs can take place for example in depression, stress, exercise, and learning (Cotman and Berchtold, 2002; Tyler et al., 2002; Hashimoto et al., 2004; Russo-Neustadt and Chen, 2005). Future characterization of the regulatory sequences and transcription factors mediating regulation of novel BDNF transcripts in different disease models is important for understanding BDNF gene regulation and its contribution to pathology.

The role of chromatin remodeling in the activity of different BDNF promoters has been investigated in several recent studies. Neuronal activity-dependent activation of BDNF gene is mediated by decreased CpG methylation of *BDNF* promoter IV and release of a repressor complex containing methyl-cytosine binding protein MeCP2, histone deacetylases HDAC1 and HDAC2, and corepressor mSin3A (Chen et al., 2003a; Martinowich et al., 2003). It has also been shown that histone modifications at specific BDNF promoters are involved in chromatin remodeling during electroconvulsive seizures (Tsankova et al., 2004) and cocaine-induced plasticity (Kumar et al., 2005) in rat and in a mouse model of depression and antidepressant treatment (Tsankova et al., 2006). In addition, zinc finger transcription factor REST/NRSF (Chong et al., 1995; Schoenherr and Anderson, 1995), which recruits multiple cofactors including HDAC1, HDAC2, and mSin3A (for review see Ballas and Mandel, 2005) to repress its target genes, negatively regulates BDNF gene expression by binding to NRSE/RE1 element in BDNF promoter II (Palm et al., 1998; Timmusk et al., 1999; Bruce et al., 2004; Ballas et al., 2005). The present study showed that the DNA demethylating agent 5AzadC evoked robust activation of BDNF gene expression in C6 rat glioma cells and more moderate activation in Neuro2A mouse neuroblastoma cells in a transcript-specific manner: induction of exon I, III, IV, V, VIII, and IXA mRNAs was observed in C6 cells, whereas only exon I and exon III mRNA levels increased in Neuro2A cells. Furthermore, in C6 cells, inhibition of histone deacetylation by TSA up-regulated the levels of BDNF exon III, exon VII, and exon IX transcripts. The results presented in this study suggest the contribution of histone modifications and methylation of BDNF promoters to the regulation of BDNF gene transcription and open up possibilities for addressing these phenomena in more detail.

Finally, we report that, in contrast with the human BDNF gene locus (Liu et al., 2005), mouse and rat BDNF gene loci do not encode antisense mRNA transcripts. These findings demonstrate that regulation of BDNF gene expression by antisense-BDNF transcripts clearly is a human- or primate-specific phenomenon and suggest that regulation of rodent and human BDNF gene differs substantially. Human-specific antisense transcripts have been reported for the tumor suppressor gene ret finger protein 2 (RFP2; Baranova et al., 2003) and for the human protocadherin (PCDH) locus (Lipovich et al., 2006). BDNF has important roles in development, particularly of the nervous system, and plays a central role in brain plasticity-related processes, underscoring the possible role of antisense BDNF gene in regulation of BDNF expression across primates manifesting in specific behavioral phenotypes.

During the preparation of this paper, an article by Liu and colleagues examining the gene structure and expression of BDNF in rodents was published (Liu et al., 2006). However, our study increases the understanding of rodent BDNF gene loci, in that we present several novel data that are complementary to the results of Liu and colleagues. 1) We identified an additional 5′ exon, exon V that was not been reported by Liu et al. Thus, both mouse and rat BDNF genes consist of at least eight 5′ exons spliced to the 3′ coding exon. In addition, we identified a novel BDNF transcript, exon IXA mRNA, consisting of only the 5′ extended protein coding exon. 2) We determined the transcription initiation sites for novel exons (III, V, VII, VIII, and IXA), showing that these exons are transcribed from distinct promoters. 3) Our data show that

exon VIII (exon VII according to Liu et al.) is driven by a separate promoter. Liu and colleagues' data argue that transcripts containing exons VII and VIII (exons VI and VII according to Liu et al.) share the same promoter. 4) Our expression analysis data for all BDNF transcripts includes a wider range of tissues and brain structures analyzed both in rat and in mouse. 5) Liu et al. studied the regulation of some BDNF transcript expression in brain upon administration of cocaine. Our data show activity-dependent regulation of rat BDNF mRNAs by kainic acid-induced seizures in rat hippocampus. Moreover, we report differential regulation of the expression of BDNF transcripts by DNA methylation and histone deacetylation. Taken together, the results of the present study on mouse and rat BDNF gene structure and tissue-specific expression provide new challenges and opportunities to identify mechanisms regulating the activity of novel BDNF promoters that contribute to the expression levels of BDNF and possibly also to the changes in BDNF expression in neurodegenerative and neuropsychiatric disorders.

ACKNOWLEDGMENTS

We thank Kaur Jaanson and Epp Väli for technical assistance and Priit Pruunsild for stimulating discussions and advice.

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PUBLICATION II

Meta-coexpression conservation analysis of microarray data: a 'subset' approach provides insight into brain-derived neurotrophic factor regulation.

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BMC Genomics (2009), 10:420

BMC Genomics

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Meta-coexpression conservation analysis of microarray data: a "subset" approach provides insight into brain-derived neurotrophic factor regulation

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Published: 8 September 2009

BMC Genomics 2009, **10**:420 doi:10.1186/1471-2164-10-420

Received: 29 December 2008
Accepted: 8 September 2009

This article is available from: http://www.biomedcentral.com/1471-2164/10/420

Abstract

Background: Alterations in brain-derived neurotrophic factor (*BDNF*) gene expression contribute to serious pathologies such as depression, epilepsy, cancer, Alzheimer's, Huntington and Parkinson's disease. Therefore, exploring the mechanisms of *BDNF* regulation represents a great clinical importance. Studying *BDNF* expression remains difficult due to its multiple neural activity-dependent and tissue-specific promoters. Thus, microarray data could provide insight into the regulation of this complex gene. Conventional microarray co-expression analysis is usually carried out by merging the datasets or by confirming the re-occurrence of significant correlations across datasets. However, co-expression patterns can be different under various conditions that are represented by subsets in a dataset. Therefore, assessing co-expression by measuring correlation coefficient across merged samples of a dataset or by merging datasets might not capture all correlation patterns.

Results: In our study, we performed meta-coexpression analysis of publicly available microarray data using *BDNF* as a "guide-gene" introducing a "subset" approach. The key steps of the analysis included: dividing datasets into subsets with biologically meaningful sample content (e.g. tissue, gender or disease state subsets); analyzing co-expression with the *BDNF* gene in each subset separately; and confirming co- expression links across subsets. Finally, we analyzed conservation in co-expression with *BDNF* between human, mouse and rat, and sought for conserved over-represented TFBSs in *BDNF* and BDNF-correlated genes. Correlated genes discovered in this study regulate nervous system development, and are associated with various types of cancer and neurological disorders. Also, several transcription factor identified here have been reported to regulate *BDNF* expression *in vitro* and *in vivo*.

Conclusion: The study demonstrates the potential of the "subset" approach in co-expression conservation analysis for studying the regulation of single genes and proposes novel regulators of *BDNF* gene expression.

Background

The accumulation of genome-wide gene expression data has enabled biologists to investigate gene regulatory mechanisms using system biology approaches. Recent developments in microarray technologies and bioinformatics have driven the progress of this field [1]. Moreover, publicly available microarray data provide information on human genome-wide gene expression under various experimental conditions, which for most researchers would be difficult to access otherwise.

BDNF (brain-derived neurotrophic factor) plays an important role in the development of the vertebrates' nervous system [2]. BDNF supports survival and differentiation of embryonic neurons and controls various neural processes in adulthood, including memory and learning [3], depression [4], and drug addiction [5]. Alterations in *BDNF* expression can contribute to serious pathologies such as epilepsy, Huntington, Alzheimer's, and Parkinson's disease [6]. Alteration in *BDNF* expression is associated with unfavorable prognosis in neuroblastoma [7], myeloma [8], hepatocellular carcinoma [9] and other tumors [10]. Apart from brain, expression of alternative *BDNF* transcripts has been detected in a variety of tissues (such as heart, muscle, testis, thymus, lung, etc.) [11,12]. Numerous studies have been conducted to unravel the regulation of *BDNF* expression in rodents and human. Data on the structure of human [11] and rodent [12]*BDNF* gene have been recently updated. Nevertheless, little is known about the regulation of human BDNF gene expression *in vivo*. Unraveling the regulation of *BDNF* expression remains difficult due to its multiple activity-dependent and tissue-specific promoters. Thus, analysis of the gene expression under various experimental conditions using microarray data could provide insight into the regulation of this complex gene.

Meta-coexpression analysis uses multiple experiments to identify more reliable sets of genes than would be found using a single data set. The rationale behind meta-coexpression analysis is that co-regulated genes should display similar expression patterns across various conditions. Moreover, such analysis may benefit from a vast representation of tissues and conditions [13]. A yeast study showed that the ability to correctly identify co-regulated genes in co-expression analysis is strongly dependent on the number of microarray experiments used [14]. Another study that examined 60 human microarray datasets for co-expressed gene pairs reports that gene ontology (GO) score for gene pairs increases steadily with the number of confirmed links compared to the pairs confirmed by only a single dataset [15]. Several studies have successfully applied meta-analysis approach to get important insights into various biological processes. For instance, microarray meta-analysis of aging and cellular senescence led to the observation that the expression pattern of cellular senescence was similar to that of aging in mice, but not in humans [16]. Data from a variety of laboratories was integrated to identify a common host transcriptional response to pathogens [17]. Also, meta-coexpression studies have displayed their efficiency to predict functional relationships between genes [18]. However, co-expression alone does not necessarily imply that genes are co-regulated. Thus, analysis of evolutionary conservation of co-expression coupled with the search for over-represented motifs in the promoters of co-expressed genes is a powerful criterion to identify genes that are co-regulated from a set of co-expressed genes [19,20].

In co-expression analysis, similarity of gene expression profiles is measured using correlation coefficients (CC) or other distance measures. If the correlation between two genes is above a given threshold, then the genes can be considered as «co-expressed» [1]. Co-expression analysis using a «guide-gene» approach involves measuring CC between pre-selected gene(s) and the rest of the genes in a dataset.

It is a common practice in meta-coexpression studies to assess co-expression by calculating the gene pair correlations after merging the datasets [20] or by confirming the re-occurrence of significant correlations across datasets [15]. However, it has been shown recently that genes can reveal differential co-expression patterns across subsets in the same dataset (e.g. gene pairs that are correlated in normal tissue might not be correlated in cancerous tissue or might be even anti-correlated) [21]. Therefore, assessing co-expression by measuring CC across merged samples of a dataset or by merging datasets may create correlation patterns that could not be captured using the CC measurement.

In this study, we performed co-expression analysis of publicly available microarray data using *BDNF* as a "guide-gene". We inferred *BDNF* gene co-expression links that were conserved between human and rodents using a novel "subset" approach. Then, we discovered new putative regulatory elements in human *BDNF* and in BDNF-correlated genes, and proposed potential regulators of *BDNF* gene expression.

Results

We analyzed 299 subsets derived from the total of 80 human, mouse and rat microarray datasets. In order to avoid spurious results that could arise from high-throughput microarray analysis methods, we applied successive filtering of genes. Then, we divided datasets into subsets with biologically meaningful sample content (e.g. tissue, gender or disease state subsets), analyzed co-expression with *BDNF* across samples separately in each subset and

confirmed the links across subsets. Finally, we analyzed conservation in co-expression between human, mouse and rat, and sought for conserved TFBSs in *BDNF* and BDNF-correlated genes (Figure 1).

Data filtering

Gene Expression Omnibus (GEO) from NCBI and ArrayExpress from EBI are the largest public peer reviewed microarray repositories, each containing about 8000 experiments. In order to avoid inaccuracies arising from measuring expression correlation across different microarray platforms [13] we used only Affymetrix GeneChips platforms for the analysis. Since ArrayExpress imports Affymetrix experiments from GEO http://www.ebi.ac.uk/microarray/doc/help/GEO_data.html, we used only GEO database to retrieve datasets.

A study examining the relationship between the number of analyzed microarray experiments and the reliability of the results reported that the accuracy of the analysis plateaus at between 50 and 100 experiments [14]. Another study demonstrated how the large amount of microarray data can be exploited to increase the reliability of inferences about gene functions. Links that were confirmed three or more times between different experiments had significantly higher GO term overlaps than those seen only once or twice ($p < 10^{-15}$) [15]. Therefore, we performed meta-coexpression analysis using multiple experiments to increases the accuracy of the prediction of the co-expression links.

Since *BDNF* served as a guide-gene for our microarray study, qualitative and quantitative criteria were applied for selection of the experiments with respect to *BDNF* probe set presence on the platform [see Additional file 1: BDNF probe sets], *BDNF* signal quality and expression levels. In addition, non-specific filtering [19] was performed to eliminate the noise (see Methods/Microarray datasets). Consequently, 80 human, mouse and rat microarray experiments (datasets) from Gene Expression Omnibus (GEO) database met the selection criteria. Each dataset was split into subsets according to the annotation file included in the experiment [see Additional file 2: Microarray datasets and Additional file 3: Subsets]. In summary, 299 subsets were obtained from 38 human, 24 mouse and 18 rat datasets. From 38 human datasets, 8 were related to neurological diseases (epilepsy, Huntington's, Alzheimer's, aging, encephalitis, glioma and schizophrenia) and contained samples from human brain; another 9 datasets contained samples from human "normal" (non-diseased) tissues (non-neural, such as blood, skin, lung, and human brain tissues); 12 datasets had samples from cancerous tissues of various origins (lung, prostate, kidney, breast and ovarian cancer). The rest 9 datasets contained samples from diseased non-neural tissues (HIV infection, smoking, stress, UV radiation etc.). Out of 24 mouse datasets, 5 datasets were related to neurological diseases (brain trauma, spinal cord injury, amyotrophic lateral sclerosis, and aging); 15 datasets contained normal tissue samples (neural and peripheral tissues); 1 dataset contained lung cancer samples; 3 datasets were related to non-neural tissues' diseases (muscle dystrophy, cardiac hypertrophy and asthma). Among 18 rat datasets, 11 datasets were related to neurological diseases (spinal cord injury, addiction, epilepsy, aging, ischemia etc), 5 datasets were with "normal tissue samples" composition and 2 datasets examined heart diseases [see Additional file 2: Microarray datasets].

According to Elo and colleagues [22] the reproducibility of the analysis of eight samples approaches 55%. Selecting subsets with more than eight samples for the analysis could increase the reproducibility of the experiment however reducing the coverage, since subsets with lower number of samples would be excluded. Thus, we selected subsets with a minimum of eight samples for the analysis, in order to achieve satisfactory reproducibility and coverage. The expression information for human, mouse and rat genes obtained from GEO database, information about BDNF probe names used for each dataset, information about subsets derived from each experiment, and data on correlation of expression between BDNF and other genes for each microarray subset has been made available online and can be accessed using the following link: http://www.bio.lmu.de/~pavlidis/bmc/bdnf.

Differential expression of BDNF across subsets

Since the study was based on analyzing subsets defined by experimental conditions (gender, age, disease state etc) it was of biological interest to examine if *BDNF* is differentially expressed across subsets within a dataset. We used Kruskal-Wallis test [23] to measure differential expression. The results of this analysis are given in the Additional files 4, 5 and 6: Differential expression of the BDNF gene in human, mouse and rat datasets.

Co-expression analysis

Since the expression of *BDNF* alternative transcripts is tissue-specific and responds to the variety of stimuli, seeking for correlated genes in each subset separately could help to reveal condition-specific co-expression. The term "subset" in this case must be understood as "a set of samples under the same condition".

We derived 119 human, 73 mouse and 107 rat subsets from the corresponding datasets. Pearson correlation coefficient (PCC) was chosen as a similarity measure since it is one of the most commonly used, with many publications describing analysis of Affymetrix platforms [13,24,25]. PCC between *BDNF* and other genes' probe sets was measured across samples for each subset separately. From each subset, probe sets with PCC $r > 0.6$ were

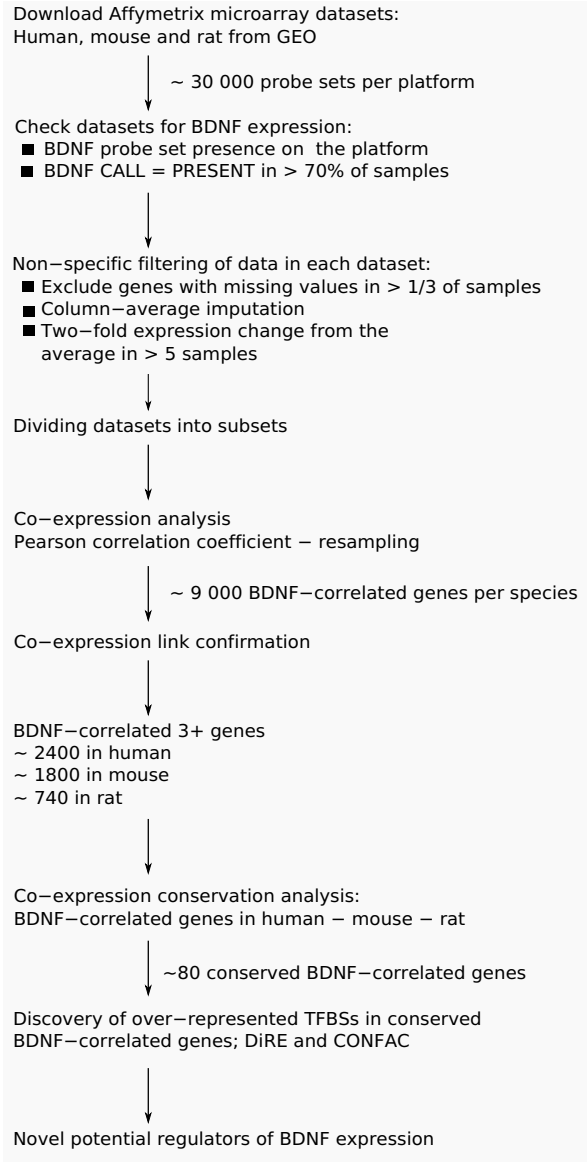

Figure 1
Microarray data analysis flowchart. Altogether, 80 human, mouse and rat Affymetrix datasets were analyzed (dataset selection criteria: > 16 samples per dataset; BDNF detection call PRESENT in more than 70% of the samples). Data was subjected to non-specific filtering (missing values and 2-fold change filtering). Thereafter, datasets were divided into 299 corresponding subsets. Co-expression analysis in human, mouse and rat subsets allowed the detection of genes that co-expressed with *BDNF* in more than 3 subsets (~1000 genes for each species). As a result of co-expression conservation analysis, 84 genes were found to be correlated with *BDNF* in all three species. Discovery of over-represented motifs in the regulatory regions of these genes and in *BDNF* suggested novel regulators of *BDNF* gene expression.

selected. It was demonstrated by Elo and colleagues [22] that in the analysis of simulated datasets a cutoff value r = 0.6 showed both high reproducibility (~0.6 for profile length equal to 10) and low error. A "data-driven cutoff value" approach has been rejected because it is based on the connectivity of the whole network, whereas we focused only on the links between *BDNF* and other genes. A lower threshold of 0.4 generated a list of genes that showed no significant similarities when analyzed using g:Profiler tool that retrieves most significant GO terms, KEGG and REACTOME pathways, and TRANSFAC motifs for a user-specified group of genes [26]. The value r = 0.6 was chosen over more stringent PCC values because the lengths of the expression profiles were not too short (mean profile length ~17, standard deviation ~12). Moreover, the PCC threshold higher than 0.6 was not justified since we performed further filtering by selecting only conserved correlated genes, thus controlling the spurious results.

Each probe set correlation with *BDNF* that passed the threshold was defined as a "link". It has been previously shown that a link must be confirmed in at least 3 experiments (3+ link) in order to be called reliable [15]. Therefore, we selected (3+) genes for evolutionary conservation analysis, narrowing the list of correlated genes to eliminate the noise. g:Profiler analysis of these genes revealed that the results are statistically significant (low p-values) and the genes belong to GO categories that are relevant to biological functions of BDNF. For example, the list of human genes produced the following results when analyzed with g:Profiler (*p*-values for the GO categories are given in the parenthess): nervous system development ($5.96 \cdot 10^{-21}$), central nervous system development ($3.29 \cdot 10^{-07}$), synaptic transmission ($4.40 \cdot 10^{-11}$), generation of neurons ($1.58 \cdot 10^{-08}$), neuron differentiation ($1.02 \cdot 10^{-06}$), neurite development ($4.11 \cdot 10^{-07}$), heart development ($1.67 \cdot 10^{-09}$), blood vessel development ($5.51 \cdot 10^{-14}$), regulation of angiogenesis ($7.16 \cdot 10^{-09}$), response to wounding ($1.32 \cdot 10^{-11}$), muscle development ($1.53 \cdot 10^{-10}$), regulation of apoptosis ($1.65 \cdot 10^{-07}$), etc.

