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Novel Transgenic Models Based on Bacterial Artificial Chromosomes for Studying BDNF Gene Regulation

KAUR JAANSON

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TALLINN UNIVERSITY OF TECHNOLOGY
FACULTY OF SCIENCE
DEPARTMENT OF GENE TECHNOLOGY

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Supervisor: Prof. Tõnis Timmusk, PhD,
Department of Gene Technology, Tallinn University of Technology, Estonia

Opponents: Assoc. Prof. Jaan-Olle Andressoo, PhD,
Institute of Biotechnology, University of Helsinki, Finland

Prof. Sulev Kõks, PhD,
Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia

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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.

/Kaur Jaanson/

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Bakteriaalsetel kunstlikel kromosoomidel põhinevad transgeensed mudelid BDNF geeni regulatsiooni uurimiseks

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* Authors have equal contribution

INTRODUCTION

As a second known member of the neurotrophin family, brain derived neurotrophic factor (BDNF) was initially purified from pig brain as a factor capable of supporting survival of sensory neurons. Regulation of BDNF signalling is complex with highly regulated transcription, translation, processing and controlled vesicular sorting, and secretion of the neurotrophin. Errors or inefficiencies at any of these steps leading to lower BDNF levels have been associated with several neurodevelopmental and -degenerative disorders.

Due to BDNF's involvement in nervous system functioning and disease, development of model systems for studying BDNF gene regulation is of interest. Previously generated transgenic mice using BDNF promoters have shown that including larger genomic areas of BDNF genomic locus in transgene helps to better mirror the endogenous BDNF expression patterns and makes models more useful for BDNF regulation studies. However, reporter genes in these transgenic mice still did not fully mimic the endogenous BDNF expression. Bacterial artificial chromosomes (BACs) are large capacity DNA vectors, able to contain entire genes together with their upstream and downstream genomic areas. Due to existence of genomic BAC libraries for human and rodents, and convenient methods for their modification, they have been increasingly used for generating transgenic models for studies of gene regulation and protein function.

In the following text I will give a brief overview of BDNF expression, processing, signalling and function. I will also review the use of BACs in studying the regulation of genes in transgenic systems.

ABBREVIATIONS

ARNT2	aryl hydrocarbon receptor nuclear translocator 2
BAC	bacterial artificial chromosome
BDNF	brain-derived neurotrophic factor
CaMKII	calmodulin-dependent protein kinase II
CAPS2	Ca^{2+}-dependent activator protein for secretion 2
CaRE1/2/3	calcium response element 1/2/3
CaRF	calcium response factor
CPB	CREB binding protein
CPE	carboxypeptidase e
CPEB-1	cytoplasmic polyadenylation element binding protein-1
CRE	cAMP response element
CREB	cAMP response element binding protein
DGK-β	diacylglycerol kinase β
eEF2K	eukaryotic elongation factor 2
EGFP	enhanced green fluorescent protein
ERK	extracellular signal regulated kinase
FISH	fluorescent in situ hybridisation
HAT	histone acetyltransferases
HDAC	histone deacetylases
HDM	histone demethylases
HMT	histone methyltransferases
hRluc	humanised Renilla luciferase
LTD	long-term depression
LTP	long-term potentiation
MAPK	mitogen activated protein kinase
MeCP2	methyl CpG binding protein 2
MEF2	myocyte enhancer factor 2
NAD	nicotinaminde adenine dinucleotide
ncRNA	non-coding RNA
NFkB	nuclear factor kappa beta
NGF	nerve growth factor
NPAS4	neuronal PAS domain protein 4
NRSF	neuron-restrictive silencing element
NT3	neurotrophin 3
NT4	neurotrophin 4
PI3K	phosphatidylinositol-3-kinase
PLC	phospholipase C
REST	repressor element 1 silencing transcription factor

RISC	RNA induced silencing complex
SNARE	soluble NSF attachment protein receptor
tPA	tissue plasminogen activator
TrkA/B/C	tropomyosin-related kinase A/B/C
TSTA	two-step transcriptional activation
USF	upstream stimulatory factor
UTR	untranslated region
YAC	yeast artificial chromosome

1. REVIEW OF THE LITERATURE

1.1. Neurotrophin family

Neurotrophin growth factor family member brain-derived neurotrophic factor (BDNF) was initially purified from pig brain as a factor capable of supporting survival of sensory neurons (Barde et al., 1982). Altogether, neurotrophin family of proteins includes BDNF, nerve growth factor (NGF), neurotrophin 3 (NT3) and neurotrophin 4 (NT4) – all of which have diverse functions in neuronal survival and synaptic plasticity related processes in developing and adult nervous system (Bibel and Barde, 2000; Park and Poo, 2013). Neurotrophin family members are synthesised as pro-neurotrophins and are processed intra- or extracellularly to produce mature neurotrophins. These two forms of neurotrophins bind to different receptors with pro forms of all neurotrophins binding with high affinity to common pro-neurotrophin receptor $p75^{NTR}$ and mature forms of neurotrophins binding to their specific tropomyosin-related kinase receptors (Trk-s) – NGF to TrkA, BDNF and NT4 to TrkB, NT3 to TrkC (Huang and Reichardt, 2001). Binding to these receptors activates distinct signalling pathways in cells, with $p75^{NTR}$ activating signalling pathways associated with apoptosis and reduction of synaptic complexity (Teng et al., 2010), and TrkB signalling activating intracellular signalling pathways that support cellular survival, neurite outgrowth and increase of synaptic plasticity (Huang and Reichardt, 2001).

1.2. BDNF expression and regulating stimuli

Importance of BDNF in development and functioning of mammalian nervous system is shown by its wide expression in central and peripheral nervous system. BDNF levels in the brain increase during the embryonic development reaching peak during first postnatal weeks (Maisonpierre et al., 1990; Timmusk et al., 1994a) with the expression appearing mainly in neurons (Ernfors et al., 1990; Maisonpierre et al., 1990; Wetmore et al., 1990; Timmusk et al., 1994a; Conner et al., 1997; Katoh-Semba et al., 1997). In addition to nervous system, BDNF is also expressed in several non-neural tissues with highest levels detected in heart and lung, but also in skeletal muscle, liver, kidney, thymus and spleen (Maisonpierre et al., 1990; Yamamoto et al., 1996; Katoh-Semba et al., 1997; Aid et al., 2007; Pruunsild et al., 2007).

BDNF expression is changed in nervous system in response to wide array of stimuli, most of which are associated with neuronal activity but also with processes like injury, stress and disorders. Direct stimulation of neural activity by kainic acid (Zafra et al., 1990; Metsis et al., 1993), electrical stimulation

(Ernfors et al., 1991; Patterson et al., 1992; Castrén et al., 1993), lesion-induced seizures (Isackson et al., 1991) or cortical application of potassium chloride (Kokaia et al., 1993) have been shown to increase BDNF expression in nervous system. BDNF regulation is also associated with sensory and memory related processes that increase neural activity. It is increased in response to whisker stimulation in rodents (Rocamora et al., 1996; Nanda and Mack, 2000). BDNF levels are increased in visual cortex in response to light (Castrén et al., 1992) and decreased in case of monocular inhibition (Bozzi et al., 1995; Rossi et al., 1999). Its levels are also increased in response to hippocampus and amygdala-dependent learning (Hall et al., 2000; Rattiner et al., 2004) and enriched environment (Falkenberg et al., 1992; Young et al., 1999). BDNF levels have also been shown to be regulated during diurnal cycle with the levels increasing during the beginning of animals activity period (Bova et al., 1998; Berchtold et al., 1999). Inhibition of neural activity by GABA reduces BDNF levels (Berninger et al., 1995).

In addition to BDNF regulation in response to neuronal activity, other stimuli also affect BDNF expression levels. BDNF has been shown to be induced in response to injuries to nervous system like ischemic and hypoglycaemic insults (Lindvall et al., 1992), and peripheral nerve axotomy (Meyer et al., 1992; Funakoshi et al., 1993). BDNF is also is involved in responses to stress with decreased levels in dentate gyrus and hippocampus due to immobilisation stress (Smith et al., 1995a, 1995b), and downregulation of its mRNAs in response to acute or chronic social defeat stress (Pizarro et al., 2004; Tsankova et al., 2006). BDNF expression is also altered in depression (Smith et al., 1995b; Berton et al., 2006; Tsankova et al., 2006; Larsen et al., 2010) with antidepressant treatments withholding its stress caused reduction (Tsankova et al., 2006) and also inducing BDNF levels (Nibuya et al., 1995; Dias et al., 2003; Berton et al., 2006; Tsankova et al., 2006; Larsen et al., 2010). Additionally, BDNF is involved in addiction related processes with its levels increased in mesolimbic system following cocaine withdrawal (Grimm et al., 2003) and in nucleus accumbens in response to cocaine self-administration (Graham et al., 2007).