We have used r = 0.6 as a "hard" threshold value for the CC. A disadvantage of this approach is that there will be no connection between *BDNF* and other genes whose correlation with *BDNF* is 0.59 in a specific dataset [27]. Using multiple datasets was expected to remedy this effect. An alternative approach would be to use "soft" threshold approaches [27]. According to the soft threshold approach, a weight between 0 and 1 is assigned to the connection between each pair of genes (or nodes in a graph). Often, the weight between the nodes *A* and *B* is represented by some power of the CC between *A* and *B*. However, other similarity measures may be used given that they are restricted in [0, 1]. A drawback of the weighted CC approach is that it is not clear how to define nodes that are directly linked to a specific node [27] because the available information is related only to how strongly two nodes are connected. Thus, if neighbors to a node are requested, threshold should be applied to the connection strengths. Alternatively, Li and Horvath [28] have developed an approach to answer this question based on extending the topological overlap measure (TOM), which means that the nodes (e.g. genes) should be strongly connected and belong to the same group of nodes. However, this analysis requires the whole network of a set of genes. In the current analysis, we did not construct the co-expression network for all the genes of microarray experiments. Instead, we focused on a small part of it i.e. the *BDNF* gene and the genes linked to *BDNF*. Therefore, TOM analysis was not possible using our approach.

To see how the "weighted CC" method would affect the results of our study we used a simplified approach. Instead of applying "hard" threshold (0.6) for the CC we measured the strength of all the connections between *BDNF* and all the genes in a microarray experiment. The connection strength $s_j = [(1 + CC_j)/2]^b$, where CC_j denotes the CC between *BDNF* and the gene j, is between 0 and 1 and b is an integer. In order to define b, analysis of the scale-free properties of the network is required. However, we used the value 6. Great b values give lower weight to weak connections. Then we calculated the average $s_j(ave(s_j))$ among all the subsets. Finally, we sorted the genes based on their $ave(s_j)$ and calculated the overlap of the top of this list with our results for each species (human mouse and rat). When restricting the top of the weighted CC list to the same number of genes that we have obtained for the 3+ list for each species, we observed that the top-weighted CC genes overlap extensively with the 3+ list (overlapping > 80%) for each species. Therefore, even though the "soft" and "hard" thresholding approaches are considerably different we observe quite extensive overlap of the results. We would like to stress that we did not apply the full weighted CC and TOM methodology since it would require the construction of the whole network which was beyond the aims of our study. However, such investigation of the whole co-expression network could contribute to the understanding of *BDNF* regulation and function.

Correlation conservation and g:Profiler analysis

Co-expression that is conserved between phylogenetically distant species may reveal functional gene associations [29]. We searched for common genes in the lists of 2436 human, 1824 mouse and 740 rat genes (3+ genes, whose expression is correlated with *BDNF*). From these genes, 490 were found to be correlated with *BDNF* in human and mouse, 210 correlated with *BDNF* in human and rat, and 207 conserved between mouse and rat [see Additional file 7: Conserved BDNF-correlated genes]. We found a total of 84 genes whose co-expression with *BDNF*

Table 1: BDNF-correlated genes conserved between human, mouse and rat.

GO category	Conserved correlated genes											
protein tyrosine kinase PW *	ANGPT1	BAIAP2	DUSP1	EPHA4	EPHA5	EPHA7	FGFR1	GAS6	KALRN	IRS2	NTRK2	
	PTPRF	FP106										
dendrite localization*	DBN1	FREQ	GRIA3	KCND2	NTRK2							
signal transduction*	ANGPT1	CREM	DUSP6	EPHA5	FGFR1	IGFBP5	KALRN	NR4A2	PDE4B	PRKAG2	PTPRF	TBX3
	BAIAP2	CXCL5	EGR1	EPHA7	GAS6	IL6ST	KLF10	NTRK2	PENK	PRKCB	RGS4	ZFP106
	COL11A1	DUSP1	EPHA4	FGF13	GRIA3	IRS2	MYH9	ODZ2	PLAUR	PRKCE	SCG2	
hsa-miR-369-3p*	COL11A1	DBC1	DCN	DUSP1	GAS6	ITF-2	KLF10	NEUROD6	PENK	TRPC4		
TF: CCCGCCCCCR CCCC (KROX) *	ATF3	ATP1B1	CCND2	COL11A1	DBN1	DLGAP4	EPHA7	GAS6	GRIA3	IL6ST	IRS2 KCND2	
	KLF10	NFIA	NPTXR	PCSK2	SNCA	THRA						
TF: GGGGAGGG (MAZ/SP1) *	ATF3	CCND2	DBC1	DUSP6	FREQ	ITF-2	MBP	NPTXR	PCSK1	PTGS2	THRA	
	BAIAP2	COL4A5	DBN1	EGR1	GRIA3	KALRN	MDM2	NR4A2	PDE4B	PTPRF	TRPC4	
	BASP1	CREM	DLGAP4	EPHA5	HN1	KLF10	NFIA	NTRK2	PRKCB1	PURA	VCAN	
	CAMK2D	CXCL5	DUSP1	EPHA7	IRS2	LMO7	NPTX1	OLFM1	PRSS23	TBX3		
NS development*	BAIAP2	EPHA4	FGF13	IRS2	MBP	NEUROD6	NR4A2	OLFM1	PTPRF	SMARCA4	TBX3	
	DBN1	EPHA7	FGFR1	KALRN	NEFL	NPTX1	NTRK2	PCSK2	PURA	SNCA		
angiogenesis	ANGPT1	BAIAP2	CYR61	MYH9	SCG2	SERPINE1	TBX3					
apoptosis/ anti-apoptosis	BIRC4	KLF10	NEFL	PLAGL1	PRKCE	SCG2	SNCA	TBX3				
cell cycle	CAMK2D	CORO1A	DUSP1	MDM2	MYH9	PPP3CA						
synaptic transmission/ plasticity	DBN1	KCND2	MBP	NPTX1	NR4A2	SNCA						

GO categories marked with a star (*) have been reported as statistically significant for this gene list by g:Profiler analysis tool. Human gene names are given representing mouse and rat orthologs whenever gene names for all three species are not the same. GO - gene ontology, PW - pathway, TF - transcription factor, NS - nervous system.

was conserved in all three organisms (Table 1) [see also Additional file 7: Conserved BDNF-correlated genes].

Due to a variety of reasons (e.g. sample size of a dataset/subset, probe set binding characteristics, sample preparation methods, etc.), when measured only in one dataset/subset, some of the co-expression links might occur by chance. Checking for multiple re-occurrence of a link is expected to reduce the number of false-positive links. More importantly, the conservation analysis should further reduce the number of artifacts. However, since our analysis comprised a multitude of subsets it was important to estimate the statistical significance of the results. To tackle this problem, we created randomized subsets similarly to what was described by Lee and colleagues [15] and calculated the distribution of correlated 3+ links for each species separately. The results showed that our co-expression link confirmation analysis resulted in a significantly higher number of links compared to the randomized data (p-value < 0.005 for each species). However, it should be mentioned that the number of 3+ links remained quite high in the randomized datasets: for human subsets it constituted about 58% of the observed 3+ links, for mouse about 43% and for rat 21%. These results justify the subsequent co-expression conservation analysis step. Indeed, in random human, mouse and rat subsets the number of correlated 3+ links was only about 9% of the discovered conserved BDNF-correlated links (that is ~7.5 genes out of 84).

Analysis of the list of 84 conserved BDNF-correlated genes using g:Profiler showed significantly low p-values for all the genes and revealed significant GO categories related to BDNF actions [see Additional file 8: g:Profiler analysis]. Statistically significant GO categories included: i) MYC-associated zinc finger protein (MAZ) targets (44 genes, $p = 1.82 \cdot 10^{-05}$); ii) signal transduction (36 genes, $p = 3.51 \cdot 10^{-06}$); iii) nervous system development (17 genes, $p = 5.27 \cdot 10^{-08}$); iv) Kruppel-box protein homolog (KROX) targets (18 genes, $p = 1.21 \cdot 10^{-04}$); v) transmembrane receptor protein tyrosine kinase pathway (7 genes, $p = 3.56 \cdot 10^{-06}$); vi) dendrite localization (5 genes, $p = 1.82 \cdot 10^{-05}$) (Table 1).

According to the Gene Ontology database, conserved BDNF-correlated gene products participate in axonogenesis (*BAIAP2*), dendrite development (*DBN1*), synaptic plasticity and synaptic transmission (*DBN1*, *KCND2*, *MBP*, *NPTX1*, *NR4A2* and *SNCA*), regeneration (*GAS6*, *PLAUR*), regulation of apoptosis (*XIAP* (known as *BIRC4*), *KLF10*, *NEFL*, *PLAGL1*, *PRKCE*, *SCG2*, *SNCA*, and *TBX3*), skeletal muscle development (*MYH9*, *PPP3CA*, and *TBX3*) and angiogenesis (*ANGPT1*, *BAIAP2*, *CYR61*, *MYH9*, *SCG2*, *SERPINE1* and *TBX3*) (Table 1). Out of 84, 24 BDNF-correlated genes are related to cancer and 14 are involved in neurological disorders (Table 2).

Interactions among correlated genes

We searched if any of the correlated genes had known interactions with BDNF using Information Hyperlinked over Proteins gene network (iHOP). iHOP allows navigating the literature cited in PubMed and gives as an output all sentences that connect gene A and gene B with a verb http://www.ihop-net.org/[30]. We constructed a "gene network" using the iHOP Gene Model tool to verify *BDNF*-co-expression links with the experimental evidences reported in the literature (Figure 2). For the URL links to the cited literature see Additional file 9: iHOP references.

According to the literature, 17 out of 84 conserved correlated genes have been reported to have functional interaction or co-regulation with BDNF (Figure 2A). *IGFBP5* [31], *NR4A2*, *RGS4* [32] and *DUSP1* [33] have been previously reported to be co-expressed with human or rodent *BDNF*. Other gene products, such as FGFR1 [34] and SNCA [35] are known to regulate *BDNF* expression. Proprotein convertase PCSK1 is implied in processing of pro-BDNF [36]. PTPRF tyrosine phosphatase receptor associates with NTRK2 and modulates neurotrophic signaling pathways [37]. Thyroid hormone receptor alpha (THRA) induces expression of BDNF receptor NTRK2 [38]. Finally, expression of such genes like *EGR1* [39], *MBP* [40], *NEFL* [41], *NPTX1* [42], *NTRK2*, *SERPINE1* [43], *SCG2* [44], *SNCA* [45] and *TCF4* (also known as *ITF2*) [46] is known to be regulated by BDNF signaling. *CCND2*, *DUSP1*, *DUSP6*, *EGR1* and *RGS4* gene expression is altered in cortical GABA neurons in the absence of BDNF [47].

iHOP reports the total of 250 interactions with human BDNF. In order to assess the probability of observing 17/84 or more functional interactions between BDNF and other genes, we had to make an assumption regarding the total number of human genes that iHOP uses. A lower number of total genes would result in higher p-values whereas a higher number of total genes would produce lower p-values. We assumed that the total number of human genes is N = 5000, 10000, 20000 or 30000. Furthermore, the total number of genes linked to BDNF is m = 250 based on iHOP data. Thus, the p-values were obtained using the right-tail of the hypergeometric probability distribution. For N = 5000, 10000, 20000 or 30000, the p-values are 1.0×10^{-07}, 1.7×10^{-12}, 1.3×10^{-17}, 1.18×10^{-20} respectively.

By analyzing the iHOP network indirect connections with BDNF could be established for the genes that did not have known direct interactions with BDNF (Figure 2B). For example, SCG2 protein is found in neuroendocrine vesicles and is cleaved by PCSK1 [48] - protease that cleaves pro-BDNF. BDNF and NTRK2 signaling affect *SNCA* gene expression and alpha-synuclein deposition in substantia nigra [49]. *ATF3* gene is regulated by EGR1 [50], which

Table 2: Conserved correlated genes are associated with various types of cancer and neurological disorders.

Disease	Associated genes	References
Schizophrenia	BDNF RGS4 NR4A2	Schmidt-Kastner et al. (2006)
Parkinson's disease	BDNF PTGS2 SNCA NR4A2	Murer et al. (2001) Chae et al. (2008) Pardo and van Duijn (2005)
Alzheimer's	BDNF KALRN	Murer et al. (2001) Youn et al. (2007)
Polyglutamine neurodegeneration	NEFL BAIAP2	Mosaheb et al. (2005) Thomas et al. (2001)
alpha-mannosidosis	MAN1A1	D'Hooge et al. (2005)
Ophthalmopathy	CYR61 DUSP1 EGR1 PTGS2	Lantz et al. (2005)
Epilepsy	BDNF DUSP6 EGR1	Binder and Scharfman (2004) Rakhade et al. (2007)
Depression	BDNF DUSP1	Russo-Neustadt and Chen (2005) Rakhade et al. (2007)
Ischemia	BDNF CD44 PTGS2	Binder and Scharfman (2004) Murphy et al. (2005)
Ovarian carcinoma	BDNF ITF2 DUSP1 RGS4	Yu et al. (2008) Kolligs et al. (2002) Puiffe et al. (2007)
Breast cancer	BDNF FGFR1 CCND2 PLAU SERPINE1 PLAUR MAZ DUSP6 EGR1 KFL10 PTRF	Tozlu et al. (2006) Koziczak et al. (2004) Grebenchtchikov et al. (2005) Cui et al. (2006) Liu et al. (2007) Reinholz et al. (2004) Levea et al. (2000)
Lung cancer	BDNF ODZ2 CCND2 GFI1	Ricci et al. (2005) Kan et al. (2006)
Prostate cancer	BDNF IGFBP5 PLAUR p75NTR	Bronzetti et al. (2008) Nalbandian et al. (2005)
Pheochromocytoma	PCSK1 PCSK2 SCG2	Guillemot et al. (2006)
Endometrial cancer	CXCL5 OLFM1	Wong et al. (2007)
Leukemia	PKCB1 CCND2	Hans et al. (2005)

expression is activated by BDNF [39]. For more interactions see Figure 2.

Motif discovery

Assuming that genes with similar tissue-specific expression patterns are likely to share common regulatory elements, we clustered co-expressed genes according to their tissue-specific expression using information provided by TiProd database [51]. Each tissue was assigned a category and the genes expressed in corresponding tissues were clustered into the following categories: i) CNS, ii) peripheral NS (PNS), ii) endocrine, iii) gastrointestinal, and iv) genitourinary. We applied DiRE [52] and CONFAC [53] motif-discovery tools to search for statistically over-represented TFBSs in the clusters and among all conserved BDNF-correlated genes. DiRE can detect regulatory elements outside of proximal promoter regions, as it takes advantage of the full gene locus to conduct the search. The

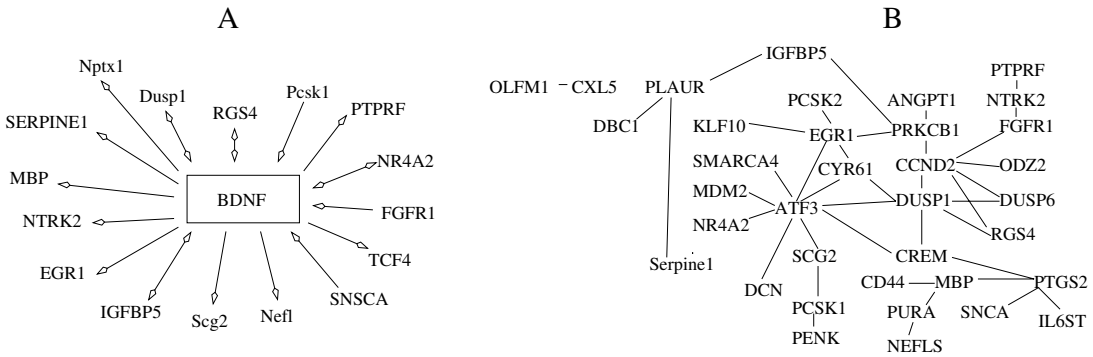

Figure 2

Reported interactions between conserved correlated genes in human. Connections between the genes were created by accessing the literature using iHOP tool. (A) Interactions between correlated genes and *BDNF*. Arrows: "↔" co-expression or co-regulation; "BDNF← "regulation of *BDNF*; "BDNF→" regulation by BDNF. (B) Connections among correlated genes.

software predicts function-specific regulatory elements (REs) consisting of clusters of specifically associated and conserved TFBSs, and it also scores the association of individual TFs with the biological function shared by the group of input genes [52]. DiRE selects a set of candidate REs from the gene loci based on the inter-species conservation pattern which is available in the form of precomputed alignments of genomic sequence from fish, rodent, human and other vertebrate lineages [54]. This type of the alignment enables the tool to detect regulatory elements that are phylogenetically conserved at the same genomic positions in different species. CONFAC software [53] enables the identification of conserved enriched TFBSs in the regulatory regions of sets of genes. To perform the search, human and mouse genomic sequences from orthologous gene pairs are compared by pairwise BLAST, and only significantly conserved (e-value < 0.001) regions are analyzed for TFBSs.

Using DiRE we discovered two regulatory regions at the human *BDNF* locus that were enriched in TFBSs (Figure 3) [see also Additional file 10: DiRE motif discovery results for BDNF and 84 conserved correlated genes]. The first regulatory region spans 218 bp and is located 622 bp upstream of human *BDNF* exon I transcription start site (TSS). The second putative regulatory region is 1625 bp long and located 2915 bp downstream of the *BDNF* stop-codon. Analysis of mouse and rat gene lists produced similar results. Significant over-representation of binding sites for WT1, KROX, ZNF219, NFkB, SOX, CREB, OCT, MYOD and MEF2 transcription factors was reported by DiRE in *BDNF* and BDNF-correlated genes when all the genes were analyzed as one cluster [see Additional file 10: DiRE motif discovery results for BDNF and 84 conserved correlated genes]. Also, the following cluster-specific over-representation of TFBSs was detected: i) CNS - KROX; ii) endocrine - TAL1beta/TCF4, ETS2, SOX5, and ARID5B (known as MRF2); iii) gastrointestinal - MMEF2, and SREBF1; iv) genitourinary - ATF4/CREB, and GTF3 (TFIII) (Table 3) [see also Additional file 11: DiRE motif discovery results for conserved BDNF-correlated genes clustered by tissue-specific expression].

To cross-check the results obtained with DiRE, we repeated the analysis using the CONFAC tool. CONFAC results overlapped with DiRE results and suggested novel regulatory elements in human BDNF promoters/exons I-IX and in BDNF 3'UTR, which were highly conserved among mammals and over-represented in the BDNF-correlated genes. Then, evolutionary conservation across mammals was checked for the core element of each TFBS discovered in the BDNF gene using UCSC Genome Browser. Based on MW test results [see Additional file 12: The results of Mann-Whitney tests (CONFAC)], on the Importance score [see Additional file 10: DiRE motif discovery results for BDNF and 84 conserved correlated genes] and on the conservation data (UCSC), we propose potential regulators of BDNF (Figure 3 and Table 3) [see also Additional file 13: Highly conserved TFBSs in the BDNF gene (according to DiRE and CONFAC)]. It is remarkable, that the TFBSs discovered in the BDNF gene are highly conserved: most of the TFBSs are 100% conserved across mammals from human to armadillo, some of them being conserved even in fish (Figure 3).

Discussion

Microarray meta-analysis has proved to be useful for constructing large gene-interaction networks and inferring evolutionarily conserved pathways. However, it is rarely used to explore the regulatory mechanisms of a single gene. We have exploited microarray data from 80 experiments for the purpose of the detailed analysis of the con-

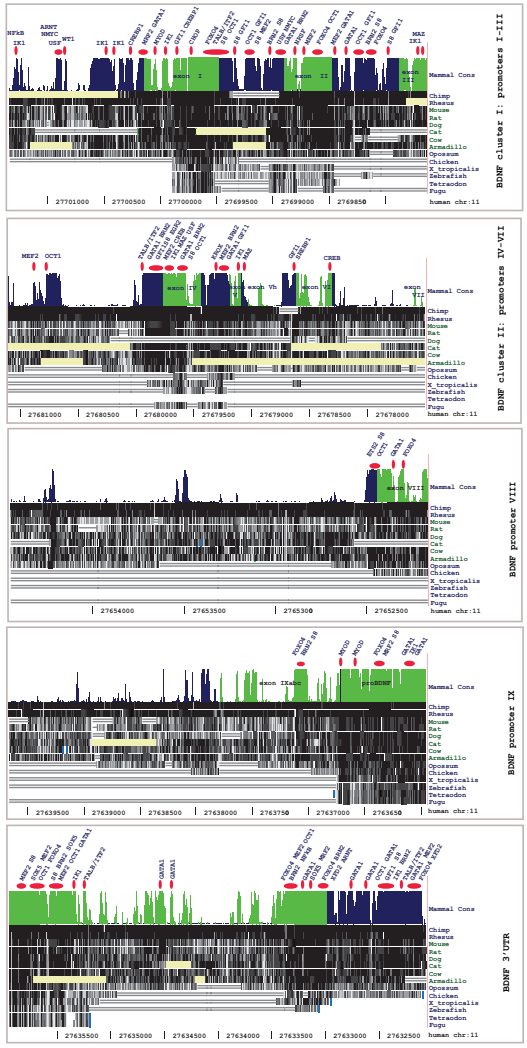

Figure 3

Novel regulatory elements in the BDNF gene. Highly conserved TFBSs in the *BDNF* locus as predicted by DiRE and CONFAC tools. Given TFBSs were also found to be over-represented in the BDNF-correlated genes. Histograms represent evolutionary conservation across 9 mammal species (adapted from UCSC Genome Browser at http://genome.ucsc.edu/) (39). The height of the histogram reflects the size of the conservation score. Conservation for each species is shown in grayscale using darker values to indicate higher levels of overall conservation. Missing sequences are highlighted by regions of yellow. Single line: no bases in the aligned species; double line: aligning species has one or more unaligned bases in the gap region. Transcribed regions (*BDNF* exons and 3'UTR) are highlighted in green; non-transcribed regions (*BDNF* promoters and introns) are highlighted in blue. Red ovals represent TFBSs mapped to the *BDNF* gene sequences. Mapped TFBSs have Matrix Similarity score >0.85 and Core Similarity score >0.99. Core elements of presented TFBSs have 100% of conservation across mammals. For the structure of human *BDNF* see Pruunsild et al., 2007 [11].

Table 3: Over-represented conserved TFBSs in human BDNF and in the BDNF-correlated genes as predicted by DiRE and CONFAC.

TFBS	*p*-value CONFAC	Target genes
ARNT	0.012	*BDNF pI-II, BDNF 3'UTR; PRKCE, USP2, CAMK2D, CCND2, NEUROD6, THRA, DUSP1, CBX6, ATP1B1, FREQ, ITF-2*
POU3F2 (BRN2)	< 0.001	*BDNF pII-V, BDNF exon II, IV, IX, BDNF3'UTR; USP2, CAMK2D, THRA, NFIA, PRSS23, CBX6, CUGBP2, EPHA5, EPHA7, BAIAP2, RKCE, CPD, EPHA4, IL6ST, CCND2, DUSP6, KCND2, MAN1A1, SCG2, GRIA3, COL11A1, TRPC4, FGF13, HN1, ANGPT1, TCF4, MYH9, PCSK1*
CHOP	NA	*BDNF I, COL11A1, CD44, BAIAP2, PPP3CA, IL6ST, NEUROD6, SCG2, CYR61, IGFBP5, THRA, NFIA, FGF13, ATP1A2, ANGPT1, DBC1, CUGBP2, EGR1*
CREB	0.013	*BDNF pI, IV, VI, BDNF exon I; BAIAP2, PRKCE, USP2, EPHA4, CAMK2D, CCND2, FGFR1, CYR61, GRIA3, THRA, DUSP1, PENK, PCSK1, PCSK2, HN1, ATP1B1, EGR1, COL4A5, KLF10, EPHA4, FGF13, CBX6, CUGBP2, EPHA5*
ETS2	NA	*BDNF pII, VIII; THRA, EPHA7, FGF13, BAIAP2 and NFIA promoters, and in COL11A1, PLAGL1, and XIAP intergenic regions*
FOXO4	< 0.001	*BDNF exon I, II, VIII, IX, BDNF pIII, IV, BDNF 3'UTR; CD44, TBX3, BAIAP2, PPP3CA, CPD, USP2, PRKCB, EPHA4, CORO1A, CAMK2D, NEUROD6, FGFR1, SCG2, CYR61, GRIA3, THRA, NFIA, COL11A1, DUSP1, TRPC4, PRSS23, PCSK2, ANGPT1, FREQ, PRKAG2, TCF4, MYH9, PCSK1, DBC1, CUGBP2, EGR1, EPHA5*
GATA1	< 0.001	*BDNF pI, III-V, BDNF exon I, II, VIII, IX, BDNF 3'UTR; CD44, TBX3, SNCA, PPP3CA, PRKCE, COL4A5, USP2, EPHA4, IL6ST, SLC4A7, CAMK2D, ATF3, CCND2, NEUROD6, DUSP6, KCND2, SCG2, CYR61, IGFBP5, THRA, NFIA, COL11A1, PENK, FGF13, PRSS23, ATP1B1, ATP1A2, ANGPT1, DBC1, CUGBP2, EGR1*
GFI1	< 0.001	*BDNF exon I, BDNF pII-VI, BDNF 3'UTR; SNCA, ATP1A2, MYH9, DBC1, CD44, BAIAP2, PPP3CA, PRKCE, COL4A5, CPD, USP2, EPHA4, IL6ST, SLC4A7, CAMK2D, CCND2, NEUROD6, KCND2, SCG2, CYR61, IGFBP5, THRA, NFIA, COL11A1, DUSP1, TRPC4, PENK, FGF13, PRSS23, PCSK2, ATP1B1, PTPRF, ANGPT1, TCF4, CUGBP2, EGR1, EPHA5, EPHA7*
IK1 (ikaros)	< 0.001	*BDNF pI, BDNF exon I-V, IX, BDNF 3'UTR; PRKCB, KLF10, KCND2, THRA, NFIA, COL11A1, FGF13, ATP1A2, MYH9, PCSK1, CUGBP2, EPHA7*
KROX family	NA	*BDNF pV, BDNF exon IV; PPP3CA, NFIA, DBN1, KCND2, IRS2, MAN1A2, CCND2, PVRL3, XIAP, DLGAP4, CYR61, ATP1B1, PURA, SMARCA4, MYH9, GRIA3, EPHA4, DUSP6, EGR1, COL4A5, TRPC4, PRKCB, NPTX1, PTGS2, EPHA5, FGFR1, CBX6, PRKCE, KLF10, THRA, ATP1A2, BAIAP2, CPD, CORO1A, CAMK2D, IGFBP5, DUSP1, PTPRF, FREQ, PRKAG2*
MAZ	NA	*BDNF pVh, BDNF exon III, IV; CD44, PPP3CA, PRKCE, COL4A5, USP2, PRKCB, KLF10, EPHA4, CAMK2D, CCND2, DUSP6, GRIA3, THRA, COL11A1, PENK, FGF13, CBX6, ATP1B1, PTPRF, ATP1A2, FREQ, DBN1, CUGBP2, EGR1, EPHA7*
MEF2	NA	*BDNF pII-V, BDNF exon II, IX, BDNF 3'UTR; CD44, TBX3, BAIAP2, PPP3CA, PRKCE, COL4A5, EPHA4, IL6ST, CAMK2D, CCND2, NEUROD6, DUSP6, MAN1A1, IGFBP5, COL11A1, TRPC4, PRSS23, ANGPT1, FREQ, PURA, MYH9, PCSK1, CUGBP2, EPHA7, SNCA, FGF13*
MYC/MAX	NA	*BDNF pI, II, IV; CD44, TBX3, PRKCE, USP2, CAMK2D, CCND2, NEUROD6, THRA, NFIA, DUSP1, CBX6, ATP1B1, FREQ, ITF-2, EGR1*
MYCN	NA	*BDNF pI, II; PRKCE, USP2, CAMK2D, CCND2, NEUROD6, THRA, DUSP1, CBX6, ATP1B1, FREQ, ITF-2*
MYOD	< 0.001	*BDNF exon I, IX; CD44, PRKCE, USP2, PRKCB, EPHA4, DUSP6, SCG2, SMARCA4, THRA, PRSS23, ATP1B1, CUGBP2*
NFkB	< 0.001	*BDNFI, BDNF 3'UTR; PPP3CA, KLF10, PCSK2, ATP1B1, ANGPT1, MYH9, USP2, DUSP6, FGF13, PURA, BAIAP2, CAMK2D, CCND2, FGFR1, CYR61, PCSK2, MYH9, CUGBP2, EGR1, EPHA7*
NRSF	NA	*BDNFII, EPHA4, IRS2, EPHA5, NPTX1, PRKCB, TRPC4, COL4A5*
S8	< 0.001	*BDNF pII-IV, BDNF exon II, IV, VIII, IX, BDNF 3'UTR; CD44, BAIAP2, PRKCE, NPTX1, EPHA4, CAMK2D, CCND2, NEUROD6, DUSP6, FGFR1, KCND2, MAN1A1, SCG2, THRA, NFIA, COL11A1, PENK, PCSK2, ANGPT1, PURA, ITF-2, MYH9, DBC1, CUGBP2, EGR1, EPHA5*

Table 3: Over-represented conserved TFBSs in human BDNF and in the BDNF-correlated genes as predicted by DiRE and CONFAC.

SOX5	0.001	*BDNF exon I, BDNF 3'UTR; EPHA4, THRA and PLAGL1 3'UTR; NFIA and OLFM1 promoters; SCG2 intergenic region; KCND2 intron*
TAL1/TCF4	NA	*BDNF pIV, BDNF exon I, BDNF 3'UTR; ATP1B1 3'UTR, MYH9 3'UTR and XIAP 3'UTR; SCG2, CD44, SERPINE1, SLC4A7, CCND2, NEUROD6, FGFR1, THRA, COL11A1, PCSK2, ANGPT1, DBC1, CUGBP2*
WT1	NA	*BDNF pI, BASP1, PPP3CA, NFIA, DBN1, EPHA7, BAIAP2, XIAP, DLGAP4, PURA, IRS2, ATP1B1, KCND2, GRIA3, HN1, EPHA4, EGR1, COL4A5, TRPC4, ATP1A2, PRKCB, NPTX1, DBC1, EPHA5*

In *BDNF*, TFBSs were found in promoters (p), exons or 3'UTR of the gene. In the correlated genes, TFBSs were searched for and discovered mostly in promoters (unless indicated otherwise). P-values are given for the TFBSs discovered using CONFAC. NA - not applicable for the TFBSs discovered using DiRE [see Additional files 10 and 11 for TFBS importance score].

servation of *BDNF* gene expression and regulation. Analysis of co-expression conservation combined with motif discovery allowed us to predict potential regulators of *BDNF* gene expression as well as to propose novel gene interactions. Several transcription factors that were identified here as potential regulators of human *BDNF* gene have been previously shown to regulate rodent *BDNF* transcription *in vitro* and *in vivo*. These transcription factors include REST (also known as NRSF) for *BDNF* promoter II [55], CREB for *BDNF* promoter I and IV [56,57], USF [58], NFkB [59], and MEF2 for *BDNF* promoter IV [60]. The support of the bioinformatics findings by experimental evidence strongly suggests that the potential regulatory elements discovered in this study in the *BDNF* locus may be involved in the regulation of *BDNF* expression.

According to g:Profiler, 44 out of 84 conserved correlated genes identified in this study (including *BDNF*) carry MYC-associated zinc finger protein (MAZ) transcription factor binding sites. Our study revealed putative binding sites for MAZ in *BDNF* promoter Vh and in exons III and IV, suggesting that MAZ could be involved in *BDNF* gene regulation from promoters III, and possibly from promoters IV, V, Vh and VI that lie in close proximity in the genome. It has been shown that MAZ is a transcriptional regulator of muscle-specific genes in skeletal and cardiac myocytes [61]. Histone deacetylation and DNA methylation might be involved in the regulation of expression of target genes by MAZ [62]. *BDNF* mRNA expression in the heart is driven by promoters IV, Vh and VI [11]. Epigenetic regulation of the *BDNF* gene expression is achieved in a cell-type and promoter-specific manner [12,63]. This could be a possible regulation mechanism of the *BDNF* gene by MAZ. Also, MAZ drives tumor-specific expression of *PPARG* in breast cancer cells, a nuclear receptor that plays a pivotal role in breast cancer [64]. Expression levels of *BDNF* and BDNF-correlated genes *CCND2, DUSP6, EGR1, KLF10* and *PTPRF* are altered in breast cancer (see Table 2). These genes were identified as putative targets of MAZ in the present study suggesting potential role for MAZ in their regulation in breast cancer cells.

Our analysis revealed that Wilms' tumor suppressor 1 (WT1) transcription factor binding sites are overrepresented in the BDNF-correlated genes. WT1 binding sites were detected in *BDNF* promoter I, in *IRS2* (insulin receptor substrate 2), *EGR1, BAIAP2* (insulin receptor substrate p53) and *PURA* promoters and in 19 other genes. WT1 acts as an oncogene in Wilms' tumor (or nephroblastoma), gliomas [65] and various other human cancers [66]. WT1 activates the *PDGFA* gene in desmoplastic small round-cell tumor, which contributes to the fibrosis associated with this tumor [67]. Puralpha (*PURA*), a putative WT1 target gene identified in this study, has also been reported to enhance transcription of the *PDGFA* gene [68]. WT1 regulates the expression of several factors from the insulin-like growth factor signaling pathway [69]. WT1 was also shown to bind the promoter of *EGR1* gene [70]. Neurotrophins and their receptors also may be involved in the pathogenesis of some Wilms' tumors [71]. Transcriptional activation of BDNF receptor *NTRK2* by WT1 has been shown to be important for normal vascularization of the developing heart [72]. Moreover, WT1 might have a role in neurodegeneration, observed in Alzheimer's disease brain [73]. We hypothesize that *BDNF* and other WT1 targets identified in this study, can play a role in normal development and tumorigenesis associated with WT1.

KROX family transcription factors' binding sites were found to be abundant in the promoters of *BDNF* and BDNF-correlated genes. KROX binding motif was detected in *BDNF* promoter V and EGR2 binding site was found in *BDNF* promoter IV. Also, *EGR1* gene expression was correlated with *BDNF* in human, mouse and rat. KROX family of zinc finger-containing transcriptional regulators, also known as Early Growth Response (EGR) gene family, consists of EGR1-EGR4 brain-specific transcription factors [74] that are able to bind to the same consensus DNA sequence (KROX motif) [75]. EGR1 is involved in the maintenance of long-term potentiation (LTP) and is required for the consolidation of long-term memory [76]. EGR3 is essential for short-term memory formation [77] and EGR2 is necessary for Schwann cell differentiation

and myelination [78,79]. Since BDNF plays a significant role in the above mentioned processes, it would be intriguing to study the regulation of BDNF by EGR factors.

Binding sites for GFI1 and MEF2 were found in *BDNF* promoters, exons and 3'UTR, and in the promoter of the *SNCA* gene. GFI1 binding sites were detected in *BDNF* promoters II-VI and in exon I. MEF2 sites were found in *BDNF* promoters II-V and in exons II and IX. *SNCA* overexpression and gene mutations that lead to SNCA protein aggregation cause Parkinson's disease (PD) [80]. *BDNF* and *SNCA* expression levels change conversely in the nigro-striatal dopamine region of the PD brain [80,81]. The myocyte enhancer factor-2 (MEF2) is known to be necessary for neurogenesis and activity-dependent neuronal survival [82,83]. Inactivation of MEF2 is responsible for dopaminergic loss *in vivo* in an MPTP mouse model of PD [84]. MEF2 recruits transcriptional co-repressor Cabin1 and class II HDACs to specific DNA sites in a calcium-dependent manner [85]. MEF2 is one of the TFs that contribute to the activity-dependent BDNF transcription from promoter IV [60]. The growth factor independence-1 (GFI1) transcription factor is essential for the development of neuroendocrine cells, sensory neurons, and blood. Also, GFI1 acts as an oncogene in human small cell lung cancer (SCLC), the deadliest neuroendocrine tumor [86]. GFI1 mediates reversible transcriptional repression by recruiting the eight 21 corepressor (ETO), histone deacetylase (HDAC) enzymes and the G9a histone lysine methyltransferase [87]. It has also been shown that GFI1 Drosophila homolog Senseless interacts with proneural proteins and functions as a transcriptional co-activator suggesting that GFI1 also cooperates with bHLH proteins in several contexts [88]. Our findings are impelling to explore inverse regulation of *BDNF* and *SNCA* genes by GFI1 and MEF2 in neurons generally and in Parkinson's disease models in particular.

BDNF promoters II-V and *BDNF* exons II, IV and IX contain BRN2 (brain-specific homeobox/POU domain POU3F2) binding sequences. BRN2 is driving expression of the *EGR2* gene - an important factor controlling myelination in Schwann cells [78,79]. BRN2 also activates the promoter of the Notch ligand Delta1, regulating neurogenesis. It also regulates the division of neural progenitors, as well as differentiation and migration of neurons [89]. Considering a prominent role of BDNF in myelination and neurogenesis, it is reasonable to hypothesize that BRN2 fulfills its tasks in part by regulating *BDNF* gene expression.

Evidence is emerging that not only proximal promoters, but also distant elements upstream and downstream from TSS can regulate transcription [90,91]. We found that *BDNF* 3'UTR contains potential binding sites for TCF4 (also known as ITF2), GFI1, BRN2, NFkB and MEF2.

Finally, we have discovered multiple binding sites in human *BDNF* promoters for the transcription factors that have been shown to participate in neuronal activity-dependent transcription of rodent *BDNF* gene. *BDNF* promoters I and IV are the most highly induced following neuronal activation. *BDNF* promoter I was shown to be regulated by cAMP-responsive element (CRE) and the binding sequence for upstream stimulatory factor 1/2 (USF) in response to neuronal activity and elevated calcium levels [92]. Several TFs (USF [58], CREB [57], MEF2 [60], CaRF [93] and MeCP2 [63]) regulate *BDNF* promoter IV upon calcium influx into neurons. Rat *BDNF* promoter II has also shown induction by neuronal activity, though to a lesser extent compared to promoters I and IV [12,94]. However, calcium responsive elements have not been yet studied in *BDNF* promoter II and it was believed that its induction is regulated by the elements located in the promoter I. Our analysis of human *BDNF* gene detected CREBP1 and USF binding sites in *BDNF* promoter I, USF and MEF2 binding sites in promoter II and USF, MEF2 and CREB binding sites in promoter IV. We suggest that MEF2 and USF elements might contribute to *BDFN* promoter II induction by neuronal activity. In addition, we have detected conserved TCF4 (ITF2) binding sequences in *BDNF* promoter IV, and in exon I. It has been shown that calcium-sensor protein calmodulin can interact with the DNA binding basic helix-loop-helix (bHLH) domain of TCF4 inhibiting its transcriptional activity [95]. Preliminary experimental evidence (Sepp and Timmusk, unpublished data) suggests that TCF4 transcription factor is involved in the regulation of *BDNF* transcription. TCF4 might play in concert with CREB, MEF2 and other transcription factors to modulate *BDNF* levels following neuronal activity.

In our study we performed the analysis of a well-known gene and it served as a good reference to evaluate the results of the "subset" approach. However, the "subset" method coupled with the analysis of evolutionary conservation of co-expression is suitable for studying poorly annotated genes as well. This approach examines co-expression across a variety of conditions, which helps to discover novel biological processes and pathways that the guide-gene and its co-expressed genes are related to. Also, searching for conserved TFBS modules in co-expressed genes helps to discover functionally important genomic regions and this does not require detailed prior knowledge of the guide-gene's structure. However, when attempting to study less known genes, additional *in silico* analysis of genomic sequences using bioinformatics tools for prediction of promoters, TSSs and exon-intron junc-

tions would be useful. Also, sequence alignment with co-expressed genes' promoters would be informative.

Conclusion

A major impediment of meta-coexpression analysis is the differences among experiments. So far, analyzing gene expression across different microarray platforms remains a challenge. Discrepancies in the expression measurements among different platforms originate from different probe sequences used, different number of genes on the platform, etc. Therefore, in order to obtain reliable results, we used only one microarray platform type for the analysis. In addition, we introduced a new approach to increase the accuracy of the analysis: we divided datasets into subsets and sought for correlated genes for each subset, implying that each subset represents an independent experimental condition. We have also performed correlation link confirmation among subsets and correlation conservation analysis to discover functionally related genes.

One of the limitations of the co-expression conservation analysis is the fact that it detects only phylogenetically conserved co-expression events. Human-specific phenomena cannot be captured by this kind of analysis. In relation to *BDNF* this means, for example, that regulation of human *BDNF* gene by antisense *BDNF* RNA (*BDNFOS* gene) [11,96] could not be studied by co-expression conservation analysis, since BDNFOS gene is not expressed in rodents [12,97]. Also, co-expression analysis using microarray experiments is limited by the number of genes included in the microarray platforms. For example, since *BDNFOS* probe sets were absent from microarray platforms, we could not study co-expression, anti-coexpression or differential expression of *BDNF* and *BDNFOS*. In addition, our list of correlated genes did not include all possible correlation links with *BDNF* due to the fact that our analysis was deliberately limited to Affymetrix microarray platforms. Moreover, in our analysis we included only those experiments that met certain requirements regarding the *BDNF* gene expression. However, biologically meaningful results justify our rigorous filtering approach: correlated genes identified in this study are known to regulate nervous system development, and are associated with various types of cancer and neurological disorders. Also, experimental evidence supports the hypothesis, that transcription factor identified here can act as potential *BDNF* regulators.

In summary, we have discovered a set of genes whose co-expression with *BDNF* was conserved between human and rodents. Also, we detected new potential regulatory elements in BDNF-correlated genes and in the *BDNF* locus using bioinformatics analysis, in which *BDNF* was playing a role of a guide-gene. The presented concept of co-expression conservation analysis can be used to study the regulation of any other gene of interest. The study provides an example of using high-throughput advancements in studying single genes and proposes hypotheses that could be tested using molecular biology techniques.