1.3. BDNF synthesis, processing and secretion

BDNF is first synthesised as a precursor protein preproBDNF, that is cleaved in endoplasmic reticulum to yield 32kDa proBDNF, which can then be cleaved further at several places along its trafficking route to yield 13kDa mature BDNF (Mowla et al., 2001). This processing is performed intracellularly in trans-Golgi network by furin, or in immature secretory vesicles by proprotein convertases (Seidah et al., 1996), and extracellularly by tissue plasminogen activator (tPA)/plasmin system (Pang et al., 2004) or matrix metalloproteinases (Lee et

al., 2001b). The proBDNF is sorted in trans-Golgi network to constitutive or regulated secretory pathway with most of the secretion going by regulated pathway in neurons (Goodman et al., 1996; Heymach et al., 1996; Mowla et al., 1999; Wu et al., 2004). Sorting of BDNF protein to dense core vesicles of regulated secretory pathway is reliant on its pro sequence (Brigadski et al., 2005; Lou et al., 2005). This process has been shown to be dependent on carboxypeptidase E (CPE) (Lou et al., 2005) and sortilin (Chen et al., 2005), both of which interact with pro region of proBDNF in trans-Golgi network and regulate its sorting. In addition to sorting BDNF to regulated secretory pathway, sortilin takes part in directing BDNF containing vesicles to lysosome (Evans et al., 2011).

Vesicular transport to axons or dendrites

BDNF-containing vesicles have been shown to be localised and bi-directionally (antero- and retrogradely) transported in axons and dendrites (Haubensak et al., 1998; Adachi et al., 2005; Park et al., 2008; Kwinter et al., 2009; Dieni et al., 2012). Anterograde BDNF vesicle transport takes place along microtubules and is dependent on assembly of huntingtin/huntingtin associated protein 1/dynactin complex (Gauthier et al 2004, Kwinter et al 2009). Phosphorylation of huntingtin enables the assembly of complex and its anterograde transport while non-phosphorylated huntingtin leads to its disassembly and retrograde transport of vesicles (Colin et al., 2008). CPE has also been shown to be necessary for assembly of motor protein complex and bi-directional transport of vesicles, possibly by recruiting kinesins and dyneins (Park et al., 2008).

Mechanisms responsible for selective axonal or dendritic localisation of BDNF are still being studied. Ca^{2+}-Dependent Activator Protein for Secretion (CAPS2) has been found to associate with BDNF-containing vesicles and be responsible for their axonal localisation (Sadakata et al., 2014). In addition to BDNF transported from soma to dendrites, overexpressed tagged BDNF has been shown to be localised to local dendritic Golgi, indicating local translation, processing, and secretion (Horton and Ehlers, 2003; Horton et al., 2005).

BDNF secretion

Regulated secretion of BDNF from cultured neurons has been shown in response to several stimuli – treatment with glutamate (Canossa et al., 2001), depolarisation (Goodman et al., 1996; Kojima et al., 2001), spontaneous synaptic activity (Kuczewski et al., 2008), and electrical stimulation (Balkowiec and Katz, 2000; Hartmann et al., 2001). Ca^{2+} necessary for secretion can be sourced extracellularly by activation of ionotropic glutamate receptors (Hartmann et al., 2001), L-type voltage-gated cation channels (VGCC) (Hartmann et al., 2001; Kolarow et al., 2007), or N-type calcium channels (Balkowiec and Katz, 2002). In addition, activation of metabotropic $GABA_B$

receptors by GABA increases intracellular Ca^{2+} leading to BDNF secretion (Fiorentino et al., 2009). Initial Ca^{2+} increase is enhanced through Ca^{2+} release from endoplasmic reticulum via activation of ryanodine receptors (Kolarow et al., 2007). Additionally, activation of calmodulin-dependent protein kinase II (CaMKII) and cAMP/protein kinase A is necessary for depolarisation-induced BDNF secretion, and activation of phospholipase C-γ (PLC-γ) pathway is necessary for glutamate or Trk receptor activation-induced BDNF release (Canossa et al., 2001; Kolarow et al., 2007). BDNF secretion has been shown to be negatively regulated by synaptotagmin IV, SNARE complex binding protein localised to BDNF-containing vesicles (Dean et al., 2009).

As previously mentioned, proBDNF can be processed at trans-Golgi network, dense-core vesicles, or extracellularly to yield mature BDNF, so there has been some controversy whether the major form of secreted BDNF is pro- or mature BDNF (Barker, 2009). Work of Matsumoto et al., showed that proBDNF is rapidly converted to mature BDNF in neurons (Matsumoto et al., 2008), and secretory vesicles have been shown to contain mostly mature BDNF and its cleaved pro-peptide together with small amount of proBDNF (Dieni et al., 2012). In alternate experimental setting, however, mostly proBDNF is secreted from neurons and subsequently cleaved extracellularly by plasmin (Yang et al., 2009b). Since tPA/plasmin is released from presynaptic terminals together with proBDNF in response to high-frequency neural activity (Pang et al., 2004) and tPA has been associated with synaptic plasticity and memory related processes (Calabresi et al., 2000), this represents one possible mechanism how opposing effects of pro- or mature BDNF could be controlled by neural activity at synaptic sites. Internalisation and recycling of proBDNF for later release has also been described in astrocytes (Bergami et al., 2008).

1.4. BDNF signalling and function

Mature BDNF dimers bind with high affinity to the tropomyosin-related kinase receptor TrkB, resulting in activation of intracellular signalling cascades – mitogen activated protein kinase/extracellular signal regulated kinase (MAPK/ERK) pathway, phosphatidylinositol-3-kinase/Akt kinase (PI3K/Akt) pathway, and activation of phospholipase Cγ1 (PLCγ1). MAPK/ERK signalling pathway promotes neuronal differentiation, PI3K/Akt leads to neuronal survival, and PLCγ1 activation leads to release of intracellular Ca^{2+} stores (Patapoutian and Reichardt, 2001). Tumor necrosis factor receptor superfamily member $p75^{NTR}$ together with its co-receptor sortilin acts as high affinity receptor for proBDNF (Teng et al., 2005, 2010). Activation of $p75^{NTR}$ leads to activation of Jun N-terminal kinase, p53, and caspases, leading to neuronal apoptosis (Dechant and Barde, 2002).

BDNF was discovered due to its survival effects on cultured spinal sensory neurons of chick embryos (Barde et al., 1982) and was soon shown to promote survival of several neural populations in central and peripheral nervous system – sensory neurons (Davies et al., 1986; Ernfors et al., 1994; Jones et al., 1994), motoneurons (Sendtner et al., 1992), retinal ganglion cells (Johnson et al., 1986; Rodriguez-Tébar et al., 1989; Frade et al., 1997), dorsal root ganglion cells (Kalcheim et al., 1987), dopaminergic neurons (Hyman et al., 1991), cerebellar granule neurons (Segal et al., 1992; Kubo et al., 1995) and septal cholinergic neurons (Alderson et al., 1990). These pro-survival effects are mostly via TrkB receptor signalling while proBDNF signalling via $p75^{NTR}$ receptor has been shown to lead to apoptosis of several neuron populations (Lee et al., 2001b), including cultured superior cervical neurons (Teng et al., 2005) and natural sympathetic neurons (Bamji et al., 1998).

BDNF signalling also affects neurite growth and synaptic development, with mature BDNF signalling leading to initiation and stimulation of axon growth (Cheng et al., 2011), increased branching of ganglion axon terminals (Cohen-Cory and Fraser, 1995), increased dendritic growth, arborisation (McAllister et al., 1995) and spine maturation (Tanaka et al., 2008; Kaneko et al., 2012). Again signalling through $p75^{NTR}$ seem to have diametrically opposite effects causing developmental axon pruning (Singh et al., 2008), retraction of presynaptic terminals (Yang et al., 2009a), decreasing dendritic complexity and spine density (Zagrebelsky et al., 2005; Yang et al., 2014). Mature BDNF has been shown to increase synaptic efficiency and neurotransmitter release (Nagappan and Lu, 2005; Lu et al., 2009), and also enhance hippocampal late long-term potentiation (LTP) (Pang et al., 2004). proBDNF acting through $p75^{NTR}$ enhances long-term depression (LTD) (Woo et al., 2005), decreases synaptic efficacy (Yang et al., 2009a), and inhibits GABAergic neurotransmission (Riffault et al., 2014).

1.5. BDNF in development and disease

Importance of BDNF in nervous system development can be seen in BDNF knock-out mice – homozygous knockout mice die during the second postnatal week in development with deficiencies in coordination of movements and balance (Ernfors et al., 1994, 1995; Liu et al., 1995). Heterozygous BDNF knock-out mice are obese and aggressive (Lyons et al., 1999), have impaired LTP (Korte et al., 1995), and display learning difficulties (Linnarsson et al., 1997) - phenotypes that are also observed in case of BDNF haploinsufficiency in humans (Gray et al., 2006). Obesity and hyperphagia phenotypes have also been described in some cases of WAGR syndrome, where genomic deletions cause BDNF haploinsufficiency in humans (Han et al., 2008). Abnormal levels of BDNF have been described in some neurodevelopmental disorders with

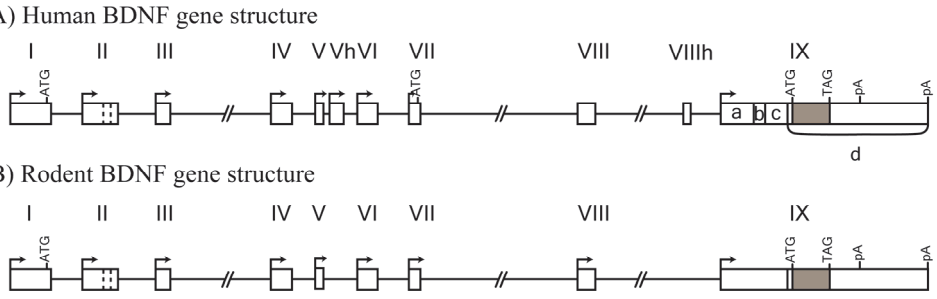

Figure 1. Structure of human (A) and rodent (B) BDNF gene structure. White boxes show exons, gray boxes protein coding regions, arrows known promoters, ATG – in-frame start codons, pA – polyadenylations sites, dashed lines – possible splice boundaries. Human BDNF gene structure adapted from Pruunsild et al., 2007, rodent gene structure adapted from Aid et al., 2007.