Methods

Microarray datasets and data filtering

Homo sapiens, *Mus Musculus* and *Rattus Norvegicus* microarray datasets were downloaded from (GEO) [98]. We selected Affymetrix GeneChips experiments that comprised a minimum of 16 samples. Datasets which contained BDNF Detection call = Absent [99] in more than 30% of the samples were not selected [see Additional file 2: Microarray datasets] for the list of datasets used in the analysis. Since the arrays contained normalized data, no additional transformation was performed. To reduce the noise, we carried out non-specific filtering of data in each dataset. Genes that had missing values in more than 1/3 of the samples of a given dataset were excluded from the analysis in order to avoid data over-imputation [100]. For the remaining genes, we followed a column-average imputation method. Totally, only 0.098% of the gene expression values were imputed with this approach. Further, we selected the genes whose expression changes were greater than two-fold from the average (across all samples) in at least five samples in a dataset [19,49]. Additionally, datasets were eliminated from the study if *BDNF* probe sets' expression failed to meet the above mentioned criteria [see Additional file 1: BDNF probe sets]. Out of 72 human datasets, only 38 passed non-specific filtering, whereas 24 out of 82 mouse and 18 out of 35 rat datasets passed the filtering and were used for the analysis.

Each dataset was split into subsets (i.e. normal tissue, disease tissue, control, treatment, disease progression, age, etc.) so that subsets of the same dataset would not have any overlapping samples [see Additional file 3: Subsets]. The division into subsets was performed manually, according to the information included in the experiment. In some cases subsets could be further subdivided into biologically appropriate sub-subsets [see Additional file 2: Microarray datasets and Additional file 3: Subsets]. Subsets that contained less than eight samples were excluded from analysis to avoid inaccuracy in the estimation of genic correlations. Biological and technical replicates were handled as equal. From all human datasets, one (GDS564 dataset) contained one technical replicate per male sample and one technical replicate for all female samples except one. For the mouse datasets no technical replicates' data accompanied the dataset information. Finally, in rat GDS1629 dataset one technical replicate has been used for each biological replicate.

Differential expression

We used Kruskal-Wallis test [23] to measure differential expression of BDNF across subsets in each dataset.

Kruskal-Wallis test is a non-parametric method for testing equality of population medians within different groups. It is similar to one-way analysis of variance (ANOVA). However, it does not require the normality assumption. Alternatively, it represents an extension of Mann-Whitney U test [101,102] for more than 2 samples. Since we used multiple datasets we applied the false discovery rate approach (FDR) at the 0.05 level as it is described by Benjamini and Hochberg (1995) [103].

Co-expression analysis

For each gene standard Pearson correlation coefficient (PCC) was calculated across samples. We followed a resampling strategy, which allows the calculation of the standard deviation of the PCC between a pair of probe sets. PCC was calculated for each subset separately. The PCC was calculated following a resampling bootstrap approach. For example, in order to calculate the CC_j between *BDNF* and gene j when data consisted of m points, we resampled the m points with replacement creating 2000 re-samples [104]. Then the CC_j was calculated as the average CC for the 2000 re-samples and the 95% bootstrap confidence interval was estimated. The average CC is very close to the sample CC. However, when m is a small number and outliers are contained in the sample then the bootstrap confidence interval may be large. The motivation behind the bootstrap approach is to avoid genes with large bootstrap confidence intervals. Thus, when we request the links between *BDNF* and the genes in the microarray experiment we ask for the genes j, whose CC_j is greater than 0.6 and the 95% bootstrap confidence interval contains only positive numbers. If instead of the bootstrapping approach we would use just the sample CC, which is more efficient computationally, then a larger set of links would be obtained which would contain some genes with very large bootstrap confidence intervals.

A threshold value of r = 0.6 was used to retrieve a list of probe sets that were co-expressed with the *BDNF* probe set [22,49]. Each probe set correlation with *BDNF* that passed the threshold was termed as a "link". It should be noted that the PCC was calculated between probe set pairs and not between gene-name pairs. Thus, when more than one probe set-pair was associated with the same gene-pair we excluded all the links except the one with the highest PCC value.

Co-expression link confirmation

We defined a "co-expression link confirmation" as a re-occurrence of links in multiple subsets. In order to avoid artifacts and biologically irrelevant links, we performed link confirmation to select the genes that were correlated with *BDNF* in three or more subsets [15]. It should be noticed that systematic differential expression within a subset could result in high PCC values. However, high PCC values in this case do not reveal any relationship between genes and represent a by-product of the differential expression of genes within a heterogeneous subset. We used a minimum between 1000 and 10% of all the probe sets within the subset as a threshold. Subsets that yielded more co-expression links between *BDNF* and other genes than an arbitrary threshold were excluded from further analysis. Thus, 5% of all the subsets were excluded.

Probe set re-annotation and ortholog search

Prior to the identification of the links that are conserved between human, mouse and rat, we transformed the probe set-pair links to gene-pair links. We used g:Profiler [26] to transform the probe set names to Ensemble gene names (ENSG). However, since many probe sets are currently related to the expressed sequence tags (ESTs), not all the probe sets could be mapped to the known genes using g:Profiler. For each dataset, we used its annotation file (see: ftp://ftp.ncbi.nih.gov/pub/geo/DATA/annotation/platforms/). To assign Ensemble gene names to the "unmapped" probe sets, we obtained the probe set sequence identifier (GI number) using the annotation file. Then, we retrieved RefSeq accession for each GI number from NCBI database. Finally, we continued with a best-hit blast approach for all three species.

Co-expression conservation and g:Profiler analysis

By performing a co-expression conservation analysis we identified the links that have passed prior filters (PCC threshold and link confirmation) and are conserved among human, mouse and rat.

Genes which co-expression with *BDNF* was found to be conserved between human, mouse, and rat constituted the input list for the g:Profiler. g:Profiler http://biit.cs.ut.ee/gprofiler/[26] is a public web server used for characterizing and manipulating gene lists resulting from mining high-throughput genomic data. It detects gene-ontology categories that are overrepresented by the input list of genes or by sorted sublists of the input. g:Profiler is using the "Set Count and Sizes" (SCS) method to calculate p-values [26].

Correlated genes' interactions

We used iHOP resource (Information Hyperlinked over Proteins, http://www.ihop-net.org/) [30] to find reports in the literature about known interaction between BDNF-correlated genes. iHOP generates a network of genes and proteins by mining the abstracts from PubMed. A link in such a network does not mean a specific regulatory relationship, but any possible interaction between two genes (such as protein activation, regulation of transcription, co-expression, etc). Each reference was verified manually to ensure the citation of valid interactions.

Motif discovery

We clustered BDNF-correlated genes according to their tissue-specific expression using gene expression information available in the TiProD database [51] (*BDNF* gene was included in every cluster). The TiProD database contains information about promoter tissue-specific expression for human genes. For each gene the list of tissues where the gene expression has been detected can be obtained from TiProD together with the tissue specificity score. For each gene we extracted information on tissue expression, selecting tissues with specificity score higher than 0.2. Each tissue was assigned a category according to its anatomy and function and the genes expressed in corresponding tissues were clustered into CNS, peripheral NS, endocrine, gastrointestinal or genitourinary cluster. Then, we searched for combinations of over-represented TFBS among the list of correlated genes, as well as the tissue clusters discovered by TiProD.

We used DiRE http://dire.dcode.org/[52] and CONFAC http://morenolab.whitehead.emory.edu/[53] tools for the discovery of TFBSs in the conserved co-expressed genes. DiRE uses position weight matrices (PWM) available from version 10.2 of the TRANSFAC Professional database [105]. In DiRE, up to 5000 background genes can be used. Only those TFBSs are extracted that occur less frequently in 95% of permutation tests than in the original distribution (corresponding to a p-value < 0.05 to observe the original distribution by chance) and that corresponds to at least a twofold increase in their density in the original distribution as compared with an average pair density in permutation tests. To correct for multiple hypothesis testing, the hypergeometric distribution with Bonferroni correction is used in the DiRE tool [106]. For each discovered TFBS DiRE defines the 'importance score' as the product of the transcription factor (TF) occurrence (percentage of tissue-specific TF with the particular TFBS) and its weight (tissue-specificity importance) in a tissue-specific set of candidate TF. Thus, the importance score is based on the abundance of the TFBS in tissue-specific TF and on the specificity of the TF that contain the particular TFBS.

Conserved transcription factor binding site (CONFAC) software [53] enables the high-throughput identification of conserved enriched TFBSs in the regulatory regions of sets of genes using TRANSFAC matrices. CONFAC uses the Mann-Whitney U-test to compare the query and the background set. It uses a heuristic method for reducing the number of false positives while retaining likely important TFBSs by applying the mean-difference cutoff which is similar to the use of fold change cutoffs in SAM analyses [107] of DNA microarray data [53]. According to the data provided by CONFAC, 50 random gene sets were compared to random sets of 250 control genes. Only one TFBS exceeded 5% false positive rate for the set of 250 random control genes that we used in our analysis with the parameters advised by the authors [53]. We used promoter sequences of BDNF-correlated genes and the sequences of *BDNF* promoters, exons, introns and the 3'UTR for the analysis. Matrix Similarity cut-off 0.85 and Core Similarity cut-off 0.95 were used for motif discovery; and the parameters recommended by authors - for Mann-Whitney tests (p-value cutoff 0.05 and mean-difference cutoff 0.5) [53].

Evolutionary conservation across mammals was confirmed manually for the 5-nucleotide core element of each TFBS discovered in the *BDNF* gene using UCSC Genome Browser [108].

Authors' contributions

TA and PP made equal contribution to conception and design of the study. PP performed computational analysis of data; TA and TT performed interpretation of the results. TA and PP were involved in drafting the manuscript; TT revised the manuscript for important intellectual content. TA, PP and TT have given final approval of the version to be published.

Additional material

Additional file 1

BDNF probe sets. *Affymetrix microarray probe sets for BDNF gene. BDNF probe set target sequences are given for each platform type that was used in the co-expression conservation analysis.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-10-420-S1.xls]

Additional file 2

Microarray datasets. *Datasets that passed non-specific filtering and were used in the analysis (38 human microarray datasets, 24 mouse datasets and 18 rat datasets). Each dataset was divided into subsets (disease state, age, agent, etc) according to experimental annotations. When possible, subsets were subdivided further (marked by *). Experiments were classified based on their description and the tissue origin. GDS refers to GEO Datasets.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-10-420-S2.xls]

Additional file 3

Subsets. *Dataset: GDS1018/1368678_at/Bdnf/Rattus norvegicus. Expression profiling of brain hippocampal CA1 and CA3 neurons of Sprague Dawleys subjected to brief preconditioning seizures. According to the dataset annotation, dataset could be divided into three subsets by cell type (A) or into two subsets by protocol (B). In addition, subsets could be subdivided further into cell type.protocol sub-subsets: CA1 pyramidal neuron.control, CA1 pyramidal neuron.preconditioning seizure, CA3 pyramidal neuron.control, etc. After filtering, subset containing less than eight samples (CA3 pyramidal neuron.preconditioning seizure) was excluded from the analysis.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-10-420-S3.pdf]

Additional file 4

Differential expression of the BDNF gene in human datasets. *Differential expression of BDNF was measured across subsets in each dataset using Kruskal-Wallis test. Only statistically significant results are presented.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-10-420-S4.pdf]

Additional file 5

Differential expression of the BDNF gene in mouse datasets. *Differential expression of BDNF was measured across subsets in each dataset using Kruskal-Wallis test. Only statistically significant results are presented.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-10-420-S5.pdf]

Additional file 6

Differential expression of the BDNF gene in rat datasets. *Differential expression of BDNF was measured across subsets in each dataset using Kruskal-Wallis test. Only statistically significant results are presented.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-10-420-S6.pdf]

Additional file 7

Conserved BDNF-correlated genes. *Genes, whose correlation with BDNF was confirmed in at least 3 subsets (3+ genes) and was conserved between i) human, mouse and rat; ii) human and rat; iii) human and mouse; iv) mouse and rat.*
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[http://www.biomedcentral.com/content/supplementary/1471-2164-10-420-S7.xls]

Additional file 8

g:Profiler analysis. *Functional profiling of the list of BDNF-correlated genes conserved between human, mouse and rat using g:G:Profiler. For details see also http://biit.cs.ut.ee/gprofiler/.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-10-420-S8.txt]

Additional file 9

iHOP references. *Interactions between conserved correlated genes in human and mouse (URL links to the literature cited in iHOP).*
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[http://www.biomedcentral.com/content/supplementary/1471-2164-10-420-S9.xls]

Additional file 10

DiRE motif discovery results for BDNF and 84 conserved correlated genes. *Over-represented TFBSs are given together with the Importance Score (cut-off 0.1 recommended by DiRE). Numbers 1 and 2 (in All 1 and 2) refer to the different ways that DiRE tool analyzes evolutionary conserved regions (ECR): 1) top 3 ECRs + promoter ECRs; 2) UTR ECRs + promoter ECRs.*
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[http://www.biomedcentral.com/content/supplementary/1471-2164-10-420-S10.xls]

Additional file 11

DiRE motif discovery results for conserved BDNF-correlated genes clustered by tissue-specific expression. *TFBSs over-represented in each tissue cluster are given together with the Importance Score (cut-off 0.1 recommended by DiRE). CNS - central nervous system, PNS - peripheral nervous system.*
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[http://www.biomedcentral.com/content/supplementary/1471-2164-10-420-S11.xls]

Additional file 12

The results of Mann-Whitney tests (CONFAC). *Overrepresented TFs in the conserved BDNF-correlated gene list. Bar graphs show the average conserved TFBS frequencies for the sample gene set (conserved BDNF-correlated genes, blue bars) and control gene set (random 250 genes, red bars). A minimum threshold for the differences in the average TFBS frequencies between the two groups was set by p-value cutoff 0.05 and a mean-difference cutoff 0.5.*
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[http://www.biomedcentral.com/content/supplementary/1471-2164-10-420-S12.pdf]

Additional file 13

Highly conserved TFBSs in the BDNF gene (according to DiRE and CONFAC). *Represented TFBSs have Matrix Similarity score >0.85 and Core Similarity score >0.99. TFBS sequences are highlighted in blue; "+" or "-" mark the DNA strand orientation; BDNF exons and 3'UTR are highlighted in green; the regulatory region in BDNF downstream from polyadenylation sites identified by DiRE is highlighted yellow.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-10-420-S13.htm]

Acknowledgements

This work was supported by the following grants: the Wellcome Trust International Senior Research Fellowship [grant number 067952]; Estonian Ministry of Education and Research [grant number 0140143]; Estonian Enterprise [grant number EU27553]; and Estonian Science Foundation [grant number 7257] to TT; the Volkswagen-Foundation [grant number 824234-1] to PP.

We thank Jüri Reimand and Marko Piirsoo for critical comments on the manuscript. Mari Sepp, Indrek Koppel, Priit Pruunsild and other members of our lab are acknowledged for useful suggestions and discussions.

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PUBLICATION III

Tissue-specific and neural activity-regulated expression of human BDNF gene in BAC transgenic mice.x

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BMC Neuroscience (2009), 10:68

BMC Neuroscience

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Tissue-specific and neural activity-regulated expression of human BDNF gene in BAC transgenic mice

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Published: 25 June 2009

BMC Neuroscience 2009, **10**:68 doi:10.1186/1471-2202-10-68

Received: 28 January 2009
Accepted: 25 June 2009

This article is available from: http://www.biomedcentral.com/1471-2202/10/68

Abstract

Background: Brain-derived neurotrophic factor (BDNF) is a small secreted protein that has important roles in the developing and adult nervous system. Altered expression or changes in the regulation of the BDNF gene have been implicated in a variety of human nervous system disorders. Although regulation of the rodent BDNF gene has been extensively investigated, *in vivo* studies regarding the human BDNF gene are largely limited to postmortem analysis. Bacterial artificial chromosome (BAC) transgenic mice harboring the human BDNF gene and its regulatory flanking sequences constitute a useful tool for studying human BDNF gene regulation and for identification of therapeutic compounds modulating BDNF expression.

Results: In this study we have generated and analyzed BAC transgenic mice carrying 168 kb of the human BDNF locus modified such that BDNF coding sequence was replaced with the sequence of a fusion protein consisting of N-terminal BDNF and the enhanced green fluorescent protein (EGFP). The human BDNF-BAC construct containing all BDNF 5' exons preceded by different promoters recapitulated the expression of endogenous BDNF mRNA in the brain and several non-neural tissues of transgenic mice. All different 5' exon-specific BDNF-EGFP alternative transcripts were expressed from the transgenic human BDNF-BAC construct, resembling the expression of endogenous BDNF. Furthermore, BDNF-EGFP mRNA was induced upon treatment with kainic acid in a promotor-specific manner, similarly to that of the endogenous mouse BDNF mRNA.

Conclusion: Genomic region covering 67 kb of human BDNF gene, 84 kb of upstream and 17 kb of downstream sequences is sufficient to drive tissue-specific and kainic acid-induced expression of the reporter gene in transgenic mice. The pattern of expression of the transgene is highly similar to BDNF gene expression in mouse and human. This is the first study to show that human BDNF gene is regulated by neural activity.

Background

Brain-derived neurotrophic factor (BDNF) [1], a member of the neurotrophin family, promotes survival and differentiation of several neuronal populations during mammalian development [2,3]. In the adult central nervous system, BDNF acts as a regulator of activity-dependent

neurotransmission and plasticity [4] and promotes survival of newborn hippocampal neurons [5]. BDNF has widespread expression in the developing and adult mammalian nervous system, its mRNA and protein levels rising dramatically in postnatal development [6-10]. In the adult, BDNF is also expressed in a number of non-neural tissues, with the highest levels of BDNF mRNA detected in thymus, heart and lung [11,12].

BDNF gene has a complex structure with multiple untranslated 5' exons alternatively spliced to one protein-coding 3' exon. The rat BDNF gene structure initially described to contain five exons [13] has been recently updated with a number of newly discovered exons for rodent [14,15] and human [16,17] BDNF. Untranslated 5' exons are linked with differentially regulated promoters directing tissue-specific expression of BDNF [13-17]. Furthermore, recently discovered BDNF antisense transcripts in human may exert additional control over BDNF transcription [16,17]. BDNF is a neural activity-dependent gene in rodents: various physiological stimuli induce its expression in neurons through excitatory neurotransmission-triggered calcium influx [18,19]. However, no data is available about activity-dependent transcription of the human BDNF gene in neurons, except one report showing that dopamine signaling increases the levels of BDNF exon IV transcripts in neuronally differentiated human embryonic teratocarcinoma NT2 cells [20].

Alterations in BDNF function have been associated with a variety of disorders of the nervous system [2]. As therapies modulating neurotrophic activity are being actively sought [21], it is of great importance to create model systems for studying the regulation of BDNF gene. BAC transgenic mice have proven useful in studying gene regulation as a) BAC clones are often long enough to contain all necessary DNA elements to recapitulate the expression patterns of endogenous genes independent of host genomic sequences flanking the transgene integration site and b) they can be easily modified with homologous recombination in *E. coli*, e.g. to introduce reporter genes under the control of promoters of interest [22]. BAC transgenes with EGFP reporter gene have been used for characterization of expression and regulatory regions of several neural genes [23-25]. Transgenic mice have been generated previously to study BDNF gene regulation *in vivo* [26,27]. Mouse lines carrying rat BDNF sequences of 10 kb range recapitulated BDNF expression only partially, suggesting that *cis*-acting regulatory elements necessary for accurate control of BDNF expression are located further away [26]. Recently, YAC-BDNF transgenic mice carrying 145 kb of human BDNF locus with BDNF coding sequence substituted for the EGFP reporter gene have been reported [27].

In this study we have generated BAC transgenic mice carrying human BDNF-EGFP fusion (hBDNF-EGFP) reporter gene under the control of 168 kb of human BDNF genomic sequences. C-terminal addition of EGFP to BDNF protein has been shown not to affect BDNF cellular localization, secretion and activation of its receptor TrkB in cultured neurons [28-30]. Therefore, to enable studying subcellular localization of the hBDNF-EGFP fusion protein *in vivo*, we specifically produced this fusion reporter gene construct. The aims of the study were to investigate a) expression of hBDNF-EGFP mRNA and protein in the brain and non-neural tissues and b) activity-dependent regulation of the hBDNF-EGFP transgene in the brain of the BAC transgenic mice.

Results

Generation of transgenic mice with 169 kb hBDNF-EGFP-BAC

A 168 kb BAC clone extending 84 kb upstream and 17 kb downstream of human BDNF gene was used to generate human BDNF-EGFP reporter transgenic mice (see Materials and Methods and Figure 1A–C). Briefly, EGFP reporter gene was inserted in-frame with BDNF coding region replacing the BDNF stop codon (Figure 1C). Resulting hBDNF-EGFP fusion protein was expected to mimic subcellular localization of endogenous BDNF, allowing fine resolution of transgene expression. hBDNF-EGFP-BAC construct was tested for integrity using PCR and restriction analysis (data not shown). Transgenic mice were generated by pronuclear injection, yielding four transgenic founders (A4, E1, E4 and C3). All founders contained one to two transgene copies as estimated by slot-blot hybridization (Figure 1D). PCR analysis of C3 genomic DNA and sequencing of the PCR products revealed tandem integration of two transgene copies and confirmed the intactness of 5' and 3' end sequences of the integrated transgene (Figure 1E). Offspring was obtained from three founders and bred for several generations to generate transgenic mouse lines E1, E4 and C3.

Expression of hBDNF-EGFP in transgenic mouse tissues

From three transgenic founder lines, C3 line showed pattern of expression of hBDNF-EGFP mRNAs that was highly similar to the expression of mouse endogenous BDNF (mBDNF) mRNA (Figure 2A). RT-PCR analysis revealed relatively high transgene expression in all brain regions of C3 mice, including cerebral cortex, hippocampus, striatum, thalamus, hypothalamus, midbrain, pons, medulla and cerebellum. In non-neural tissues, high levels of transgene mRNA were detected in testis, moderate levels in thymus and lung and low levels in skeletal muscle. BDNF mRNA is endogenously expressed in all these tissues both in mouse and human [14,16]; (Figure 2A). However, dissimilarly from mouse endogenous BDNF mRNA, hBDNF-EGFP mRNA was not detected in heart and kidney, where relatively high levels of mBDNF mRNA were detected. Low expression of hBDNF-EGFP transgene in the mouse kidney correlates with the finding that BDNF is expressed at low levels in human kidney [8,16].

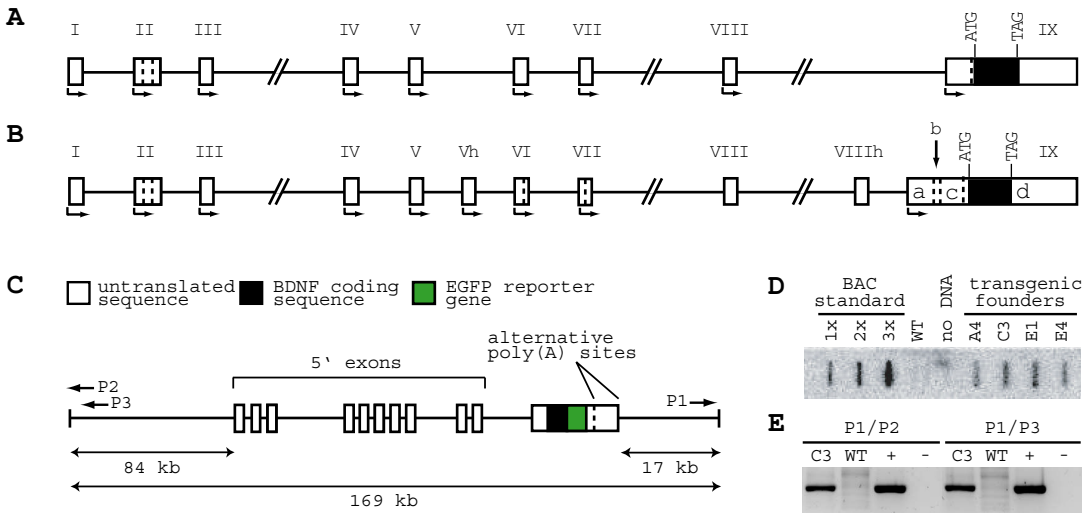

Figure 1

Schematic drawings of rodent and human BDNF genes and the BAC transgenic construct used in this study. Rodent (**A**) and human (**B**) BDNF gene structures. Rodent BDNF gene consists of a number of 5' exons (I-VIII) spliced together with a common protein-coding sequence in exon IX (transcriptional start sites are indicated with arrows). BDNF transcription can also start from exon IX introducing a unique 5' UTR sequence. Hatched lines indicate sites of alternative splicing. Although the human BDNF gene has a similar structure and splicing pattern, it has additional exons Vh and VIIIh, longer and more complexly spliced 5'UTR of exon IX. Furthermore, human BDNF exons VIII and VIIIh are not used as 5'exons, but are always spliced with exon V. For detailed description see [14,16]. (**C**) Schematic drawing of the modified BAC construct used in this study containing the human BDNF locus. EGFP reporter gene was inserted in-frame with the BDNF coding region before the BDNF stop codon creating a fused BDNF-EGFP open reading frame within 168 kb of human BDNF locus. Arrows P1-3 indicate PCR primers used for analysis of transgene integration. (**D**) Slot-blot hybridization analysis of transgene copy number in hBDNF-EGFP transgenic founder mice (A4, C3, E1 and E4). BAC standard contains hBDNF-EGFP-BAC DNA in amounts equivalent to 1–3 copies of transgene in the blotted genomic DNA. WT- wild type mouse DNA. (**E**) PCR analysis of genomic DNA from transgenic mouse line C3 with primers detecting tandem integration of hBDNF-EGFP-BAC constructs. WT – wild type mouse DNA as a negative control; (+) – circular hBDNF-EGFP-BAC DNA as a positive control; (-) – PCR without DNA as a negative control.

In E1 mice, transgene expression recapitulated that of the endogenous BDNF mRNA in thymus, lung, kidney and testis, but not in other non-neural tissues that express BDNF. In the adult brain of E1 mice, transgene mRNA expression was detected in midbrain, cerebellum, pons and medulla at levels that were lower than in the respective brain regions of C3 mice. In E4 line, hBDNF-EGFP mRNA was detected only in testis and thymus (Figure 2A).

Expression of transgenic hBDNF-EGFP mRNA was further examined in different brain regions of C3 mice since this line largely recapitulated endogenous BDNF expression and expressed the transgene at the highest levels. Quantification of hBDNF-EGFP transcripts in C3 hippocampus and cortex using ribonuclease protection assay (RPA) revealed that transgene mRNA levels were about tenfold lower than endogenous mBDNF mRNA levels (Figure 2B). Analysis of transcription from the alternative human BDNF promoters in C3 mice confirmed the expression of all transcripts with different 5' exons described to date (exons I-IXe) both in hippocampus (Figure 2C) and cerebral cortex (data not shown).

In situ hybridization of C3 mice adult brain sections revealed hBDNF-EGFP mRNA expression in the hippocampus, particularly in the pyramidal neurons of CA1 and CA3 regions and in the polymorphic neurons in the hilus of the dentate gyrus, and also in several cortical areas, including neurons of frontal, sensorimotor and piriform cortex (Figure 3, 4). Endogenous mBDNF mRNA was detected in all brain areas where hBDNF-EGFP mRNA labeling was observed. However, hBDNF-EGFP labeling was absent or below the detection limit of our *in situ* hybridization assay in several areas expressing mBDNF mRNA, e.g. claustrum, amygdala, thalamic, hypothalamic and pontine nuclei. Furthermore, *in situ* hybridization

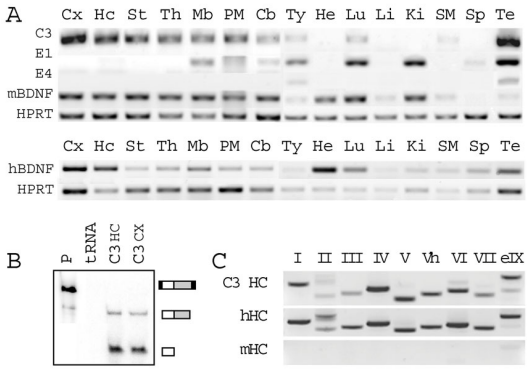

Figure 2
hBDNF-EGFP mRNA expression in tissues of three transgenic mouse lines. (**A**) RT-PCR analysis of hBDNF-EGFP mRNA expression in tissues of three transgenic BAC mouse lines – C3, E1, E4. mBDNF – mouse BDNF; hBDNF – human BDNF in human tissues; HPRT – reference gene hypoxanthine phosphoribosyltransferase. *Cx* – cortex; *Hc* – hippocampus; *St* – striatum; *Th* – thalamus; *Mb* – midbrain; *PM* – pons/medulla; *Cb* – cerebellum; *Ty* – thymus; *He* – heart; *Lu* – lung; *Li* – liver; *Ki* – kidney; *SM* – skeletal muscle; *Sp* – spleen; *Te* – testis. (**B**) Analysis of hBDNF-EGFP mRNA expression levels in C3 mouse brain by RNase protection assay. hBDNF-EGFP probe was used to determine both transgenic and endogenous BDNF mRNA levels as protein coding sequences of mouse and human BDNF share a high degree of similarity. *P* – probe without RNase; *tRNA* – yeast tRNA; *HC* – hippocampus; *CX* – cortex. On the right, black boxes denote vector-derived sequences, white boxes BDNF and gray boxes EGFP sequences. (**C**) Expression of alternative hBDNF-EGFP transcripts in C3 mouse hippocampus (HC), analyzed by RT-PCR. PCR primers used were specific for human BDNF transcripts as shown by control reactions with human (hHC) and mouse (mHC) hippocampal cDNA. eIX – transcript containing 5'-extended exon IX.

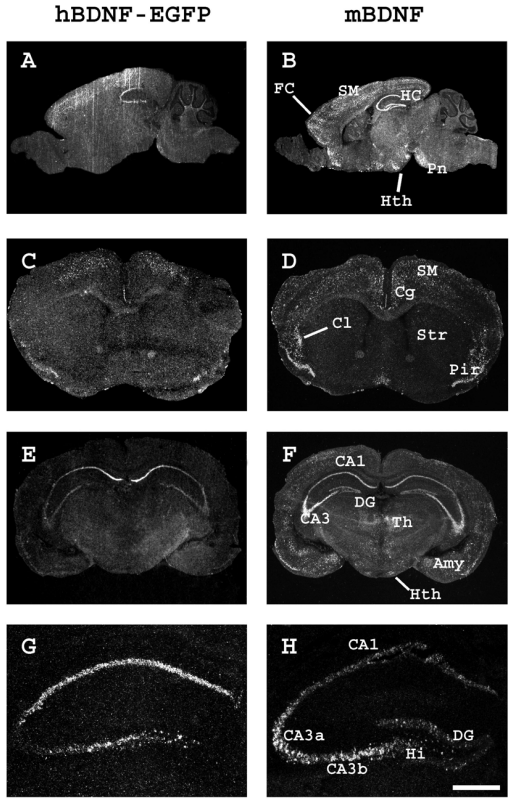

Figure 3
Overlapping patterns of BAC-driven hBDNF-EGFP and mBDNF mRNA expression in C3 mouse brain. *In situ* hybridization analysis, photoemulsion autoradiographs of 16 μm sagittal (**A**,**B**) and coronal (**C**-**H**) sections. (**C**) and (**D**) are sections taken at striatal level; (**E**) and (**F**) are sections taken at posterior hippocampal levels; (**G**) and (**H**) show enlarged hippocampal area (scale bar: 0,5 mm). *FC* – frontal cortex;*SM* – sensorimotor cortex; *HC* – hippocampus; *Pn* – pontine nuclei; *Hth* – hypothalamus; *Cg* – cingulate cortex; *Pir* – piriform cortex; *Cl* – claustrum; *Str* – striatum; *CA1*, *CA3* – hippocampal subfields; *DG* – dentate gyrus of hippocampus; *Hi* – hilar area of dentate gyrus; *Th* – thalamus; *Amy* – amygdala.

showed differential expression of hBDNF and mBDNF in cortical and hippocampal subfields. While mBDNF mRNA was expressed at high levels throughout the cerebral cortex, hBDNF-EGFP labeling was more prominent in the frontal cortex and in the sensorimotor area extending along the longitudinal fissure (Figure 3C, D and Figure 4K–N). In the hippocampus, hBDNF-EGFP labeling was observed over the CA1 and hilar subfields and part of the CA3 subfield (CA3b in Figure 3G, H and Figure 4C, D), mimicking the pattern of expression of endogenous mBDNF mRNA. On the other hand, hBDNF-EGFP mRNA was expressed at considerably lower levels in the part of CA3 subfield that showed high levels of mBDNF mRNA expression (CA3a in Figure 3G, H and Figure 4E, F). In addition, no hBDNF-EGFP labeling was detected in the granule neurons of dentate gyrus where endogenous mBDNF mRNA was highly expressed (Figure 3G, H and Figure 4I, J).

Since the BDNF gene in the transgenic construct was of human origin, we also analyzed the expression of BDNF in the human hippocampus using *in situ* hybridization. In agreement with earlier findings [31,32], our results showed that the highest levels of hBDNF mRNA were

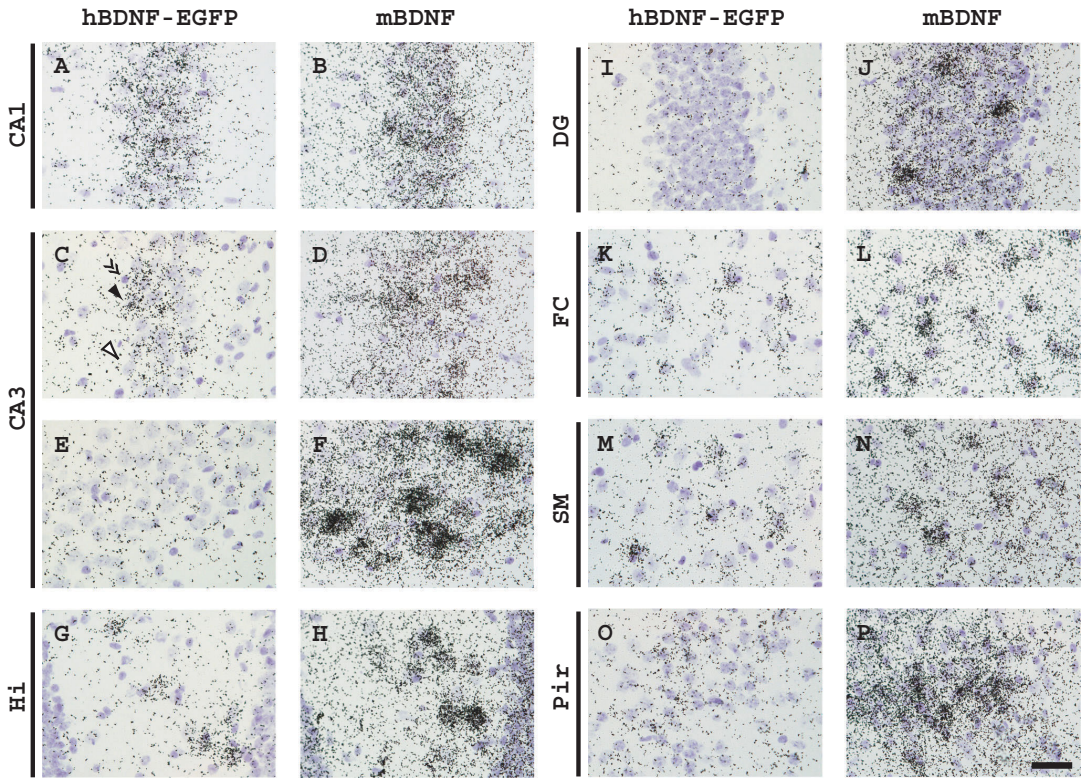

Figure 4

Cellular expression of hBDNF-EGFP mRNA in adult C3 mouse brain. *In situ* hybridization analysis, shown are bright-field autoradiographs of emulsion-dipped sections. Hybridization probes are indicated above the columns. Filled arrowhead indicates a neuron with strong labeling, empty arrowhead indicates a neuron with weak or absent labeling and double arrowheads indicate a glial cell showing no labeling. *CA1*, *CA3* – hippocampal subfields; *DG* – dentate gyrus of hippocampus; *Hi* – hilar area of dentate gyrus; *FC* – frontal cortex; *SM* – sensorimotor cortex; *Pir* – piriform cortex. Scale bar: 20 μm.

present in the granule cells of dentate gyrus, whereas other hippocampal regions showed relatively weaker expression (Figure 5). However, strong hBDNF labeling was detected over majority of CA3 and CA1 neurons using high magnification (Figure 5B, C), indicating that these areas show much weaker signal in the dark-field image partly because of the scarcity of neuronal cell bodies in the CA1 and CA3 subfields of the human hippocampus.

Next we examined expression of hBDNF-EGFP fusion protein across tissues in C3 mice. No EGFP fluorescence was observed in brain sections or cultured primary embryonic (E18) hippocampal neurons. In addition, hBDNF-EGFP protein was not detected in the hippocampus, cortex and testis by Western blot analysis with anti-EGFP or anti-BDNF antibodies (data not shown). hBDNF-EGFP open reading frame in C3 genomic DNA was analyzed for possible mutations by sequencing and was found to be intact. Together with mRNA expression data these results suggest that hBDNF-EGFP protein was either not translated in the brain and testis of C3 mice or was expressed at levels below the detection limits of our methods.

Kainic acid induces hBDNF-EGFP mRNA expression in transgenic mouse brain

Kainic acid (KA), agonist of the KA subtype ionotropic glutamate receptor, has been shown to induce BDNF mRNA levels in adult rodent hippocampus and cerebral cortex [13,19,33,34]. KA induction of transgenic hBDNF-EGFP transcripts in the hippocampus and cerebral cortex of C3 mice largely followed the induction pattern of endogenous mBDNF transcripts (Figure 6A). KA markedly

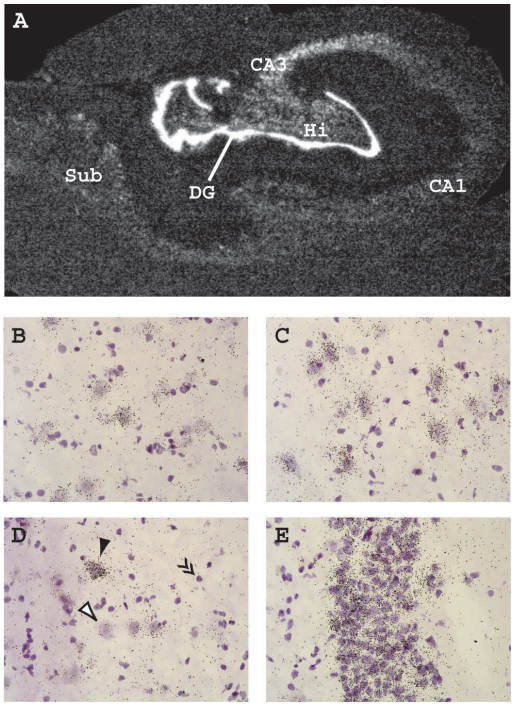

Figure 5
Expression of BDNF mRNA in the human hippocampus. (**A**) *In situ* hybridization autoradiograph of a 16 μm coronal section.*DG* – granular layer of dentate gyrus; *Hi* – hilar area of dentate gyrus; *Sub* – subiculum; *CA1, CA3* – hippocampal subfields. (**B-E**) High magnification bright-field photomicrographs of hematoxylin-counterstained neurons in subfields CA1 (**B**) and CA3 (**C**), the hilus (**D**) and granular layer of dentate gyrus (**E**). Filled arrowhead indicates a neuron with strong labeling, empty arrowhead indicates a neuron with weak or absent labeling and double arrowheads indicate a glial cell showing no labeling.

upregulated both endogenous mouse and transgenic hBDNF-EGFP transcripts containing exons I, IV and 5'-extended exon IX (eIX) in the hippocampus and cortex. hBDNF-EGFP and mBDNF mRNAs containing other 5' exons were induced to a lesser extent. Of note, recently described human-specific exon Vh-containing transcripts were not induced by KA in transgenic mice in the context of 169 kb hBDNF-EGFP BAC construct (Figure 6A).

Levels of BDNF transcripts showing the most robust induction by kainic acid were analyzed further using quantitative real-time RT-PCR analysis (Figure 6B). Transgenic hBDNF-EGFP exon I, exon IV and 5'-extended exon

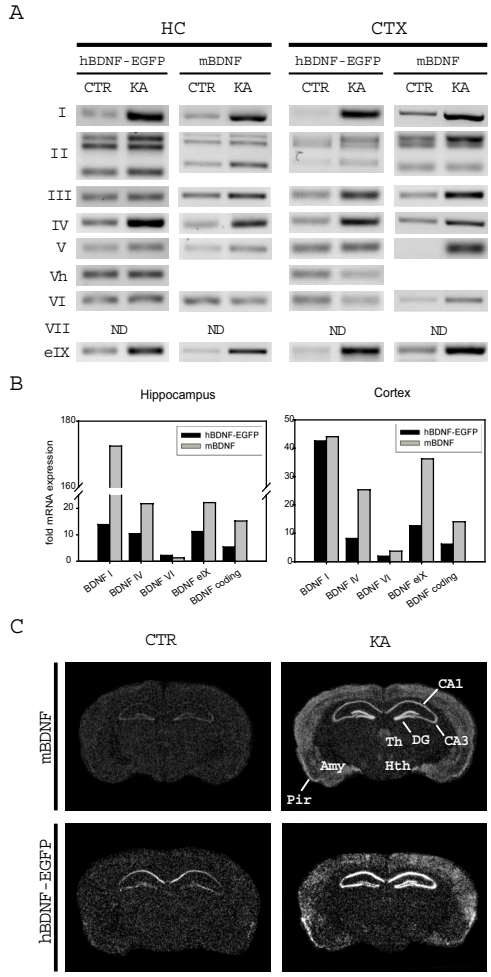

Figure 6
Kainic acid (30 mg/kg) induces transgenic hBDNF-EGFP mRNA expression in brains of C3 line transgenic mice. (**A**) Induction of alternatively spliced hBDNF-EGFP transcripts in C3 mouse hippocampus (HC) and cerebral cortex (CTX), analyzed with RT-PCR. mBDNF – mouse transcripts; ND – not determined; KA – kainic acid treated mice; CTR – control mice. Three BDNF-II bands correspond to alternatively spliced transcripts. (**B**) Quantitative real-time RT-PCR analysis of selected BDNF transcripts, normalized to HPRT1 levels and expressed as fold difference relative to mRNA levels in untreated mice. (**C**) *In situ* hybridization autoradiographs of C3 mouse coronal brain sections. *Pir* – piriform cortex; *CA1, CA3* – hippocampal subfields; *DG* – dentate gyrus of hippocampus; *Hi* – hilar area of dentate gyrus; *Th* – thalamus; *Hth* – hypothalamus; *Amy* – amygdala.