BDNF levels being altered in mental retardation, autism and Rett syndrome (Miyazaki et al., 2004; Kasarpalkar et al., 2014; Katz, 2014). Altered BDNF expression has also been shown in several neurodegenerative disorders with mRNA and protein levels being altered in patients of Alzheimer's Disease (Phillips et al., 1991; Narisawa-Saito et al., 1996; Ferrer et al., 1999), reduced in substansia nigra of Parkinson's Disease patients (Mogi et al., 1999; Howells et al., 2000), and in cortices of Huntington's Disease patients (Ferrer et al., 2000; Zuccato et al., 2008). BDNF has also been associated with sensitisation and development of neuropathic pain (Coull et al., 2005).

Due to its involvement in nervous system development and functioning, with its altered levels being associated with serious dysfunctions, it is of great interest to study BDNF gene structure and regulation to understand its involvement in these pathologies and possibly develop treatments.

1.6. BDNF gene structure

To this date, BDNF gene has been described in human (Pruunsild et al., 2007), mouse and rat (Aid et al., 2007), chicken (Yu et al., 2009), pond turtle (Ambigapathy et al., 2013, 2014), sea bass (Tognoli et al., 2010) and zebrafish (Heinrich and Pagtakhan, 2004). In most of the organisms where BDNF gene structure has been described, it has a characteristic structural conservation with several 5' untranslated exons, under the control of different promoters, spliced to single protein coding 3' exon.

The human BDNF gene (Figure 1A) consists of 11 different exons, with two of the exons (Vh and VIIIh) being human specific. Promoters preceding exons I, II, III, IV, V, Vh, VI, VII and IX direct tissue specific expression of different transcripts containing these exons as 5' exons. Exons II, V and VI contain alternative splice sites that can lead to transcripts with different 5' UTR lengths.

The exon IX consists of four different regions “a”, “b”, “c” and “d” with transcripts containing 5’ exons I to VIIIh preferentially including protein coding region “d” only. Exon VI-containing transcripts rarely include also region “b”. Occasionally transcription starts from the beginning of region “a” of exon IX. In those cases there is usually no splicing and all parts of exon IX are included in the final transcript. Sometimes region “c” is spliced out of transcripts that start from the beginning of exon IX. Exon IX also contains two different polyadenylation sites that lead to mature transcripts with different 3’ UTR lengths. Translation from all the transcripts starts from start codon in the “d” region of exon IX. Exons I, VII and VIII contain an additional in-frame ATG that could be used to synthesize BDNF protein with a longer N-terminus. However, usage of these start-codons has not been shown *in vivo*. Altogether this leads to transcription of 17 transcripts with different 5’ and 3’ UTR-s from human BDNF gene (Pruunsild et al., 2007).

The mouse and rat BDNF gene (Figure 1B) has eight untranslated 5’ exons and one protein coding 3’ exon. Each 5’ exon has a separate promoter and can be alternatively spliced to the 3’ protein coding exon (Timmusk et al., 1993; Aid et al., 2007). As human BDNF exon II, rat BDNF exon II undergoes cryptic splicing producing three splice variants of different lengths. Alternative usage of BDNF promoters leads to transcription of eleven different rodent BDNF transcripts with different 5’ exons and a common 3’ protein coding exon (Aid et al., 2007). In addition, tripartite transcript containing exons VII and VIII has also been described (Liu et al., 2006). Rodent exon IX also contains two different polyadenylation signals leading to transcripts with different 3’ UTR lengths (Timmusk et al., 1993). Exon I also contains in-frame ATG, leading to translation of BDNF protein with longer N-terminus (Koppel et al., 2015).

BDNF gene in chicken (Yu et al., 2009), pond turtle (Ambigapathy et al., 2013, 2014), sea bass (Tognoli et al., 2010) and zebrafish (Heinrich and Pagtakhan, 2004) is structurally similar to that of human and rodent genes with 5’ untranslated exons being spliced to single 3’ protein coding exon. It is interesting to note that in pond turtle there have been described existence of transcript with truncated 3’ end leading to truncated BDNF protein expression (Ambigapathy et al., 2014). This is only the second described splicing event known to take place in BDNF protein coding region with the first one being rare in-frame deletion in rat BDNF possibly also leading to expression of truncated protein (Liu et al., 2006).

1.7. BDNF gene regulation

Complex structure of the BDNF gene also allows its complex regulation at epigenetic, transcriptional, mRNA trafficking and translational level. Many regulatory mechanisms at these steps are responsive to neural activity, starting

from neural activity activated transcription, chromatin modifying complexes, and culminating with increased mRNA transport, stability and translation.

Regulatory elements and binding factors at BDNF promoters

Multitude of regulatory elements have been mapped to different BDNF promoters (recently reviewed in West et al., 2014), some of them act by local chromatin modification, some by directly recruiting core transcription machinery. Out of different characterised BDNF promoters, expression from promoters I and IV is highly induced in response to neural activity, and regulation of both have been extensively studied. Promoter I has been shown to contain *cis*-regulatory elements PasRE, binding basic helix-loop-helix (bHLH)-PAS transcription factor neuronal PAS domain protein 4 (NPAS4) and aryl hydrocarbon receptor nuclear translocator 2 (ARNT2) heterodimer (NPAS4-ARNT2) (Pruunsild et al., 2011); CRE, binding cAMP response element binding protein (CREB) (Tabuchi et al., 2002); E-box elements binding upstream stimulatory factors (USFs) (Tabuchi et al., 2002); and NFkB regulatory elements, binding nuclear factor kappa beta (NFkB) (Lubin et al., 2007). Transcription from promoter I is also influenced by neuron-restrictive silencing element (NRSE) in promoter II that binds repressor element 1 silencing transcription factor (REST) and represses transcription from promoters I and II (Timmusk et al., 1999; Zuccato et al., 2003). Promoter IV contains regulatory elements CaRE1, binding calcium response factor (CaRF) (Tao et al., 2002); CaRE2, binding upstream stimulatory factors 1/2 (USFs 1/2) (Chen et al., 2003b; Pruunsild et al., 2011); CaRE3/CRE, binding CREB (Shieh et al., 1998; Tao et al., 1998); hHLHB2-RE binding basic helix-loop-helix transcription factor bHLHB2 (Jiang et al., 2008); PasRE, binding NPAS4-ARNT2 heterodimer (Lin et al., 2008; Pruunsild et al., 2011) and NFkB-RE, bound by NFkB (Lipsky et al., 2001). Promoter IV also binds transcription factors myocyte enhancer factor 2 (MEF2) (Lyons et al., 2012) and methyl CpG binding protein 2 (MeCP2) (Martinowich et al., 2003). All these elements and transcription factors are to lesser or greater extent involved in calcium-dependent induction of transcription from promoters I and IV with PasRE/NPAS4-ARNT2 control at promoter I (Pruunsild et al., 2011) and CaRE3/CREB control at promoter IV (Hong et al., 2008) having critical role in transcriptional regulation of these promoters. In addition to these experimentally shown transcription factors, Aid-Pavlidis et al. have predicted *in silico* a number of transcription factor binding sites in BDNF promoters that are conserved in genes co-expressed with BDNF in different tissues/conditions/organisms using publicly available microarray data (Aid-Pavlidis et al., 2009).

Transcriptional control from gene promoters is also affected by distal regulatory elements like enhancers and insulators (Smallwood and Ren, 2013). Work with transgenic animals using genomic regions from BDNF gene locus

have shown that BDNF gene has regulatory regions outside of areas covered by these transgenes (Timmusk et al., 1995; Guillemot et al., 2007). Enhancer element 6.5 kb upstream of promoter I, binding transcription factor MEF2D, has been shown to control activity dependent transcription from this promoter (Flavell et al., 2008). A distal regulatory locus controlling BDNF expression has been mapped 850 kb upstream of human and mouse gene. Disruption of this locus leads to BDNF haploinsufficiency phenotype in human and mouse (Gray et al., 2006; Sha et al., 2007).