IX transcripts, and total hBDNF-EGFP mRNA were potently induced in both hippocampus and cortex following 3 hours of kainate treatment, similarly to respective endogenous mBDNF mRNAs. Exon VI-containing hBDNF-EGFP and endogenous mBDNF transcripts showed no induction, which is consistent with previous findings [13,14,33].

In situ hybidization analysis showed marked induction of transgenic hBDNF-EGFP mRNA by KA in the pyramidal neurons of CA1-CA3 layers, in the hilar region of hippocampus and also in the layers II – VI of cerebral cortex (Figure 6C). Importantly, kainic acid induced transgene expression also in the granular layer of dentate gyrus of hippocampus, whereas control animals did not show any detectable expression in this area. Endogenous mBDNF was induced in the same neuronal populations, suggesting that the 169 kb hBDNF-EGFP BAC construct contains all the regulatory elements that mediate kainic acid induction. We also examined expression of the hBDNF-EGFP protein in the brains of kainic acid treated C3 mice by direct EGFP fluorescence and Western blot analysis but no fusion protein was detected (data not shown).

Discussion

In this study, BAC transgenic mice carrying 168 kb of the human BDNF locus and encoding human BDNF-EGFP fusion protein were generated and analyzed. Out of three analyzed founder lines, one line (C3) largely recapitulated human BDNF mRNA expression in the brain, thymus, lung, skeletal muscle and testis. Founder line E1 mimicked human BDNF mRNA expression in some brain regions, and also in thymus, lung and kidney. Founder line E4 expressed transgene only in the thymus and testis. These results showed that although all three founder lines expressed hBDNF-EGFP mRNA at different levels, the 169 kb BAC construct, carrying 67 kb of human BDNF gene, 84 kb of 5' and 17 kb of 3' sequences, contains regulatory elements necessary for hBDNF mRNA expression in many brain regions and non-neural tissues. However, integration site-dependent expression of transgene in different founder lines suggests that the BAC construct may not contain necessary insulator elements to protect it from the influence of genomic regions flanking the transgene integration site. It has been shown for many genes that insulators can functionally isolate neighboring genes and block their interactions [35].

In several non-neural tissues, the 169 kb hBDNF-EGFP BAC recapitulated endogenous expression of both mouse and human BDNF. Transgenic mRNA was expressed in the thymus and testis in three mouse lines, expression in the lung was seen in two lines and only one line expressed hBDNF-EGFP in the kidney and skeletal muscle. All these tissues have been shown to express BDNF both in mouse and human [7,14,16]. Of note, all three founder lines expressed relatively high levels of hBDNF-EGFP in adult testis, in contrast to the very low expression levels of endogenous mBDNF in the testis. This transgene expression pattern can be explained by human origin of the BDNF gene as relatively high levels of BDNF mRNA, comparable to the levels in the brain, have been detected in the human testis [16]. In the adult human testis, expression of BDNF and its receptor TrkB has been reported in Leydig, Sertoli and germ cells [36], while in the adult mouse testis, BDNF expression has been detected in Sertoli cells and expression of its receptor TrkB in germ cells [37]. These findings indicate differences in BDNF expression between human and mouse and are further supported by the present study. On the other hand, none of the founder lines expressed hBDNF mRNA in the heart, a tissue with high levels of BDNF expression both in human and rodents [8,11,12,14]. This suggests that distinct heart-specific regulatory elements are located outside of the genomic DNA fragment that was included in the BAC construct.

Detailed analysis of hBDNF-EGFP expression in the C3 mouse brain by *in situ* hybridization showed that the transgene mimicked mBDNF expression in many neuron populations, including neurons of the CA1-CA3 and hilar regions of the hippocampus and the cerebral cortex. However, hBDNF-EGFP failed to recapitulate endogenous BDNF expression in several neuron populations, including the granule cells of dentate gyrus of hippocampus where BDNF mRNA is expressed both in human and rodents. hBDNF-EGFP expression was detected in all analyzed brain regions by RT-PCR, but not by *in situ* hybridization, indicating that transgene mRNA levels in several brain structures were below the detection limit of our *in situ* hybridization analysis.

BDNF transcription is regulated by neuronal activity through calcium-mediated pathways [18,38]. Systemic treatment of rodents with kainic acid (KA) has been used to model activity-dependent induction of BDNF mRNA in the nervous system [13,19,33,34]. Here we show that KA differentially induced alternative hBDNF-EGFP transcripts in the cortex and hippocampus (for comparison with mouse and rat see Table 1). Pronounced induction of transgenic hBDNF-EGFP transcripts containing exons I, IV, and 5'-extended exon IX (eIX), moderate induction of transcripts containing exons II, III and absence of induction of transcripts containing exon VI is consistent with the induction pattern of respective BDNF mRNAs in mouse and rat [13,14,33]. To our knowledge, this is the first time to report neural activity-dependent regulation of the human BDNF gene *in vivo*. Real-time PCR showed that total transgenic mRNA, as well as transcripts containing exons I, IV and 5'-extended exon IX were induced to a

lesser extent than the respective endogenous mBDNF mRNAs. This is consistent with earlier results reported for shorter rat BDNF transgenes [26] and could be caused by increased stability of transgenic BDNF-reporter mRNAs as compared to the mouse endogenous BDNF mRNAs. Alternatively, the absence of important regulatory elements in the transgenic construct may underlie the reduced induction of the transgene by kainic acid. *In situ* hybridization analysis of KA-treated C3 mouse brains showed induction of hBDNF-EGFP mRNAs in several neuronal populations where endogenous BDNF mRNA levels were also increased. These results show that, similarly to rodent BDNF, expression of the human BDNF gene is induced by neural activity and that regulatory elements mediating the induction are included in the 168 kb of the human BDNF locus contained in the BAC transgene. Several regulatory elements located in the rat BDNF proximal promoter IV and the transcription factors mediating activity-dependent activation of this promoter have previously been characterized [39]. Among these elements, CRE (cAMP-response element) was found to be the most important for Ca^{2+}-mediated activation of rodent BDNF promoter IV [40-42]. However, the respective regulatory elements and transcription factors responsible for the activity-dependent regulation of the human BDNF gene have not been characterized. Transgenic mice described here can be used to study the regulation of human BDNF gene *in vivo* using a variety of methods successfully applied in the studies of rodent BDNF [39].

Table 1: Regulation of human, mouse and rat BDNF exon-specific mRNAs by kainic acid in the hippocampus and cerebral cortex.

	human[1]		mouse[2]		rat[3]
exon	HC	CTX	HC	CTX	HC
I	**	**	**	**	**
II	*	*	*	*	*
III	*-	*	*	*	-
IV	**	**	**	**	**
V	*	*	*	**	**
Vh	-	-	X	X	X
VI	-	-	-	-	-
VII	ND	ND	ND	ND	**
VIII	X	X	ND	ND	*
eIX	**	**	**	**	**

- no induction; * weak induction; ** strong induction; ND – not determined; X – transcript containing this exon as the 5' exon does not exist in this organism; [1,2] based on data from the present study; [3] based on data from [14]; HC – hippocampus; CTX – cerebral cortex.

Previously, transgenic mice carrying shorter fragments of the BDNF locus have been generated and characterized [26,27]. Mice expressing the CAT reporter gene under the control of 9 kb of rat BDNF genomic sequences covering promoters I-III or promoters IV-VI showed relatively high CAT activity in most tissues and brain regions expressing endogenous BDNF mRNA. *In situ* hybridization analysis showed that these constructs carrying either BDNF promoters I-III or IV-VI were able to drive CAT mRNA expression in adult rat brain in a pattern largely overlapping with mouse BDNF mRNA expression. Nevertheless, recapitulation of endogenous BDNF expression had a number of shortcomings in these transgenes: both constructs were not expressed or were expressed at low levels in the dentate granule cells and granule cells of cerebellum; BDNF IV-VI did not mimic BDNF expression in the heart; both constructs displayed relatively high reporter activity in the striatum where rat BDNF is virtually not expressed [43]. It was assumed that these transgenic constructs lacked important regulatory elements, which could be present in a much longer gene fragment than the BAC clone used here. Although BAC transgenic mouse lines generated in this study showed improved recapitulation of expression as compared to that of the BDNF-CAT transgenic mice [26], we could not detect transgene expression in several tissues and neuron populations that express endogenous BDNF mRNA.

A recent study reported generation of human BDNF-EGFP transgenic mice using a 145 kb YAC clone including 45 kb of 5' and 33 kb of 3' flanking sequences of hBDNF gene with the protein coding sequence partially replaced with EGFP reporter gene [27]. Three out of five transgenic founder lines obtained in that study expressed transgenic mRNA in the brain and only one of these showed expression of transgenic hBDNF transcripts containing exons IV and VI in the heart. Out of three lines analyzed, EGFP fluorescence was detected in the brain of only one line, specifically in the claustrum, intermediate layer of parietal cortex, pyramidal cell layer of CA3 hippocampal subfield and a population of neurons in the granule cell layer of the dentate gyrus. However, EGFP fluorescence was not detected in other cortical neuron populations and in the CA1 region of hippocampus where rodent and also human BDNF mRNA are expressed [27]. Differences in the tissue- and neuron-specific expression of transgenic hBDNF-EGFP mRNA and protein between the study by Guillemot et al. [27] and this study can be explained with different lengths of the BDNF gene-flanking genomic regions in the transgenic constructs used: the hBDNF-BAC

Table 2: PCR primers used in this study

Primer/application	Sequence
BAC modification	
hBDNFcod_rpsL_neo_s	5' GGATAGACACTTCTTGTGTATGTACATTGACCATTAAA AGGGGAAGATAGGGCCTGGTGATGATGGCGGGATCG 3'
hBDNF_rpsL_neo_as	5'AATAGATAATTTTTGTCTCAATATAATCTAATCTATACAACATAAATCCATCAGAAGAACTCGTCAA GAAGG 3'
hBDNFcod_linker_EGFP_s	5' TAAGGATAGACACTTCTTGTGTATGTACATTGACCAT TAAAAGGGGAAGACGGGATCCACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAGCTG 3'
hBDNF_EGFP_as	5' AATAGATAATTTTTGTCTCAATATAATCTAATCTATAC AACATAAATCCATTACTTGTACAGCTCGTCCATGCCGA 3'
genotyping/slot-blot hybridization/expression analysis	
hBDNF_s	GTACGTGCGGGCCCTTACCATGGATAGC
EGFP_as	TGGTGCAGATGAACTTCAGGGTCAGC
expression analysis	
mBDNF_s	GTATGTTCGGGCCCTTACTATGGATAGC
mBDNF_as	AAGTTGTGCGCAAATGACTGTTTC
HPRT1_s	CTTTGCTGACCTGCTGGATTAC
HPRT1_as	GTCCTTTTCACCAGCAAGCTTG
hBDNF_I_s	GATGCCAGTTGCTTTGTCTTCTGTAG
hBDNF_II_s	GGGCGATAGGAGTCCATTCAGCACC
hBDNF_III_s	AGTTTCGGGCGCTGGCTTAGAG
hBDNF_IV_s	GCTGCAGAACAGAAGGAGTACA
hBDNF_V_s	TCGCGTTCGCAAGCTCCGTAGTG
hBDNF_Vh_s	GGCTGGAACACCCCTCGAA
hBDNF_VI_s	GGCTTTAATGAGACACCCACCGC
hBDNF_VII_s	GAACTGAAAGGGTCTGCGACACTCT
hBDNF_IXb_s	GCTGCTAAAGTGGGAAGAAGG
hBDNF_IX_as1	GTCCTCATCCAACAGCTCTTCTATC
hBDNF_IX_as2 (with VII_s)	GAAGTGTACAAGTCCGCGTCCTTA
expression analyis (qPCR)	
EGFPq_s	CAGAAGAACGGCATCAAGGTG

Table 2: PCR primers used in this study *(Continued)*

EGFPq_as	TGGGTGCTCAGGTAGTGGTTG
hBDNFq_I_s	CAGCATCTGTTGGGGAGACGAGA
hBDNFq_IV_s	GAAGTCTTTCCCGGAGCAGCT
hBDNFq_VI_s	ATCGGAACCACGATGTGACT
hBDNFq_IXc_s	AACCTTGACCCTGCAGAATGGCCT
hBDNFq_IX_as1 (with I, IV_s)	ATGGGGGCAGCCTTCATGCA
hBDNFq_IX_as2 (with VI_s)	ACCTTGTCCTCGGATGTTTG
hBDNFq_IX_as3 (with IXc_s)	GATGGTCATCACTCTTCTCACCT
mBDNFq_I_s	TTGAAGCTTTGCGGATATTGCG
mBDNFq_IV_s	GAAATATATAGTAAGAGTCTAGAACCTTG
mBDNFq_VI_s	GCTTTGTGTGGACCCTGAGTTC
mBDNFq_IXa_s	GGACTATGCTGCTGACTTGAAAGGA
mBDNFq_IX_as1 (with I, IV, VIs)	AAGTTGCCTTGTCCGTGGAC
mBDNFq_IX_as2 (with IXa_s)	GAGTAAACGGTTTCTAAGCAAGTG
mBDNFq_coding_s	GGCCCAACGAAGAAAACCAT
mBDNFq_coding_s	AGCATCACCCGGGAAGTGT
HPRT1q_s	CAGTCCCAGCGTCGTGATTA
HPRT1q_as	AGCAAGTCTTTCAGTCCTGTC
transgene integrity	
pBACe3.6_SP6 (5'end)	TATTTAGGTGACACTATAG
rp11_5'_as (5'end)	GGACAACAGACCCAAGGAGA
rp11_3'_s (3'end)	GTAGGGTGTCTGGGTTGGTG
pBACe3.6_T7 (3'end)	TAATACGACTCACTATAGGG
transgene tandem integration	
rp11_3'_s (P1)	GTAGGGTGTCTGGGTTGGTG
pBACe_11326_s (P2)	CGGTTACGGTTGAGTAATAAATGGATG
pBACe_11365_s (P3)	GGGGCACATTTCATTACCTCTTTCTC

used in the present study contained 39 kb longer 5' and 16 kb shorter 3' genomic regions of hBDNF gene than the reported hBDNF-YAC construct [27]. In addition, part of BDNF coding sequence had been replaced with EGFP reporter gene in the hBDNF-YAC transgene [27], possibly removing *cis*-elements with regulatory function. In contrast to the present study, hBDNF-YAC transgenic mRNA expression was not analyzed in different brain regions and expression of transgenic mRNAs containing exons III, V, Vh, VII and 5'-extended exon IX was not analyzed. More detailed comparison of hBDNF-EGFP expression in the two hBDNF transgenic mouse models would allow narrowing down genomic regions containing enhancer elements for tissue-specific expression of human BDNF. For example, on the basis of current data it can be hypothesized that a *cis*-element promoting heart-specific expression of hBDNF mRNA is located within the 3' terminal 16 kb of hBDNF-YAC construct (17–33 kb downstream of the hBDNF gene; chr11:27,600,000–27,616,000; UCSC Genome Browser, Mar 2006 Assembly). Recently, a BDNF regulatory locus has been discovered 850 kb upstream of the human and mouse BDNF genes that causes obesity, cognitive impairment and hyperactivity when disrupted [44,45]. Therefore, it is possible that in addition to regulatory elements included in the hBDNF-BAC of this study and the hBDNF-YAC described before [27], others can be found hundreds of kilobases away from the BDNF gene.

EGFP reporter gene has been successfully used to visualize BAC-driven expression of neural genes in a number of studies [23-25]. In the BAC construct that was used to generate transgenic mice in the present study, EGFP reporter gene was fused C-terminally with the human BDNF coding sequence to allow detailed characterization of human BDNF expression in the nervous system. Unfortunately, we could not detect EGFP protein in the brain of C3 mice neither with fluorescence microscopy nor with Western blot analysis. This could be explained with low levels of hBDNF-EGFP protein expressed in the C3 mouse brain as transgenic hBDNF-EGFP mRNA levels were about tenfold lower than these of endogenous BDNF. It is also possible that founder mice with higher levels of BDNF-EGFP expression died during embryonic development due to overactivation of BDNF receptor TrkB. This hypothesis is supported by a study showing that embryonic overexpression of BDNF from nestin promoter results in gross abnormalities in brain architecture and perinatal death [46]. Although the hBDNF-EGFP fusion protein can be expressed in cultured cells *in vitro* [28-30], it is conceivable that it is not translated or has poor translatability and/or stability when expressed in transgenic mice *in vivo*.

Conclusion

Human genomic region covering 67 kb of the BDNF gene, 84 kb of upstream and 17 kb of downstream sequences is able to drive tissue-specific and kainic acid-induced expression of reporter gene in transgenic mice that largely overlaps with BDNF gene expression and regulation in mouse and human. This is the first study to directly show that human BDNF gene is regulated by neural activity. The BDNF-BAC transgenic mice are useful for studying the transcription regulation of human BDNF gene *in vivo*. In addition, these mice could be used for screening therapeutic agents modulating human BDNF transcription.

Methods

Generation of transgenic mice

BAC clone (RP11-651M4) containing the human BDNF locus [GenBank:AC087446.13] was purchased from Chori BACPAC Resources (USA). Red®/ET® homologous recombination in *E. coli* (Counter-Selection BAC Modification Kit, Gene Bridges GmbH, Germany) was used to delete BDNF stop codon and to insert EGFP reporter gene with the linker sequence (CGG GAT CCA CCG GTC GCC ACC) into the 3' end of BDNF. For sequences of primers used for insert synthesis see Table 2. Modified BAC was tested for the absence of rearrangements using EcoRV restriction analysis and pulsed field gel electrophoresis. Integrity of the hBDNF-EGFP reading frame was confirmed by sequencing. In order to validate the reporter activity, BAC DNA was purified using the Large Construct Purification Kit (Qiagen, USA) and transfected into COS-7 cells using DEAE-dextran mediated transfection system [47]. Five days after transfection EGFP expression and distribution in COS-7 cells was visualized using fluorescence microscopy (Eclipse 80i upright microscope, Nikon).

hEGFP-BDNF BAC DNA was purified for microinjection by alkaline lysis and linearized with PI-SceI enzyme (NEB, USA). Restriction solution was separated in low-melt agarose gel (Fermentas, Lithuania) using CHEF-DR II Pulsed Field Electrophoresis System (Bio-Rad, USA). Linearized BAC DNA was excised from the gel and purified from agarose using Gelase enzyme (NEB, USA). Transgenic mice were generated by pronuclear injection of linearized hBDNF-EGFP-BAC into CBA × C57Bl/6 mouse pronuclei in the Karolinska Center for Transgene Technologies (Sweden). Founder mice carrying the BAC transgene were identified by PCR analysis of genomic DNA. Transgene copy number was analyzed by slot-blot hybridization of genomic DNA with a [α-^{32}P]dCTP-labeled probe generated with HexaLabel DNA Labeling Kit (Fermentas, Lithuania) using pEGFP-N1 (Clontech, USA) plasmid as a template. Genomic DNA of the C3 mouse founder line was analyzed by PCR for the presence of 5' and 3' ends of the linearized transgene. Tandem insertion of transgene into the C3 line genomic DNA was analyzed by PCR with primers pBACe_11326_s or pBACe_11365_s in combination with rp11_3'_s (see Table 2) and sequencing of the PCR product. All animal experiments were performed in

agreement with the local Ethical Committee of Animal Experimentation.

Cell culture, antibodies and animal experiments

African green monkey kidney fibroblast COS-7 cells were grown in DMEM with 10% fetal calf serum and antibiotics. Primary neuronal cultures from embryonic day 18 cerebral cortex were prepared as described [48]. For Western blots and immunohistochemistry the following antibodies were used: mouse anti-GFP monoclonal antibodies (Roche Applied Science), mouse anti-GFP monoclonal antibodies (Clontech, USA); rabbit anti-BDNF (Santa Cruz Biotechnology, USA). For kainic acid treatment, adult mice weighing 20–25 g were injected intraperitoneally with 30 mg/kg of kainic acid or 1× PBS. 3 hours later mice were decapitated, hippocampus and cortex dissected, frozen on dry ice and stored at -70°C. For *in situ* hybridization whole brains were embedded in Shandon Cryomatrix™ (Thermo Fisher Scientific, USA). Four kainic acid-treated C3 mice and two control mice were used for quantitative RT-PCR analysis of total hBDNF-EGFP mRNA expression in the cerebral cortex and hippocampus. Total hBDNF-EGFP mRNA was induced 2,5–6 fold in the hippocampus of kainic acid-treated C3 mice and the mouse displaying highest induction of hBDNF-EGFP and mBDNF mRNA was analyzed further with RT-PCR for expression of exon-specific transcripts. Five kainic acid-treated C3 mice and two control mice were used for *in situ* hybridization analysis and the mouse showing highest induction of hBDNF-EGFP and mBDNF mRNA was further analyzed in more detail.