Regulation of BDNF expression by chromatin modifications

Transcription from genomic locus is influenced by chromatin structure surrounding the gene and its promoters. Acetylation of lysine residues at histones H3 and H4 is mostly associated with transcriptional activation and open chromatin. Histone methylation effect is more dependent on the target lysine residue and nucleosome positioning on genome, with H3 lysine 4 and 14 methylation associated with transcriptional activation and H3 lysine 9 associated with repression. The histone modification-dependent effect to gene expression is modulated by chromatin modifying proteins – histone acetyltranferases (HATs) and deacetylases (HDACs); histone methyltransferases (HMTs) and demethylases (HDMs) (Kouzarides, 2007). In addition, DNA methylation of cytosine residues in CpG islands at promoters is associated with transcriptional repression (Moore et al., 2013).

Several stimuli that influence BDNF transcription are associated with change in acetylation status at corresponding BDNF promoters. Seizure (Huang et al., 2002; Tsankova et al., 2004), membrane depolarisation (Chen et al., 2003a; Martinowich et al., 2003), treatment with antidepressants (Tsankova et al., 2006; Yasuda et al., 2007), cocaine administration (Kumar et al., 2005) and its forced abstinence (Sadri-Vakili et al., 2010), extinction of conditioned fear (Bredy et al., 2007), light deprivation (Karpova et al., 2010), and exercise (Tsankova et al., 2006) have been shown to increase histone acetylation at BDNF promoters together with increased BDNF expression.

Neural activity-induced changes in histone methylation have also been described at BDNF promoters. Membrane depolarisation increases transcription-activating histone methylation and reduces repressive methylation at promoter IV (Chen et al., 2003a; Martinowich et al., 2003). Similarly, environmental enrichment increases activating histone methylation at promoters II and IV and decreases repressive ones at promoters III and IV (Kuzumaki et al., 2011). Chronic social defeat stress (Tsankova et al., 2006), light deprivation (Karpova et al., 2010) and increasing NAD levels (Chang et al., 2010) increase repressive histone methylations at BDNF promoter IV. Activity-dependent changes in DNA methylation at BDNF promoters have been shown in response to membrane depolarisation (Martinowich et al., 2003), exercise (Tsankova et

al., 2006), light-deprivation (Karpova et al., 2010), early-life abusive behaviour (Roth et al., 2009), contextual fear learning (Lubin et al., 2008) and change in NAD levels (Chang et al., 2010).

Several mechanisms responsible for regulating histone and DNA modifications at BDNF promoters have been described. Quite often these modifications are interdependent on each other and are associated with neural activity-induced upregulation of BDNF expression. Neural activity-induced phosphorylation of CREB recruits histone acetyltransferase CBP (CREB binding protein) to BDNF promoter IV (Hong et al., 2008). MeCP2, binding to methylated CpGs, recruits mSin3A/HDAC1 repressor complex at promoters I, IV (Chen et al., 2003a; Martinowich et al., 2003; Tian et al., 2010) and VI (Rousseaud et al., 2015) thereby facilitating repression of these promoters at resting conditions. Upon calcium/neural activity-dependent phosphorylation of MeCP2, it is released from promoters, allowing binding of activating factors to these sites, for example CREB to promoters I and IV (Chen et al., 2003a; Martinowich et al., 2003; Tian et al., 2010). Loss of CTCF/cohesin binding to promoter IV also leads to increased DNA methylation, MeCP2 binding and increased repressive histone methylation at the promoter (Chang et al., 2010). HDAC2, HDAC4 and HDAC5 have also been shown to regulate BDNF expression with HDAC2 being associated with promoters I, II and IV (Guan et al., 2009; Gräff et al., 2012) and HDAC4 as well as HDAC5 being responsible for repression of promoter IV (Koppel and Timmusk, 2013). Neural activity-dependent DNA demethylation at promoter IX has been shown to be mediated by Gadd45b and TET1 (Ma et al., 2009; Guo et al., 2011). Changes in chromatin modifications at BDNF locus have also been associated with age and Alzheimer's Disease, with lower levels of histone acetylation and increased repressive methylation leading to lower BDNF expression in both cases (Walker et al., 2012).

BDNF mRNA localisation, stability and translational control

BDNF gene expression is also regulated post-transcriptionally by regulation of mRNA localisation, stability and translatability. This is made possible in part by combination of alternating 3' and 5' untranslated regions in BDNF mRNAs that have been shown to contain sequences responsible for localisation, stability or translation of BDNF mRNAs in response to various stimuli.

Different BDNF transcripts have been demonstrated to have markedly different localisation within cell compartments. This subcellular localisation of BDNF transcripts is in part based on 5' noncoding exons with transcripts containing exons II and VI localised in soma and proximal dendrites and transcripts containing exons I and IV restricted to soma (Pattabiraman et al., 2005; Aliaga et al., 2008; Chiaruttini et al., 2008). BDNF 3' UTR has been shown to be partly responsible for localisation of mRNAs containing short 3'

UTR to soma and transcripts with long 3' UTRs to dendrites (An et al., 2008). Targeting of BDNF mRNAs to dendrites is also controlled by neural activity (Tongiorgi et al., 1997, 2004; An et al., 2008). Exercise and antidepressant treatment have been shown to increase BDNF exon VI-containing mRNA trafficking to dendrites (Baj et al., 2012). Some mechanisms responsible for targeting of BDNF mRNAs to distinct cellular compartments have been characterised. Protein coding region in BDNF mRNAs contains constitutively active dendritic targeting signal bound by RNA trafficking protein translin that regulates trafficking of BDNF mRNAs at resting and depolarised conditions (Chiaruttini et al., 2009; Wu et al., 2011). BDNF short 3' UTR also contains two cytoplasmic polyadenylation element like elements that interact with CPEB-1 and are responsible for constitutive and activity dependent targeting of BDNF mRNAs (Oe and Yoneda, 2010). There is however some doubt whether the 3' UTR is responsible for the dendritic localisation of BDNF mRNAs because recently both were seen to be expressed at low levels, localised to soma and to be induced the same amount in rat hippocampal neurons (Will et al., 2013).

Two elements have been found in BDNF transcripts that have effect on their stability. A conserved AU-rich element in BDNF long 3' UTR interacts with HuD protein, leading to stabilisation of these transcripts (Lim and Alkon, 2012; Allen et al., 2013) and a stem-loop secondary structure in BDNF short 3' UTR have been shown to be responsible for activity-dependent control of BDNF mRNA stability (Fukuchi and Tsuda, 2010).

The different BDNF UTRs also have effect on activity-dependent translation of BDNF mRNAs. This was first seen in increased association of short 3' UTR containing mRNAs with polysomes (Timmusk et al., 1994b). Long BDNF 3' UTR represses translation of BDNF protein at rest, while short 3' UTR containing BDNF transcripts are translated to keep basal BDNF levels. Upon neuronal activity, long 3' UTR-containing BDNF transcripts are localised to polysomes and translation is induced (Lau et al., 2010). Different 5' and 3' UTRs also affect the translatability of BDNF mRNAs in response to treatment with different stimulators of BDNF expression suggesting the existence of quantitative code for regulated protein expression (Vaghi et al., 2014). Transcripts containing exon I have an additional in-frame AUG that functions as a more efficient translation initiation codon than the conventional start-codon at exon IX (Koppel et al., 2015). Activity-dependent dendritic BDNF synthesis has also been shown to be regulated by eukaryotic elongation factor 2 (eEF2K) (Verpelli et al., 2010). In addition to increasing transcript stability, neural activity-dependent HuD binding to BDNF 3' UTR relieves basal repression of BDNF mRNA translation in dendrites, leading to increased BDNF synthesis (Vanevski and Xu, 2015).

Non-coding RNA regulation of BDNF

Non-coding RNA species (ncRNAs) exert influential control over gene expression by having effect on chromatin structure and transcript stability. miRNAs mediate their effects through degradation of target mRNAs by RISC complex or also through their effects on chromatin, while many long ncRNAs transcribed from antisense strand of mammalian protein coding genes (natural antisense transcripts) have been shown to repress expression of gene by remodelling chromatin or associating with gene transcripts as dsRNA (Tushir and Akbarian, 2014).

Importance of miRNA-based regulation for neural differentiation have been demonstrated with conditional Dicer knockout mice, where forebrain specific knockdown of Dicer led to loss of brain specific miRNAs and also increased BDNF expression (Konopka et al., 2010). Several miRNAs have been shown to regulate BDNF mRNAs – miR-30a-5p (Mellios et al., 2008; Müller, 2014), miR-15a (Friedman et al., 2009), miR-22 (Muiños-Gimeno et al., 2011), miR-206 (Lee et al., 2012; Miura et al., 2012), miR-10b-5p (Müller, 2014), miR-1, miR-10b, miR-155 and miR-191 (Varendi et al., 2014). BDNF locus in human (Liu et al., 2005; Pruunsild et al., 2007) and mouse (Modarresi et al., 2012) has been shown to transcribe natural antisense ncRNA gene (BDNFOS) starting from promoter on opposite DNA strand downstream from exon IX. Transcripts from the antisense BDNF gene form dsRNA duplexes with BDNF transcripts (Pruunsild et al., 2007) and regulate BDNF levels *in vivo* through polycomb repressive complex 2 mediated repressive chromatin remodelling (Modarresi et al., 2012).

1.8. Transgenic mice used for studying BDNF regulation

Several transgenic mice have been developed for studies of BDNF gene regulation, providing insight into genomic regions responsible for its tissue and activity-specific expression. Plasmid based mini-gene constructs using 9kb rat BDNF gene regions containing exons I-III or exons IV-VI together with CAT reporter gene were used to study tissue-specific, axotomy and neuronal-activity induced BDNF regulation in transgenic mice (Timmusk et al., 1995). While transgene expression in these mice recapitulated endogenous BDNF expression in several brain regions and peripheral tissues, there were shortcomings in expression patterns across tissues, possibly due to the lack of distal regulatory elements and transgene positional effects from random insertion into the mouse genome. These effects could be avoided using larger genomic fragments for generation of transgenic mice as was done by Guillemot et al., who used yeast artificial chromosome (YAC) containing 145 kb of human BDNF locus modified to encode EGFP reporter gene. The obtained mice still did not fully recapitulate endogenous BDNF expression patterns, indicating that more distal

elements regulating the gene were still missing from the transgene (Guillemot et al., 2007).

1.9. Bacterial Artificial Chromosomes

Ideal transgenic model for use in studying gene regulation would contain all the endogenous regulatory elements of the gene under study and also should be isolated from transgene positional effects in the genome. Transgenic animals and cell lines using plasmid or mini-gene constructs containing limited regulatory regions of gene under study have shown that these vectors are usually restricted by their capacity, positional effects and only partially recapitulate endogenous expression of the transgene. Increasing the size of the included genomic area surrounding the transgene increases faithfulness of reporter gene expression and avoids positional effects in transgenic animals and cell lines. This has been done using large capacity vectors systems like yeast artificial chromosomes, P1 artificial chromosomes, and bacterial artificial chromosomes (BACs) (Giraldo and Montoliu, 2001). Out of these, BAC vector system was developed as a high-capacity cloning system based on *E. coli* F plasmid for genomic library construction in Human Genome Project (Shizuya and Kouros-Mehr, 2001).

BAC is capable of stably containing genomic fragments up to 300 kb in size (Shizuya et al., 1992), meaning it is capable of containing most mammalian genes together with possible upstream and downstream regulatory regions. In addition, compared to YACs that need careful handling and sometimes display chimaerism and instability, working with BACs is similar to working with traditional plasmid constructs and BACs are highly stable in *E. coli*. Use of traditional molecular cloning methods to introduce modifications to BAC DNA is, however, challenging due to their large size. Therefore, alternative methods have been developed to facilitate introduction of deletions, insertions or mutations. Most common of these is homologous recombination in *E. coli* using either RecE and RecT proteins from prophage (Zhang et al., 1998) or functionally analogous system based on Redα, Redβ and Redγ proteins from λ phage Red operon (Muyrers et al., 1999; Poteete, 2001). Homologous recombination allows site-directed modification of BAC using selection cassette with homologous arms to target region. Additionally, it can be used for seamless BAC modification by using counter-selection targeting cassette (Bird et al., 2012). Commercial kits (from Genebridges GmbH) and non-commercial plasmid-based systems (Hartwich and Nothwang, 2012) for recombineering are available and well established protocols for the use of the method for generating transgenic constructs have been developed (Hollenback et al., 2011). In addition to introducing changes to BACs, recombination system have been used for subcloning of BAC regions to smaller plasmids using gap repair (Lee et al.,

2001a; Hartwich and Nothwang, 2012), combine regions from overlapping BACs to assemble a larger gene locus (Zhang and Huang, 2003; Kotzamanis and Huxley, 2004) and rapid addition of multiple mutations to BACs using oligonucleotides (Swaminathan et al., 2001). Recombination has also been optimised for high-throughput BAC modification (Poser et al., 2008; Gong et al., 2010). In addition to homologous recombination, Cre/loxP and Flpe/FRT recombinase systems have been used for BAC modifications, for example removal of selection cassettes (Parrish et al., 2011), introduction of resistance/episomal maintenance cassette (Magin-Lachmann et al., 2003), and truncation of BAC ends (Shakes et al., 2005).

Due to the use of BACs in genome sequencing projects, large BAC libraries covering entire genomes for human, rat, mouse and other organisms are available (https://bacpac.chori.org/). This, in addition to size capacity, convenient modification and handling methods, have made BACs popular in generation of transgenic mice using pronuclear injection (Yang et al., 1997) or embryonic stem cell transgenesis (Kaufman et al., 1999). One of the best examples of using BACs to study gene expression in central nervous system is GENESAT project, which used BACs of nervous system expressed genes to generate over 10000 transgenic mouse lines expressing EGFP reporter gene under the control of various gene regulatory regions (Gong et al., 2010; Schmidt et al., 2013). This annotated repository of BAC transgenic mouse lines has been subsequently used for purification and functional analysis of specific cell types in nervous system (Gong et al., 2003; Zhang et al., 2014) and generation of Cre driver lines for targeting specific CNS cell types (Gong et al., 2007). Use of multiple BAC transgenesis into single mouse locus has also been demonstrated, significantly speeding up generation of transgenic mice with multiple marked cell populations and making it possible to better study functional interactions between different cell types (Dougherty et al., 2012).

In addition to generation of transgenic mice, BACs have also been used in cell culture systems for gene regulation and protein function studies, quite often for the same reasons that made them useful in mice. Delivery and expression of transgenes from BACs in mammalian cells have been demonstrated using lipid or polyamine-based transfection (Montigny et al., 2003; White et al., 2003; Magin-Lachmann et al., 2004; Schwank et al., 2013), electroporation (Abranches et al., 2013), nucleofection (Placantonakis et al., 2009), gene gun (Smith-Hicks et al., 2010), viral delivery (Wade-Martins et al., 2003; Inoue et al., 2004) or bacterial transfer (Narayanan and Warburton, 2003; Laner et al., 2005). Also, BAC vectors have been retrofitted with oriP/EBNA-1 sequences allowing episomal maintenance in mammalian cells (Wade-Martins et al., 1999; Magin-Lachmann et al., 2003; Eeds et al., 2007). Compared to small plasmid expression vectors, expression from BAC transgenes seems to take longer time to develop (3-5 days) but is more persistent (Montigny et al., 2003). This has

led to use of BACs in development of expression vectors based on genomic regions with known open chromatin context (for example housekeeping genes) for recombinant protein production in mammalian cells. These vectors provide higher protein yields and transgene stability than most traditional plasmid based protein expression systems (Blaas et al., 2009; Bian and Belmont, 2010; Mader et al., 2013; Zboray et al., 2015).

Using BACs as transgenic vectors, several elegant models for gene regulation and functional studies in mammalian cells have been developed. Large scale BAC modification and transgenesis has allowed their use in protein function studies in tissue culture and mouse embryonic stem cells (Poser et al., 2008). In addition, it has been used in gene function studies for complementation of genetic deficiency in mammalian cells (Wade-Martins et al., 2000, 2003; Inoue et al., 2004), studies of Cdc6 protein function during cell-cycle (Illenye and Heintz, 2004), effect of single nucleotide polymorphisms on RNA splicing and processing in whole gene context (Eeds et al., 2007), and to elucidate the necessity of a specific transcription factor binding site in Arc gene locus for its functioning in establishing late phase of LTD in cerebellar Purkinje cells (Smith-Hicks et al., 2010). Furthermore, BAC transgenesis has been used in human and mouse embryonic stem cell cultures to mark cell pluripotent state (Abranches et al., 2013) and study cell fate and signalling during differentiation (Placantonakis et al., 2009) as well as in intestinal epithelial organoid cultures to mark cell lineage (Schwank et al., 2013). In addition to these works, cell lines using BAC transgenes have been established for disease related genes which allow high-throughput screening of drug candidates for therapeutic purposes (Li et al., 2013). All these experimental systems have benefitted from inclusion of entire genomic locus in inserted BAC transgene, which provides close to endogenous gene environment aimed at native functioning of chromatin, transcriptional and RNA processing mechanisms for faithful reporter gene expression.

2. AIMS OF THE STUDY

The purpose of this study was to develop and study transgenic model systems for elucidating the regulation of BDNF gene. For this the following aims were set:

1) Generation and characterisation of BAC transgenic mouse and cell lines carrying human or rat BDNF gene locus.

2) Studying BDNF gene regulation using the generated BAC transgenic mouse and cell lines.

3. MATERIALS AND METHODS

3.1. Semiquantitative RT-PCR analysis

Publications I and II

3.2. PCR genotyping

Publications I and II

3.3. Quantitative RT-PCR analysis

Publications I and II

3.4. Cell culture, transfection and stable cell line generation

Publication III

3.5. Luciferase reporter assay

Publication III

3.6. FACS analysis of reporter expression

Publication III

3.7. Quantitative qPCR analysis of transgene copy number

Publication III

3.8. Fluorescent *in situ* hybridisation (FISH) analysis

Publication III

3.9. rBDNF-lacZ copy number analysis by slot-blot hybridisation

Transgene copy number was analysed in rBDNF-lacZ mouse line by slot-blot hybridisation of genomic DNA with a [α-^{32}P]dCTP-labelled probe generated with DecaLabel DNA labelling kit (Fermentas, Lithuania) using rBDNF-lacZ BAC specific probe generated with primers 3p_II_s (5'-CCGGGGAGATGTGTTTCTAA-3') and 3p_II_as (5'-CACTCAGAAGCCTTGGGAAG-3') as a template.