RT-PCR

Total RNA was isolated from mouse and human tissues using TRI reagent (Ambion, USA). All experiments with human tissues were approved by the local Ethical Committee for Medical Research. Two mice from each transgenic line were analyzed for tissue-specific expression of hBDNF-EGFP mRNA in brain regions and non-neural tissues and they showed identical transgene expression pattern. RNA was treated with DNase (DNA-*free*, Ambion, USA) following manufacturer's instructions and five micrograms of total RNA was used for cDNA synthesis with oligo-dT primer (Microsynth, Switzerland) and SuperScript III reverse transcriptase (Invitrogen, USA). PCR amplification was carried out with HotFire DNA polymerase (Solis Biodyne, Estonia) according to the manufacturer's instructions. Quantitative real-time PCR was performed on a LightCycler 2.0 instrument (Roche Applied Science) using qPCR Core kit for SYBR® Green I No ROX (Eurogentec, Belgium). Melting curve analysis was carried out at the end of cycling to confirm amplification of a single PCR product. All qPCR reactions were performed in triplicate and normalized to hypoxanthin phosphoribosyltransferase 1 (HPRT1) mRNA levels.

Ribonuclease protection assay

For cRNA synthesis 624 bp BDNF-EGFP fragment containing 452 bp of BDNF, 21 bp linker sequence and 151 bp of EGFP sequence was amplified with PCR from modified BAC clone RP11-651M4 and cloned into pBluescript SK+ vector (Stratagene, USA). [α-^{32}P]UTP-labeled cRNA probe was *in vitro* transcribed from linearized plasmid template using MAXIscript Kit and T3 polymerase (Ambion, USA). 10 μg of total RNA and 2.5 ×10^5 CPM of radiolabeled probe were used for RPA hybridization and the assay was performed with the RPA III Kit from Ambion as suggested by the manufacturer. The protected fragments were separated in 4% acrylamid-urea gel and detected autoradiographically using BioRad Molecular Imager FX.

In situ hybridization

cRNA probe complementary to the coding region was used to mouse BDNF mRNA and probe complementary to EGFP was used to detect hBDNF mRNA. Probes were synthesized from DNA fragments subcloned into pCR4-TOPO vector (Invitrogen, USA). [α-^{35}S]UTP-labeled probes were generated with MAXIScript In Vitro Transcription Kit (Ambion, USA) using linearized DNA template and T3 or T7 RNA polymerase. 16 μm sections of fresh-frozen C3 mouse brain were processed according to the protocol described in [13]. Slides were exposed to either BioMax MR X-ray film for one week or NTB-2 photoemulsion for 2 months, developed with D19 developer and fixed with a general-purpose fixer (all from Eastman Kodak, USA). Slides exposed to NTB-2 were counterstained with hematoxylin (Vector Laboratories Inc., USA).

Authors' contributions

IK bred and analyzed the transgenic mice, performed in situ hybridization and RT-PCR analysis. TAP prepared the BAC-BDNF-EGFP construct, carried out transfection experiments and initial characterization of the transgenic mice. KJ performed transgene integration analysis, RT-PCR experiments and contributed to the breeding of founder lines. MS performed RNase protection assay, Western blot analysis and fluorescence microscopy. PP contributed to the initial characterization of the transgenic mice, cultured embryonic neurons and performed in situ hybridization analysis of BDNF mRNA expression in human hippocampus. KP conceived and coordinated the preparation of the transgenic construct. TT conceived and coordinated the study. IK and TT co-wrote the manuscript, all authors contributed to the analysis of the results and preparation of the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank Epp Väli for technical assistance and Enn Jõeste and Liina Kiho from North Estonian Regional Hospital, Tallinn, for collaboration. This work was supported by the following grants to TT: Wellcome Trust Inter-

national Senior Research Fellowship (Grant 067952), Estonian Ministry of Education and Research (Grant 0140143), Estonian Enterprise (Grant EU27553) and Estonian Science Foundation (Grant 7257).

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PUBLICATION IV

BAC transgenic mice reveal distal cis-regulatory elements governing BDNF gene expression.

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Genesis (2010), **48**, 214-219

genesis 48:214–219 (2010)

LETTER

BAC Transgenic Mice Reveal Distal Cis-Regulatory Elements Governing BDNF Gene Expression

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Received 7 July 2009; Revised 10 December 2009; Accepted 21 December 2009

Summary: Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family of neurotrophic factors, has important functions in the peripheral and central nervous system of vertebrates. We have generated bacterial artificial chromosome (BAC) transgenic mice harboring 207 kb of the rat *BDNF* (*rBDNF*) locus containing the gene, 13 kb of genomic sequences upstream of *BDNF* exon I, and 144 kb downstream of protein encoding exon IX, in which protein coding region was replaced with the *lacZ* reporter gene. This *BDNF*-BAC drove transgene expression in the brain, heart, and lung, recapitulating endogenous *BDNF* expression to a larger extent than shorter rat *BDNF* transgenes employed previously. Moreover, kainic acid induced the expression of the transgenic *BDNF* mRNA in the cerebral cortex and hippocampus through preferential activation of promoters I and IV, thus recapitulating neuronal activity-dependent transcription of the endogenous *BDNF* gene. genesis 48:214–219, 2010. © 2010 Wiley-Liss, Inc.

Key words: neurotrophin; transcription; promoter; BAC; transgenic mouse; kainic acid

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family of proteins, supports the survival and differentiation of certain neuronal populations during development (Bibel and Barde, 2000; Binder and Scharfman, 2004). In the adult, BDNF regulates long-term potentiation of synapses, thus playing a key role in long-term memory formation (Lu *et al*., 2008). BDNF was originally isolated from the brain, but it is also expressed in the peripheral nervous system and non-neural tissues (Binder and Scharfman, 2004). Changes in *BDNF* gene expression accompany and contribute to the development of various disorders of the nervous system (Bibel and Barde, 2000).

The *BDNF* gene contains multiple promoters that initiate the transcription of a number of distinct mRNAs, each of which contains an alternative 5′ untranslated exon spliced to a common 3′ protein coding exon. In addition, the protein coding exon employs two different polyadenylation sites that give rise to mRNA species with 3′ untranslated regions (UTRs) of different lengths. Alternative promoter usage, differential splicing, and the use of two different polyadenylation sites within each of the transcription units generate at least 22 different *BDNF* mRNAs in rodents and 34 *BDNF* mRNAs in human that encode the same mature BDNF protein (Aid *et al*., 2007; Pruunsild *et al*., 2007). It has been shown that the subcellular localization of *BDNF* mRNAs and its regulation by neuronal activity depends on the 5′ exon and 3′ UTRs used in the transcript (An *et al*., 2008; Chiaruttini *et al*., 2008). In addition, it has been shown that *BDNF* mRNAs containing the short 3′ UTRs are more enriched in polysomal fraction isolated from total brain than *BDNF* mRNAs with the long 3′ UTRs suggesting that they are more efficiently translated (Timmusk *et al*., 1994). Numerous regulatory elements involved in the regulation of *BDNF* expression in vitro and in vivo have been identified and characterized in different *BDNF* promoters. Transcription factors such as REST (Timmusk *et al*., 1999; Zuccato *et al*., 2003), CREB (Shieh *et al*., 1998; Tao *et al*., 1998), NFkB (Lipsky *et al*., 2001), MEF2 (Flavell *et al*., 2008), NPAS4 (Lin *et al*., 2008), bHLHB2 (Jiang *et al*., 2008), and MeCP2 (Chen *et al*., 2003; Martinowich *et al*., 2003) have been shown to regulate *BDNF* expression in a promoter-specific manner. However, the genomic regions including all necessary *cis*-acting elements responsible for the tissue-specific and activity-dependent *BDNF* gene regulation in vivo remain poorly characterized. A few studies have addressed these issues using transgenic mouse models (Funakoshi *et al*., 1998; Guillemot *et al*., 2007; Koppel *et al*., 2009; Timmusk *et al*., 1995, 1999).

In the present study, we have generated a transgenic mouse line using a bacterial artificial chromosome (BAC) clone containing 207 kb of rat *BDNF* (*rBDNF*) locus,

Indrek Koppel and Tamara Aid-Pavlidis contributed equally to this work.

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Contract grant sponsor: Wellcome Trust International Senior Research Fellowship, Contract grant number: 067952, Contract grant sponsor: Estonian Ministry of Education and Research, Contract grant number: 0140143, Contract grant sponsor: Enterprise Estonia, Contract grant number: EU27553

Published online 22 February 2010 in
Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/dvg.20606

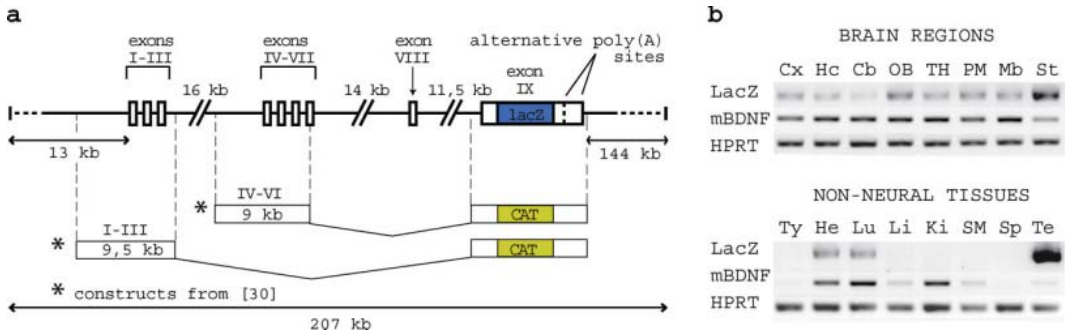

FIG. 1. (**a**) Schematic diagram of the BAC construct used for generating *rBDNF-lacZ*-BAC transgenic mice (thick lines). White boxes represent untranslated sequences and the blue filled box represents *lacZ* reporter gene that replaces the *BDNF* coding sequence. *rBDNF-CAT* constructs (I–III and IV–VI) used by Timmusk *et al*. (1995) to generate *rBDNF* transgenic mice are shown with asterisks. (**b**) RT-PCR analysis of *rBDNF-lacZ* mRNA expression driven by *rBDNF* promoters in transgenic mouse tissues. Abbreviations: *mBDNF*, mouse *BDNF*; *HPRT*, hypoxanthine phosphoribosyltransferase 1; Cx, cortex; Hc, hippocampus; Cb, cerebellum; OB, olfactory bulb; TH, thalamus and hypothalamus; PM, pons/medulla; Mb, midbrain; St, striatum; Ty, thymus; He, heart; Lu, lung; Li, liver; Ki, kidney; SM, skeletal muscle; Sp, spleen; Te, testis. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

encompassing the genomic region from 13 kb upstream of *rBDNF* exon I to 144 kb downstream of *rBDNF* coding exon. Neighboring genes of the *rBDNF* gene lie 151 kb upstream (*Ifna4*) and 190 kb downstream (*Sqrdl*) from it and therefore no additional genes/promoters were included in the BAC construct. To facilitate detection of transgene expression, we replaced the protein coding region of exon IX in the *rBDNF*-BAC with *lacZ* reporter gene (Fig. 1a). This should lead to the expression of functional β-galactosidase protein but not a BDNF-lacZ fusion protein. Functional β-galactosidase protein encoded by the *lacZ* reporter gene in *rBDNF-lacZ*-BAC was detected by transient expression in COS-7 cells (data not shown).

In the *rBDNF-lacZ*-BAC transgenic line, the expression of *rBDNF-lacZ* mRNA was detected by RT-PCR in several brain regions and peripheral organs expressing endogenous mouse *BDNF* (*mBDNF*) mRNA (Fig. 1b). Specifically, the expression of *rBDNF-lacZ* mRNA was detected in the brain regions of cortex, hippocampus, cerebellum, olfactory bulb, thalamus/hypothalamus, pons/medulla, midbrain, striatum, and also in the heart and lung. *rBDNF-lacZ* mRNA expression levels were not detected by RT-PCR in the thymus, liver, kidney, spleen, and skeletal muscle. Particularly high expression of the transgene was observed in the testis.

In the adult brain of the *rBDNF-lacZ*-BAC transgenic mice, in situ hybridization analysis revealed intense labeling of both *rBDNF-lacZ* and endogenous *mBDNF* mRNAs in the cerebral cortex (Figs. 2a–f and 3g,h), olfactory nucleus (Fig. 2a,b), hippocampus (Figs. 2e,f and 3a–f), amygdala (Fig. 2e–f), nucleus of the lateral olfactory tract (Fig. 2i,j), and hypothalamic nuclei (Fig. 2e,f and 2k–n) including mamillary nuclei (Fig. 2k,l). In the granular cell layer of the olfactory bulb (Fig. 2a,b), caudate putamen, and nucleus accumbens (Fig. 2c,d), high levels of *rBDNF-lacZ* mRNA were detected, whereas labeling of the endogenous *mBDNF* mRNA was indistinguishable from background signal. In the claustrum (Fig. 2c,d) and hypothalamus (Fig. 2e,f), *rBDNF-lacZ* mRNA expression levels were relatively lower than *mBDNF* mRNA levels. In the hippocampus, intensive *rBDNF-lacZ* labeling over scattered neurons in the CA1 and CA3 subfields (Fig. 3a,c) mirrored the expression of the endogenous *mBDNF* (Fig. 3b,d). However, in the granule cells of dentate gyrus that showed high expression of *mBDNF* mRNA (Figs. 2f and 3f) no expression of *rBDNF-lacZ* was detected (Figs. 2e and 3e). In the cortex, *rBDNF-lacZ* expression was observed in cingulate and somatosensory areas in layers II–III and V–VI (Figs. 2c,e and 3g), whereas endogenous *mBDNF* was expressed throughout layers II–VI (Figs. 2d,f and 3h). Expression of *rBDNF-lacZ* (Fig. 2g,o) and *mBDNF* (Fig. 2h,p) mRNA was detected also in cardiac blood vessels but not in ventricular myocardium (Fig. 2g,h). In lung tissue, the levels of both *rBDNF-lacZ* and *mBDNF* mRNA were below detection limits of our in situ hybridization analysis (data not shown).

We also analyzed the expression and enzymatic activity of β-galactosidase protein in *rBDNF-lacZ*-BAC mouse tissues. Reporter activity was not detected in the brain or testis of the analyzed *rBDNF-lacZ*-BAC mouse line using X-gal staining assay. In addition, no expression of β-galactosidase protein was detected in the hippocampus, cortex, and testis of the transgenic animals using Western blot analysis (data not shown). These results suggest that β-galactosidase protein was either not translated from BAC-driven *rBDNF-lacZ* mRNAs or the levels of expression of the reporter protein remained below detection limits of the methods used in this study.

Kainic acid has been shown to induce *BDNF* mRNA expression in the adult rodent hippocampus and cerebral cortex (Zafra *et al*., 1990) in a promoter-specific

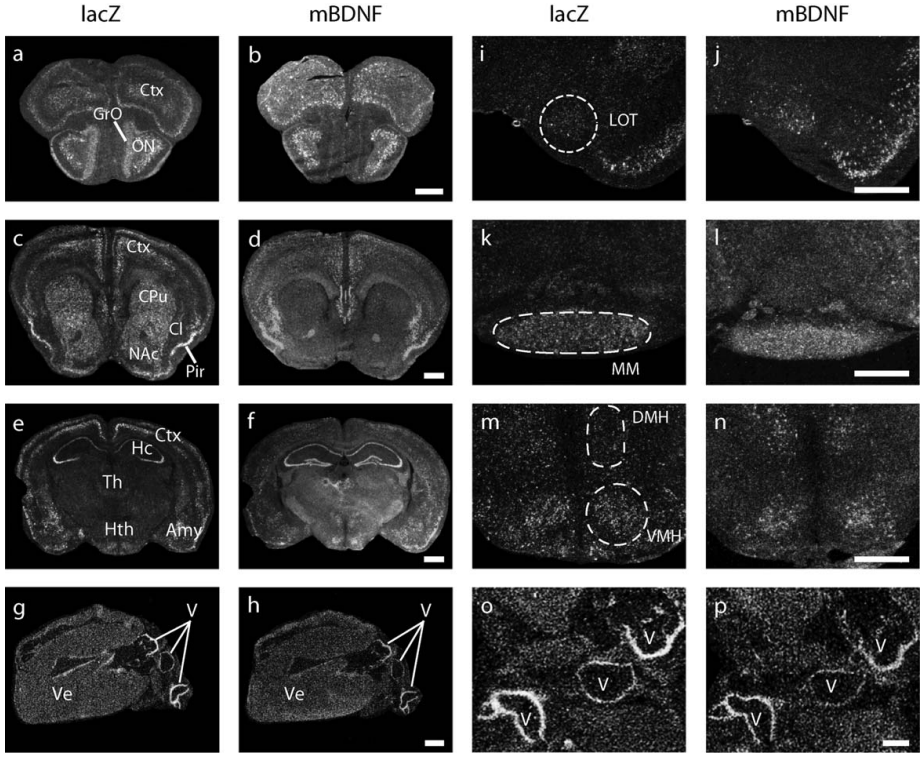

FIG. 2. In situ hybridization analysis of *rBDNF-lacZ* mRNA expression in adult *rBDNF-lacZ*-BAC transgenic mouse brain and heart. Photomicrographs of 16 μm coronal brain (**a–f; i–n**) and transverse heart sections (**g,h,o,p**) hybridized with ^{35}S-labeled *lacZ* or mouse endogenous *BDNF* (*mBDNF*) cRNA. The brain sections shown are at the levels of olfactory bulb (**a**,**b**), striatum (**c**,**d**), and hippocampus (**e**,**f**). (**i–n**) Magnifications of selected brain regions: LOT, nucleus of the lateral olfactory tract; MM, medial mammillary nucleus; DMH, dorsomedial hypothalamic nucleus; VMH, ventromedial hypothalamic nucleus. (**o,p**) Magnifications of cardiac blood vessels. Scale bars: 1 mm (**a–h**) and 0.5 mm (**i–p**). Abbreviations: Ctx, cortex; GrO, olfactory bulb, granular cell layer; ON, olfactory nuclei; CPu, caudate putamen; Cl, claustrum; NAc, nucleus accumbens; Pir, piriform cortex; Hc, hippocampus; Th, thalamus; Hth, hypothalamus; Amy, amygdala; Ve, ventricle; V, cardiac blood vessel.

manner (Aid *et al*., 2007; Timmusk *et al*., 1993). Three hours after systemic injection of kainic acid, the levels of transgenic *rBDNF-lacZ* mRNA were increased in *rBDNF-lacZ*-BAC mice similarly to endogenous *mBDNF* mRNA (see Fig. 4). The elevated levels of *rBDNF-lacZ* and *mBDNF* mRNA expression were observed in cortical layers II–III and V–VI, hippocampal subfields CA1 and CA3, and in the amygdala. However, in contrast to endogenous *mBDNF*, induction of *rBDNF-lacZ* mRNA expression in the granule cells of the dentate gyrus was not observed (Fig. 4e,f). Quantitative real-time PCR analysis showed that induction pattern of different *rBDNF-lacZ* transcripts by kainic acid largely followed that of the endogenous *BDNF*: both transgenic and endogenous exon I and exon IV mRNAs transcribed from promoters I and IV, respectively, showed higher levels of induction than exon VI mRNAs transcribed from promoter VI (Fig. 4g,h). Similarly to untreated mice, β-galactosidase activity and protein expression was not detected in the cortex, hippocampus, and testis of kainate-treated *rBDNF-lacZ*-BAC mice (data not shown).