3.10. Genome walking

Genome walking was used to map transgene integration sites in rBDNF-lacZ transgenic mouse line using protocol described in GenomeWalker Universal Kit (Clontech Laboratories Inc., Cat. No. 638904) with primers and adaptor oligos ordered from Microsynth (Switzerland). Transgene and adaptor-specific primers used for genomic fragment isolation are shown in Table 1. Fragments were amplified using HotFire DNA polymerase (Solis Biodyne, Estonia). Fragments amplified by genome walking were sequenced and resulting sequences were searched against mouse genome using NCBI Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi). After mapping the integration site to DGK-β locus, it was confirmed with locus specific primers mDGKB_ctr1_s and mDGKB_ctr1_as. Tandem integration of several transgenes was also confirmed by PCR with primers ch230_3p_gw_2 together with ch230_5p_gw_1. PCR products were confirmed by sequencing.

Table 1. Primers used for integration site mapping by genome walking, integration site confirmation.

Primer name	Primer sequence (5'-3')	Description
Adp1	GTAATACGACTCACTATAGGGC	Adaptor primer 1
Adp2	ACTATAGGGCACGCGTGGT	Adaptor primer 2
ch230_3p_w17_n1	ATGTTACTGGTGATGGACTTAGTTAGC	Rat BAC specific primers for Genome Walking
ch230_3p_w17_n2	CTTAGTTAGCAGGGAGTATCTGAGTTG	
ch230_3p_w27_n1	CTGATTTGATACAAAGAGGAGACAGAC	
ch230_3p_w27_n2	TACAAAGAGGAGACAGACTGAACTAAG	
ch230_3p_w31_n1	AAGGCAGGGTGCTGTAAATCTCAG	
ch230_3p_w49_n1	CTATACACATAGGAAGCCTAACATGG	
ch230_3p_w49_n2	GAGAGGTTGTAGAAACAAATCTCCAC	
ch230_5p_w11_n1	GAGTATTCATATGCACCCCTAAAGAG	
ch230_5p_w11_n2	GAATACCTTTCACCAAGTACAGTCAC	
ch230_5p_w5_n1	ATCGTGACTACTAAGTATTGAGCACTG	
ch230_5p_w5_n2	GGTTAGCAACTGATAGGATGAGAACTA	
mDGKB_CTR_F	CAGAGATGTGTGAGATGATTCCA	Integration site control primers
mDGKB_CTR_R	CCATGCTGCCAAAAGAAAAGTAGCATA	
ch230_3p_w31_n1	AAGGCAGGGTGCTGTAAATCTCAG	
ch230_5p_w11_n2	GAATACCTTTCACCAAGTACAGTCAC	
ch230_3p_gw_2	TATGAAGAAGGCAGTTCCACAGAGTGAT	Tandem site control primers
ch230_5p_gw_1	AGCTGAGAATTCCCTATGAAGATCCTTC	

3.11. DGK-β RT-PCR analysis

To probe DGK-β expression in rBDNF-lacZ transgenic mouse line tissues, RT-PCR was performed on cortex cDNAs using primers mDGKb_ex20s/mDGKb_ex22as for transcripts containing DGK-β exons 20-22

and mDGKb_ex18s/mDGKb_ex20as for transcripts containing DGK-β exons 18-20.

Table 2. Primers used for RT-PCR analysis of DGK-β transcripts.

Primer name	Primer sequence (5'-3')
mDGKb_ex18s	GACTGGCAATGACTTAGCAAGG
mDGKb_ex20as	GTGCTTTTCTCTCATGATGTGG
mDGKb_ex20s	ATTCCACATCATGAGAGAAAAGC
mDGKb_ex22as	AAACTTCAGCTCTTTTGCATCTG

4. RESULTS AND DISCUSSION

4.1. BDNF BAC transgenic mice display reporter mRNA expression similar to endogenous gene (publications I and II)

To study BDNF regulation, BAC transgenic mice were generated using BAC constructs containing human and rat BDNF gene that were modified to contain reporter proteins. The hBDNF-EGFP BAC contained 168 kb of the human BDNF gene together with regions 84 kb upstream and 17 kb downstream of the gene and the protein coding region was modified to encode for hBDNF-EGFP fusion reporter gene (publication I). The rBDNF-lacZ BAC contained 207 kb of rat BDNF gene together with genomic regions 13 kb upstream and 144 kb downstream with BDNF coding sequence replaced with β-galactosidase (lacZ) reporter gene (publication II). Using BACs as vectors for generation of transgenic mice was expected to negate positional effects of genomic insertion site and also provide sufficient native context for the endogenous like expression of the reporter gene. Altogether, pronuclear injection yielded four transgenic founder lines – hBDNF-EGFP BAC transgenic lines C3, E1 and E4 and one rBDNF-lacZ BAC transgenic mouse line.

Out of three transgenic cell lines established with hBDNF-EGFP BAC, C3 transgenic mouse line expressed reporter mRNA most similarly to endogenous mouse BDNF in different parts of the brain and in thymus, lung, skeletal muscle and testis. In two founder lines E4 and E1 transgene mRNA expression pattern deviated more from endogenous mouse BDNF mRNA with E1 showing expression only in midbrain, medulla, cerebellum and in thymus, lung and kidney, and E4 showing reporter expression only in thymus and testis (publication I). In C3 line, all transgenic transcripts with different human 5' exons were transcribed in the hippocampus of transgenic mice (publication I).

In rBDNF-lacZ BAC transgenic mice lacZ reporter mRNA expression resembled endogenous BDNF expression in the brain, heart and lung. However, there were also differences from endogenous BDNF expression including no detectable transgene expression in thymus, liver, kidney, skeletal muscle, and dentate granule cells of the hippocampus. Transgene was also expressed in striatum, olfactory bulb granular layer, caudate putamen, nucleus accumbens, and testis – regions where endogenous mouse BDNF mRNA was not detected (publication II).

In both hBDNF-EGFP-C3 and rBDNF-lacZ mouse lines, treatment with kainic acid induced transgene mRNA levels correspondingly to respective endogenous BDNF mRNAs in hippocampus and cortex (publication I and II), indicating that regulatory elements for activity-dependent BDNF expression

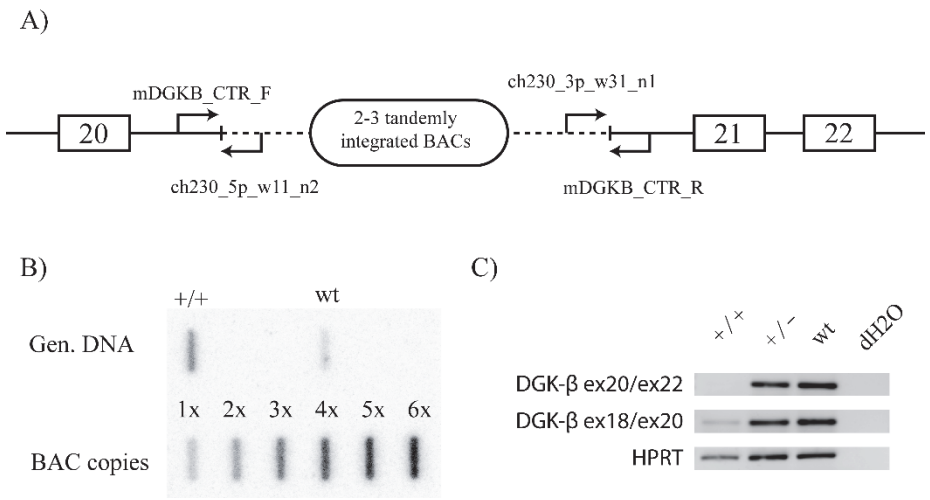

Figure 2. rBDNF-lacZ transgene integration into DGK-β gene intron in rBDNF-lacZ mouse line disrupting endogenous DGK-β expression. (A) Genome walking placed transgene insertion into intron of DGK-β gene between exons 20 and 21. Primers used for confirming integration site are marked with arrows. Dashed line shows integrated BAC sequences. (B) Slot-blot hybridisation analysis of transgene copy number in rBDNF-lacZ mouse line. BAC standard contains rBDNF-lacZ BAC DNA in 1-6 copies in the blotted genomic DNA. (+/+) homozygous rBDNF-lacZ mouse DNA, wt – wild-type mouse DNA. (C) RT-PCR analysis of DGK-β transcripts containing exons 18-20 in cortex of homozygous (+/+), heterozygous (+/-) rBDNF-lacZ transgenic and wild-type (wt) mice.

were included in the genomic regions covered by the BACs. Induction of reporter mRNA in the hBDNF-EGFP-C3 mouse line is the first demonstration of neural-activity dependent activation of transcription from the human BDNF gene. This mouse line has been used in further studies, that are not part of this thesis, showing that this line is useful for studies of human BDNF gene regulation (Pruunsild et al., 2011).

The varying expression patterns of reporter mRNA in different hBDNF-EGFP BAC transgenic mice lines with similar transgene copy numbers (publication I) suggests that the genomic region in the BAC is not sufficient to overcome the positional effects of transgene insertion. Positional effects influencing reporter expression were also seen in the rBDNF-lacZ transgenic mouse line. In rBDNF-lacZ mouse line, 2-3 copies of BAC construct had integrated into the mouse genome between exons 20 and 21 of diacylglycerol kinase β (DGK-β) gene (Figure 2A and B). Integration of the transgene into this locus disrupted the expression of the endogenous DGK-β transcripts (Figure 2C). It is possible that high expression of reporter mRNA in caudate putamen and nucleus accumbens (and maybe also granular layer of olfactory bulb), areas where endogenous mouse BDNF mRNA is not expressed or is expressed at low

levels, is due to the integration site-specific effects because DGK-β has been shown to be strongly expressed in these brain regions (Goto and Kondo, 1993). Altogether, this suggests that while BAC constructs used in this work contain many of the regulatory elements necessary for reproducing the tissue-specific expression of the BDNF transgene, these are not sufficient to fully protect transgenes from position effects.

Neither hBDNF-EGFP fusion protein nor β-galactosidase reporter protein was detected in hBDNF-EGFP-C3 or rBDNF-lacZ mouse brain, respectively, in basal or kainic acid induced conditions, while mRNAs encoding for the transgenic transcripts were readily detected. When compared to endogenous BDNF mRNA levels, transgene expression was about ten-fold lower in hBDNF-EGFP-C3 transgenic line (publication I). Copy numbers of integrated BAC transgenes were low in both transgenic mouse lines and it has been shown in GENSAT project that integration of fewer than five BAC transgene copies into genome may lead to very low expression levels that can cause problems detecting reporter expression (Gong et al., 2003). It is possible that low transgene copy numbers together with missing regulatory elements lead to very low reporter protein levels that were below the detection limit in both hBDNF-EGFP-C3 and rBDNF-lacZ transgenic lines.

Several transgenic mouse lines have been developed for studies of BDNF gene regulation. Transgenic mice containing different rat BDNF promoter regions fused to the CAT reporter gene, while recapitulating BDNF expression in some tissues, also showed minimal or lack of transgene expression in dentate granule cells of hippocampus and heart (Timmusk et al., 1995). Five transgenic mouse lines were generated by Guillemot et al. using YAC containing 145 kb of human BDNF gene with regions 45 kb upstream and 33 kb downstream sequences, where BDNF protein coding region was replaced with EGFP reporter gene (Guillemot et al., 2007). Some indications of tissue-specific regulatory regions can be made based on the expression patterns of these previously developed mice and transgenic lines characterised by us. Similarly to previously generated rat promoter CAT transgenic mice, where reporter was not expressed (or was expressed at low levels) in dentate gyrus granule cells (Timmusk et al., 1995), rBDNF-lacZ mouse line did not replicate the BDNF expression in hippocampal dentate gyrus granule cells, while in hBDNF-EGFP-C3 mouse line reporter expression in these cells was detectable after kainate treatment (publication I). However, expression of the reporter gene in some dentate granule cells in human YAC transgenic mice lines (Guillemot et al., 2007) suggests that at least some of the regulatory regions responsible for BDNF expression are located in the hBDNF YAC transgene used. It is possible that hBDNF-EGFP BAC used here and hBDNF YAC transgene contained some of the regulatory regions needed for BDNF expression in dentate gyrus cells while in rBDNF-lacZ BAC these elements were missing or masked due to

position effects. While endogenous BDNF is expressed at extremely low levels in rat striatum and at slightly higher levels in mouse striatum (Timmusk et al., 1994b, 1995), rat BDNF-CAT, hBDNF-EGFP-C3 and rBDNF-lacZ BAC transgenic mouse lines all showed strong reporter expression in striatum (Timmusk et al., 1995, publication I and II). The hBDNF-EGFP-C3 mouse line did not express reporter gene mRNA in the heart where endogenous BDNF is expressed, while rBDNF-lacZ line did. This suggests that elements responsible for heart-specific BDNF expression are located 17-144 kb downstream of BDNF gene. One human YAC transgenic mouse line also displayed reporter gene expression in the heart (Guillemot et al., 2007) suggesting that heart-specific regulatory region lies somewhere between 17-33 kb downstream of human BDNF gene.

Previous indications of distal BDNF regulatory areas have come from studies associating disruption of genomic region about 850 kb upstream of BDNF gene with phenotype similar to BDNF haploinsufficiency in human (Gray et al., 2006) and mice (Sha et al., 2007). High-resolution genome interaction data shows two increased association peaks near this genomic region, one 811 kb (chr11:28,550,000-28,555,000, GRCh37/hg19) and another 976 kb (chr11:28,710,000-28,720,000, GRCh37/hg19) upstream of BDNF exon I, both interacting with region comprised of second cluster of BDNF promoters/exons (IV-VI, chr11:27,720,000-27,725,000, GRCh37/hg19) in GM12878 lymphoblastoid cell line (Rao et al., 2014). These peaks reflect the presence of chromatin loops that are often associated with enhancer-promoter interactions. Additional regulatory elements between these distal enhancers and BDNF promoters may exist as evidenced by disruption of genomic region 80 kb upstream of BDNF in some of obese patients of WAGR syndrome (Han et al., 2008) and existence of MEF2D interacting region 6.5 kb upstream of promoter I, which possibly acts as a proximal enhancer taking part in activity-induced BDNF transcription from promoter I (Flavell et al., 2008).

The high level of similarity between transgene and endogenous BDNF expression in basal and neural activity-induced conditions in both hBDNF-EGFP-C3 and rBDNF-lacZ BAC transgenic lines developed here shows that they are useful models for *in vivo* studies of BDNF gene regulation.

4.2. BAC transgenic cell lines for screening of modulators of BDNF expression (publication III)

Transgenic cell lines are often used for studies of gene expression regulation. Transgenic mice generated using rat BDNF BAC showed that it contains regulatory elements for mostly faithful reporter gene mRNA expression under basal and induced Ca^{2+} signalling conditions (publication II). For more convenient screening of factors regulating BDNF expression, we developed

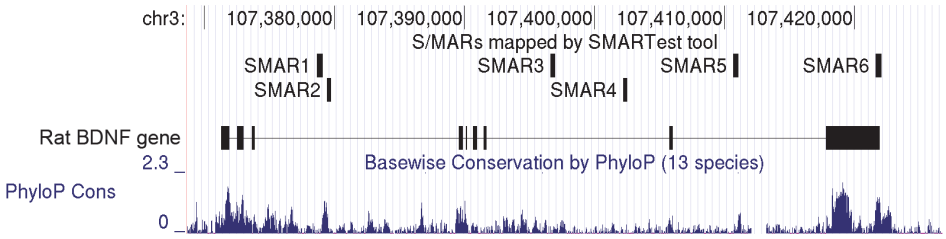

Figure 3. S/MAR elements predicted with SMARTest tool mapped to rat BDNF gene. Coordinates of the displayed rat genomic locus are chr3:107368728-107426727 (RGSC 5.0/rn5).

BAC transgenic cell lines in HeLa cell background using rat BDNF BAC, where the BDNF protein coding region was replaced by hRluc-EGFP reporter gene using homologous recombination (publication III). Previously, use of BACs as transgene vectors for generating cell lines for drug screening have shown that these cell lines faithfully express reporter genes and can be used for large scale screening of possible modulators of gene under study (Li et al., 2013).

In all HeLa hBDNF-hRluc-EGFP BAC cell lines we generated, detection of EGFP fluorescence and hRluc luminescence showed that hRluc-EGFP reporter gene was expressed (publication III). Expression of most of the transgenic transcripts also showed that entire rat BDNF gene was maintained in transgenic cell lines (publication III). High copy numbers of transgenes in all established cell lines led to suggest that transgenic construct was maintained episomally in transgenic cell lines, which was confirmed by FISH analysis (publication III). In all established cell lines, the reporter gene under the control of rat BDNF promoter regions was induced in response to ionomycin-mediated Ca^{2+} signalling (publication III), treatment with histone deacetylase inhibitors (publication III) and by overexpression of VP16-CREB and NPAS4+ARNT2 heterodimer transcription factors (publication III), all known to induce BDNF expression (Pruunsild et al., 2011).

Given the previous reports that plasmids containing matrix attachment regions (MARs) are maintained episomally as double minute chromosomes in HeLa cells (Shimizu et al., 2001), episomal maintenance of transgene in our reporter cell lines suggests presence of possible scaffold/matrix attachment regions (S/MARs) in the genomic locus contained in rat BDNF BAC. S/MARs are regions on DNA that have been associated with multitude of functions such as anchoring of DNA and maintenance of the nuclear architecture, but also regulation of replication and transcription. The six *in silico* predicted S/MAR elements were all positioned inside the BDNF gene with SMAR1 and 2 positioned in the intron between exons I and II, SMAR 3 and 4 in the intron between exons VII and VIII, SMAR 5 in the intron between exons VIII and IX

and SMAR6 in the 3' UTR of exon IX overlapping the polyA site (Figure 3). Interestingly, SMAR1, SMAR2 and SMAR6 are all located at or near sites of high conservation between different species.