Transgenic mice expressing reporter genes under the control of various regulatory regions of the *rBDNF* gene have been described previously. *rBDNF-CAT* transgenic mice carrying 9 kb of genomic sequence comprising one or more *BDNF* 5′ untranslated exons were reported in (Timmusk *et al*., 1995). These transgenic mice (Fig. 1a) recapitulated *BDNF* expression in most brain regions and in the thymus. However, *BDNF* IV–VI construct failed to recapitulate *BDNF* expression in the cerebellum, heart, and other peripheral tissues (Timmusk *et al*., 1995) where *BDNF* transcripts IV and VI are endogenously expressed (Aid *et al*., 2007; Pruunsild *et al*., 2007; Timmusk *et al*., 1993). Here we demonstrate that *rBDNF-lacZ*-BAC including 50 kb of the *rBDNF* gene, 13 kb of upstream and 144 kb of downstream sequences

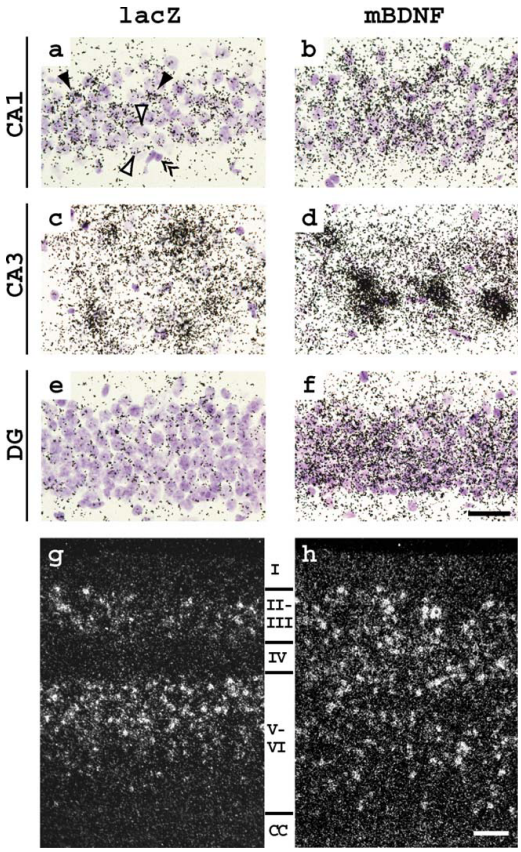

FIG. 3. Cellular expression of *rBDNF-lacZ* mRNA in adult transgenic mouse brain: in situ hybridization analysis. (**a–f**) Bright-field photomicrographs of hippocampal subfields CA1, CA3, and dentate gyrus (DG). Hybridization probes are indicated above the columns; closed arrowheads indicate neurons with strong labeling; open arrowheads indicate neurons with weak or absent labeling; double arrowheads indicate a glial cell showing no labeling. (**g**,**h**) Distribution of *lacZ* and mouse *BDNF* labeling in cortical layers I–VI. Abbreviation: CC, corpus callosum. Scale bars: 20 μm (**a–f**) and 100 μm (**g**,**h**).

contains regulatory elements necessary for recapitulation of endogenous *BDNF* expression in the brain, heart, and lung, indicating that regulatory elements governing *BDNF* mRNA expression in these tissues are located within the 207 kb rat genomic sequence of the transgene. In addition, neuronal activity induced expression of *rBDNF-lacZ* mRNA in a promotor-specific manner in the *rBDNF-lacZ*-BAC mice, mimicking induction of the respective 5′ exon-specific transcripts of endogenous *BDNF*.

Recently, we have shown that human *BDNF-EGFP*-BAC covering 67 kb of the human *BDNF* (*hBDNF*) gene, 84 kb of upstream and 17 kb of downstream sequences are not sufficient to drive *EGFP* (enhanced green fluorescent protein) reporter gene expression in the heart (Koppel *et al*., 2009). Expression of *rBDNF-lacZ* mRNA in the heart of *rBDNF-lacZ*-BAC transgenic mice reported here (with 144 kb region 3′ of the *r*BDNF

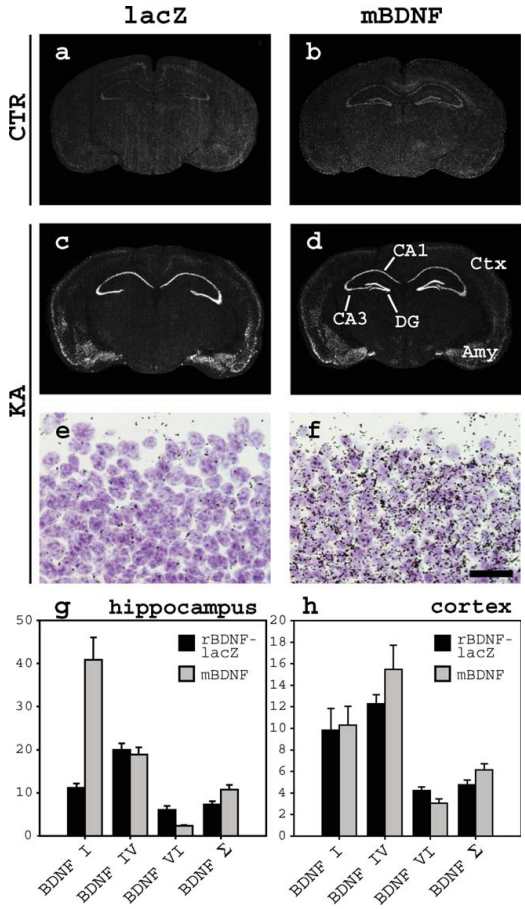

FIG. 4. Induction of *rBDNF-lacZ* mRNA in transgenic mouse brain by kainic acid treatment. (**a–f**) In situ hybridization analysis with probes for transgenic *rBDNF-lacZ* and mouse endogenous (*mBDNF*) mRNA. Autoradiographs of sections from vehicle-treated (**a**,**b**) and kainate-treated animals (**c–f**) are shown. Dark-field autoradiographs of coronal sections (**a–d**); high magnification bright-field photomicrographs of the dentate gyrus (**e**,**f**). Scale bar: 20 μm (**e**,**f**). (**g**,**h**) Quantitative real-time PCR analysis of *rBDNF-lacZ* and endogenous *mBDNF* mRNA expression in the hippocampus (**g**) and cerebral cortex (**h**) of transgenic mice, expressed as fold difference relative to mRNA levels in vehicle-treated mice. Shown are transcripts containing exons I, IV, VI, and total *BDNF* mRNA (BDNF Σ). Error bars represent standard deviation of three RT-PCR experiments. Abbreviations: CTR, vehicle-treated control mice; KA, kainate-treated mice; CA1, CA3, hippocampal subfields; DG, dentate gyrus; Ctx, cortex; Amy, amygdala.

Table 1
PCR Primers Used in This Study

BAC modification	
mrBDNF_rpsLneo_F	TGTCTGTCTCTGCTTCCTTCCCACAGTTCCACCAGGTGAGAAGAGTGGGCCTGGTGATGATGGCGGGATCG
rBDNF_rpsLneo_R	ATACAAATAGATAATTTTTGTCTCAATATAATCTATACAACATAAATCCATCAGAAGAACTCGTCAAGAAGG
BDNF_lacZ_300_F	GCCGTCACTTGCTTAGAAACCGTT
BDNF_lacZ_300_R	GAGTACTAACAAGAACGAAGATACT
Genotyping/RT-PCR	
rBDNF_LacZ_F	CCCTGCAGCTGGAGTGGATCAGTAAG
rBDNF_LacZ_R	GAAGATCGCACTCCAGCCAGCTTTCC
mBDNF_F	GTATGTTCGGGCCCTTACTATGGATAGC
mBDNF_R	AAGTTGTGCGCAAATGACTGTTTC
HPRT1_F	CTTTGCTGACCTGCTGGATTAC
HPRT1_R	GTCCTTTTCACCAGCAAGCTTG
Quantitative real-time RT-PCR	
Mouse endogenous mRNAs	
mBDNFq_I_F	TTGAAGCTTTGCGGATATTGCG
mBDNFq_IV_F	GAAATATATAGTAAGAGTCTAGAACCTTG
mBDNFq_VI_F	GCTTTGTGTGGACCCTGAGTTC
mBDNFq_RT_IXcod_R	AAGTTGCCTTGTCCGTGGAC
mBDNFq_cod_F	GGCCCAACGAAGAAAACCAT
mBDNFq_cod_R	AGCATCACCCGGGAAGTGT
HPRT1q_F	CAGTCCCAGCGTCGTGATTA
HPRT1q_R	AGCAAGTCTTTCAGTCCTGTC
Rat BDNF-lacZ mRNAs	
rBDNFq_I_F	AGTCTCCAGGACAGCAAAGC
rBDNFq_IV_F	GAAATATATAGTAAGAGTCTAGAACCTTG
rBDNFq_VI_F	GCTTTGTGTGGACCCTGAGTTC
LacZq_F	CGAAGTGACCAGCGAATACCTGT
LacZq_R1	CAACTGTTTACCTTGTGGAGCGACA
LacZq_R2 (with I_F)	CAAGGCGATTAAGTTGGGTAAC
LacZq_R3 (with IV,VI_F)	GTTTTCCCAGTCACGACGTT

gene) suggests that a heart-specific regulatory element is located within 18–144 kb 3′ of *BDNF* gene. However, this prediction should be treated with caution as regulatory regions of *BDNF* genes of different species are compared. On the other hand, neither *bBDNF-EGFP*-BAC (Koppel *et al*., 2009) nor *rBDNF-lacZ*-BAC could direct transgene expression to hippocampal dentate granule cells suggesting that the respective regulatory regions are located in genomic regions further than 84 kb upstream of *BDNF* exon I and 144 kb downstream of *BDNF* coding exon. Existence of remote cis-acting elements controlling *BDNF* transcription has been demonstrated by recent studies describing a regulatory region 850 kb upstream of human and mouse *BDNF* genes, disruption of which causes obesity, cognitive impairment, and hyperactivity (Gray *et al*., 2006; Sha *et al*., 2007).

In conclusion, we have generated transgenic mice containing *rBDNF-lacZ*-BAC transgene that recapitulated the expression of endogenous *BDNF* mRNA in the brain and peripheral tissues and neuronal activity-dependent regulation of *BDNF* mRNA in the adult cerebral cortex and hippocampus. This mouse model represents a useful tool for further mapping of proximal and distal regulatory elements in rodent *BDNF* gene in vivo.

METHODS

rBDNF-lacZ-BAC transgenic mice were generated using BAC clone CH230-106M15 (Chori BACPAC Resources, Oakland, CA) modified to replace *rBDNF* coding sequence with the *lacZ* reporter gene (Red®/ET® homologous recombination technology, Gene Bridges, Heidelberg, Germany) (Muyrers *et al*., 1999). The BAC clone contains 207 kb of the *rBDNF* genomic locus (GenBank: AC108236) including 50 kb of *rBDNF* gene, 13 kb of 5′ and 144 kb of 3′ flanking sequences (Fig. 1a). Purified *rBDNF-lacZ*-BAC was transfected into COS-7 cells by DEAE-dextran and tested for reporter activity using β-galactosidase assay. Transgenic mice were generated at the Karolinska Center for Transgene Technologies (Stockholm, Sweden) by injection of NotI-linearized *rBDNF-lacZ*-BAC into CBA x C57Bl/6 mouse pronuclei. One transgenic founder mouse was obtained and bred to establish a transgenic mouse line. Integration of two copies of *rBDNF-lacZ*-BAC transgene was estimated by slot-blot hybridization of genomic DNA with [α-^{32}P]dCTP-labeled *lacZ*-specific probe.

RNA isolation and analysis of *rBDNF-lacZ mRNA* expression in transgenic mouse tissues with RT-PCR was performed as described (Pruunsild *et al*., 2007). Quantitative real-time PCR was performed on LightCycler 2.0 (Roche Diagnostics, Mannheim, Germany) using qPCR Core Kit for SYBR(r) Green I No ROX (Eurogentec, Liège, Belgium). qPCR reactions were processed in triplicate and all expression data were normalized to hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) mRNA levels. For primer sequences see Table 1. In situ hybridization analysis with [α-^{35}S]UTP-labeled cRNA probes for *rBDNF-lacZ* and endogenous mouse *BDNF* mRNA was performed as described in Timmusk *et al*. (1993). Kainic

acid (KA; 30 mg/kg) or phosphate-buffered saline was administered intraperitoneally to adult *rBDNF-lacZ*-BAC mice weighing 20–25 g. Two kainic acid-treated and two vehicle-treated animals were used for qRT-PCR analysis. Four kainic acid-treated animals and one vehicle-treated animal were used for in situ hybridization analysis. Only animals with induced tonic-clonic seizures were selected for analysis and results are shown for individuals showing highest induction of transgenic and endogenous *BDNF* mRNA. All animal procedures were carried out in compliance with the local ethics committee.

ACKNOWLEDGMENTS

We thank Epp Väli for technical assistance and Priit Pruunsild for critical comments on the manuscript.

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ABSTRACT

Structure and regulation of BDNF gene

Brain-derived neurotrophic factor (BDNF) is essential for development and functioning of the nervous system in vertebrates. BDNF role in numerous neurological disorders has brought a lot of attention to this gene in the last two decades. However, many aspects of the BDNF gene expression still remain poorly characterized. This thesis is focused on BDNF gene structure and its transcriptional regulation *in vivo* in transgenic mice. Here I present revised BDNF gene organization in rodents and describe the regulation of BDNF alternative promoters by neuronal activity and by chromatin remodeling drugs. Furthermore, I propose novel potential regulators of BDNF expression based on the bioinformatics co-expression analysis of microarray data. And finally, I describe the transgenic BAC mouse models generated for studying BDNF regulation *in vivo*.

First, I showed that mouse and rat BDNF gene as well, contains novel 5′ untranslated exons and introduced a new numbering for mouse and rat BDNF exons. According to the new nomenclature, mouse and rat BDNF gene consist of eight 5′ untranslated exons (I-VIII) and one protein coding 3′ exon (IX). BDNF transcripts contain either one of the eight 5′ exons spliced to the common protein coding exon IX or only 5′ extended protein coding exon (IXA). We report tissue-specific expression of rodent BDNF transcripts in different brain regions and non-neural tissues. Kainic acid-induced seizures as well as inhibition of DNA methylation and histone deacetylation differentially affect the expression of alternative BDNF transcripts. Importantly, in contrast to human BDNF gene, mouse and rat BDNF gene loci do not encode BDNF antisense RNAs.

Second, a bioinformatics meta-coexpression analysis of publicly available microarray data using BDNF as a ‘guide-gene’. The key steps of the analysis using a novel ‘subset’ approach included i) dividing datasets into subsets with biologically meaningful sample content (e.g. tissue, gender or disease state subsets); ii) analyzing co-expression with the BDNF gene in each subset separately; iii) and confirming co- expression links across subsets. Then we analyzed conservation in co-expression with BDNF between human, mouse and rat, and finally, sought for conserved over-represented transcription factor binding sites (TFBSs) in BDNF and

BDNF-correlated genes. Correlated genes discovered in this study regulate nervous system development, and are associated with various types of cancer and neurodegenerative disorders. We report that BDNF promoters and exons contain highly conserved TFBSs for WT1, MAZ, KROX, CREB, OCT, MYOD, MEF2, TCF4 (ITF2), and BRN2 whose binding sites were also enriched in the BDNF-correlated genes. Several transcription factors identified here have been shown to regulate BDNF expression *in vitro* and *in vivo*. Our study demonstrates a potential of the 'subset' approach for studying the regulation of single genes and proposes novel regulators of the BDNF gene expression.

And finally, we generated BDNF-BAC transgenic mice for studying proximal and distal regulatory *cis*-elements in the BDNF gene *in vivo*. Bacterial artificial chromosome (BAC) construct containing 207 kb of the rat BDNF locus was modified to replace BDNF coding exon with the lacZ reporter gene. Transgenic mouse line containing rBDNF-lacZ-BAC transgene recapitulated the expression of endogenous BDNF mRNA in mouse brain and peripheral tissues as well as upon neuronal activity in the adult cerebral cortex and hippocampus. Also, we generated and analyzed BAC transgenic mice carrying 168 kb of the human BDNF locus. In this transgene, BAC construct was modified to insert the enhanced green fluorescent protein (EGFP) reporter gene into the C-terminus of the human BDNF gene, generating hBDNF-EGFP fusion protein. All hBDNF-EGFP alternative transcripts were expressed from the transgenic hBDNF-EGFP-BAC construct, resembling the expression of endogenous BDNF. Furthermore, hBDNF-EGFP mRNA was induced in a promotor-specific manner following treatment with kainic acid similarly to that of the endogenous mouse BDNF. These results show that the genomic region covering 84 kb upstream of hBDNF exon I to 17 kb downstream of hBDNF coding exon is sufficient to drive tissue-specific and kainic acid-induced expression of the reporter gene in transgenic mice. This is the first study to show that the human BDNF gene is regulated by neural activity. Taken together, our mouse models represent a useful tool for studying proximal and distal regulatory elements in the rodent and human BDNF gene *in vivo*.

KOKKUVÕTE

BDNF geeni struktuur ja regulatsioon

Ajust pärinev neurotroofne faktor (*brain derived neurotrophic factor*, ehk BDNF) täidab olulisi rolle nii arenevas kui täiskasvanud närvisüsteemis selgroogsetel. Möödunud paarikümne aasta jooksul avastatud seosed mitmete neuroloogiliste ja psühhiaatriliste haigustega on toonud selle faktori suure teadusliku tähelepanu alla, kuid BDNF geeni regulatsiooni mitmeid aspekte pole tänaseks piisavalt põhjalikult iseloomustatud.

Käesolevas doktoritöös esitan ma uuendatud näriliste BDNF geeni struktuuri, kirjeldan BDNFi alternatiivsete promootorite regulatsiooni neuronaalse aktiivsusega ning kromatiini epigeneetilisi modifikatsioone mõjutavate ravimitega, esitan mikrokiipide andmestiku bioinformaatilisel töötlusel leitud uued potentsiaalsed BDNFi transkriptsiooni reguleerivad faktorid ning kirjeldan BAC transgeenseid hiiri BDNF geeniregulatsiooni uurimiseks *in vivo*.

Töö esimeses etapis kirjeldasin mitmeid uusi näriliste BDNF geeni 5′ mittetransleeritavaid eksoneid ning juurutasime nende tähistamiseks uue numeratsiooni. Selle kohaselt koosneb hiire ja roti BDNF geen kaheksast 5′ mittetransleeritavast eksonist (I-VIII) ning ühest valku kodeerivast 3′ eksonist (IX). BDNF transkriptid koosnevad ühest või kahest eksonist kas kaheksa alternatiivse hulgast valitud ühest 5′ eksonist ja eksonist IX või ainult 5′ suunal pikema mittetransleeritava alaga eksonist IX (IXA). Olulise erinevusena inimese BDNF lookusega võrreldes ei sisalda näriliste lookus antisense transkripte tootvat anti-BDNF geeni. Järgnevalt kirjeldasime kõikide BDNF transkriptide ekspressiooni hiire ja roti ajuosades ning mitteneuraalsetes kudedes ning uurisime nende diferentsiaalset regulatsiooni eksitatoorset neuraalset aktiivsust, DNA metülatsiooni ning histoonide atsetülatsiooni mõjutavate farmakonide poolt.

Töö teises osas analüüsisime bioinformaatiliselt meta-koekspressiooni meetodil avalikus kasutuses olevat mikrokiipide andmestikku uudsel alamhulkade meetodil kasutades BDNFi juhtgeenina. Selle analüüsi võtmeetappideks olid: i) andmehulkade jagamine alamhulkadeks bioloogilise sisu järgi (näiteks koe, soo või haiguste järgi); ii) koekspressioonanalüüs BDNF geeniga eraldi igas alamhulgas; iii) koekspressiooni seoste kinnitamine alamhulkade omavahelisel võrdlusel. Järgnevalt analüüsisime koekspressioonimustrite konserveerumist inimese, hiire ja roti vahel ning viimases etapis otsisime BDNFi ja BDNF-korreleeritud geenide hulkades üleesindatud transk-

riptsionifaktorite sidumissaite. BDNF-korreleeritud geenide hulgas on närvisüsteemi arengut reguleerivaid, mitmete vähitüüpidega ning neurodegeneratiivsete haigustega seotud geene. BDNFi promootorites ja eksonites leiti kõrgelt konserveerunud sidumissaidid WT1, MAZ, KROX, CREB, OCT, MYOD, MEF2, TCF4 (ITF2), BRN2 ja mitmetele teistele transkriptsioonifaktoritele, mille saidid on rikastunud ka BDNF-korreleeruvates geenides. Me oletame, et need transkriptsioonifaktorid võivad reguleerida alternatiivsete BDNF promootorite aktiivsust. Mitmed selle analüüsi käigus leitud transkriptsioonifaktorid on varasemalt näidatud reguleerivat BDNF ekspressiooni *in vitro* ja *in vivo*. Meie töö näitab ekspressiooniandmestiku alamhulkade meetodi potentsiaali üksikute geenide regulatsiooni uurimisel ning pakub mitmeid uudseid faktoreid kandidaatidena BDNF geenieksпressiooni reguleerimisel.

Töö viimaseks osaks on BDNF-BAC transgeensete hiirte tegemine uurimaks inimese ja roti BNDF geeni reguleerivaid proksimaalseid ja distaalseid cis-elemente *in vivo*. Selleks valmistasime järgnevad transgeensed hiireliinid:

1. 168 kb inimese BDNF lookust sisaldavad BAC-BDNF-EGFP liinid, kus transgeen kodeerib EGFP-BDNF liitvalku (EGFP C-terminuses)

2. 207 kb roti BDNF lookust sisaldav liin, kus transgeen kodeerib BDNF raami viidud β-galaktosidaas reportervalku.

Transgeenseid BDNF-reporter mRNAsid ekspresseerisid hiire endogeense BDNF transkriptidega võrreldaval tasemel üks inimese BDNF-BAC liin ning roti BDNF-BAC liin. Mõlemates hiireliinides järgis BDNF-reporter transkriptide ekspressioonimuster suurel määral endogeense hiire BDNF geeni ekspressiooni ajus ja mitteneuraalsetes kudedes. Lisaks rekapituleerisid mõlemad transgeenid BDNFi promootorspetsiifilist induktsiooni neuronaalse aktiivsusega hiirte töötlemisel kainaathappega. Teadaolevalt on see esimeseks uurimuseks, kus näidatakse otseselt inimese geeni regulatsiooni neuronaalse aktiivsusega. Loodud hiireliinid on kasutatavad väärtuslike tööriistadena BDNF geeni regulaatorelementide uurimisel.

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Publikatsioonid

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