In established transgenic cell lines, BAC transgene was maintained episomally in high copy numbers and therefore both luminescence and fluorescence of hRluc-EGFP reporter protein was readily detected. This is in contrast to our BDNF-BAC transgenic mice where we could not detect reporter protein expression. It is possible that high copy numbers of transgene in reporter cell lines led to readily detectable levels of hRluc-EGFP reporter expression. However low reporter induction in response to different known activators of BDNF expression (~1.5-2 fold) shows that while high transgene copy number provides sufficient levels of reporter protein expression, it may have effect on induction of the reporter. This is best seen with ionomycin treatment on high and low copy number cell lines, with lower copy number cell lines showing higher reporter induction (publication III). This may be due to depletion of transcription factors by their response elements in BDNF regulatory regions in high copy number cell lines. The lack of copy number effect on reporter induction by HDAC inhibitor treatments is probably due to the already open chromatin context of double minute chromosomes and more general effect of increased histone acetylation leading to opening of chromatin at transgenic DNA regions.

The choice of using hRluc-EGFP fusion protein as the reporter enabled to use both fluorescence and luminescence methods for reporter detection. EGFP fluorescence makes it possible to monitor the expression and regulation of transgene at single cell levels using flow cytometry or fluorescent microscopy, while *Renilla* luciferase (hRluc) luminescence allows sensitive detection of reporter using either conventional luminescence detection kits for end point signal detection, or live cell luciferase substrates for continuous monitoring of reporter expression. One drawback of the used hRluc-EGFP protein may be its increased stability due to the EGFP. The half-life of the EGFP has been shown to be 26 hours in mammalian cells (Corish and Tyler-Smith, 1999) and the half-life of hRluc is about 3-4 hours (Promega, personal communication). While the half-life of the hRluc-EGFP fusion protein is not known, it is possible that part of the low induction in response to the tested treatments is due to the high stability of the reporter protein. This effect could be alleviated by the inclusion of a protein destabilising sequence in the reporter gene in future studies.

Another approach to increase the induction fold of the transgene reporter protein in future cell lines would be to control the copy numbers of the BDNF BAC transgene. However, this may lead to a trade-off – low (or single) copy numbers of the transgene may not be enough for the detection of the reporter protein while increasing the transgene copy number may lead to decreased induction fold in response to a modulator. This problem might be solved by

using two-step transcriptional activation (TSTA) system where BDNF locus in BAC vector would encode a strong artificial transcriptional activator, for example Gal4-VP16 or VP16-E2, that would activate the reporter expression from a second promoter controlling the expression of suitable reporter gene. TSTA system have been used for such reporter induction systems and have shown to greatly enhance reporter (or therapeutic) gene expression under the control of weak promoter (Iyer et al., 2001; Arendt et al., 2009). This approach could allow expression from low copy numbers of weakly expressing BAC transgene that would reliably reflect the endogenous BDNF gene and at the same time increase the reporter expression to detectable levels. The exponential effect of reporter induction in TSTA system might even be beneficial for detection of lower effect modulators, when the effects of included noise are accounted for in subsequent analysis.

The robust nature of the HeLa background, readily detectable hRluc-EGFP reporter expression and its induction by ionomycin, HDAC inhibitors and transcription factors known to induce BDNF, make these cell lines well suited for initial screening of factors regulating BDNF gene.

CONCLUSIONS

1) Generation and characterisation of BAC transgenic mouse and cell lines carrying human or rat BDNF gene loci.

   - BAC transgenic mice and cell lines carrying 168 kb of the human or 207 kb of the rat BDNF genomic locus were generated and characterised.

   - Transgenic BAC cell lines carrying 207 kb of the rat BDNF genomic locus were generated and characterised.

2) Studying BDNF gene regulation using the generated BAC transgenic mouse and cell lines.

   - The BDNF BAC transgenic mice developed in this study largely recapitulated endogenous BDNF expression pattern in different tissues and its regulation by neural activity in the brain.

   - Analysis of reporter expression patterns in transgenic mice generated here indicated location of a possible heart-specific regulatory region downstream of BDNF gene.

   - Effect of transgene integration site on reporter expression in BAC transgenic mouse lines indicated that some insulating regulatory elements exist outside of the genomic regions covered by human and rat BDNF BACs.

   - Episomal maintenance of BAC transgene in rat BDNF BAC cell lines indicated presence of possible S/MAR elements in the genomic region included in the studied rat BDNF gene locus.

   - Transgenic BDNF BAC cell lines responded to known regulators of BDNF gene transcription.

   - Established cell lines could be used for screening of BDNF regulators *in vitro* and transgenic mouse lines for further studies of BDNF gene regulation *in vivo.*

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ABSTRACT

Brain derived neurotrophic factor (BDNF), a member of the neurotrophin family, has important functions in development and functioning of the nervous system. In early development, BDNF promotes survival and differentiation of various neuronal populations in the central and peripheral nervous system. In later development and adulthood, it plays important roles in the regulation of synaptic plasticity. Given its involvement in several nervous system disorders, studying BDNF gene regulation provides insight for developing therapeutics for these disorders.

We generated transgenic mouse lines using large capacity bacterial artificial chromosome (BAC) vectors carrying human or rat BDNF genomic loci. Human BDNF BAC transgenic mice were generated using a BAC containing 168 kb of human BDNF genomic region that was modified to encode the BDNF-EGFP fusion reporter gene. Rat BDNF BAC transgenic mice were generated using a BAC containing 207 kb of rat BDNF genomic locus that was modified to encode the β-galactosidase (*lacZ*) reporter gene. Reporter mRNA expression patterns in established transgenic mouse lines largely recapitulated endogenous BDNF expression patterns in the brain and peripheral tissues. Transgenic transcripts were also upregulated in response to neural activity similarly to endogenous BDNF in different brain regions. These results show that BAC transgenes used in generating these transgenic mouse lines contain most of the regulatory regions responsible for endogenous BDNF transcription and its regulation by neural activity. In addition, transgenic mice containing human BDNF BAC open up the possibility to study regulation of the human BDNF gene *in vivo*. The developed transgenic mouse lines are useful for future studies of BDNF gene regulation.

To generate a screening system for regulators of BDNF gene expression, we established several BAC transgenic cell lines in HeLa cell background using a BAC containing 207 kb of rat BDNF genomic region, where the BDNF protein-coding sequence was replaced with the hRluc-EGFP fusion reporter gene. These cell lines had high episomal transgene copy numbers and displayed increased reporter protein expression in response to treatment with known activators of BDNF expression. These cell lines are useful for further studies of BDNF gene regulation and screening of compounds and transcription factors regulating BDNF expression.

Altogether, mouse and cell lines generated and characterised in this work provide useful experimental models for studying BDNF gene regulation and screening regulators of its expression both *in vivo* and *in vitro*.

KOKKUVÕTE

Neurotrofiinide perekonna liige, ajust pärinev neurotroofne tegur (BDNF), omab tähtsat rolli organismi varajases arengus erinevate kesk- ja piirdenärvisüsteemi neuronite populatsioonide elulemuses ja diferentseerimises. Lisaks osaleb BDNF hilisemas arengus ja täiskasvanud organismis ka sünaptilise plastilisusega seotud protsessides. Tulenevalt BDNF valgu tasemete häirumisest erinevate neurodegeneratiivsete ja psühhiaatriliste häirete korral, on BDNF geeni uurimine oluline nende haiguste mehhanismide väljaselgitamiseks ja võimalike ravimeetodite väljatöötamiseks.

Antud töö käigus loodi transgeensed hiire- ja rakumudelid BDNF geeni regulatsiooni uurimiseks. Transgeensed hiireliinid tehti kasutades bakteriaalse kunstliku kromosoomi (BAC) konstrukte, mis sisaldasid inimese või roti BDNF geeni. Inimese BDNF BAC sisaldas 168 kb BDNF geeni genoomset lookust, kus valku kodeeriv osa oli muudetud kodeerimaks BDNF-EGFP liitvalku. Roti BDNF BAC sisaldas 207 kb roti BDNF geeni genoomset lookust, kus valku kodeeriv osa oli asendatud β-galaktosidaasi kodeeriva järjestusega. Loodud transgeensetes hiireliinides oli reporter mRNA-de ekspressioon erinevates kudedes suurel määral sarnane endogeensele BDNF geeni ekspressioonimustrile. Transgeenide ekspressioon reguleerus ka neuraalse aktiivsuse tagajärjel erinevates ajuosades sarnaselt endogeensele BDNF geenile. Inimese BDNF BAC-i sisaldavas hiireliinis transgeeni reageerimine neuraalsele aktiivsusele näitab, et seda hiireliini on edaspidi võimalik kasutada inimese BDNF geeni regulatsiooni uurimiseks *in vivo*. Kokkuvõtvalt võib öelda, et genoomsed regioonid, mis sisaldusid transgeensete hiireliinide loomiseks kasutatud BAC konstruktides, sisaldavad enamikke regulatoorseid elemente, mis on vajalikud BDNF geeni ekspressiooniks. Väljatöötatud transgeensed hiireliinid sobivad BDNF geeni regulatsiooni uurimiseks.

BDNF geeni reguleerivate tegurite sõeluuringuks sai loodud mitu BAC transgeenset HeLa rakuliini, kasutades 207 kb roti BDNF BAC konstrukti, milles valku kodeeriv osa oli asendatud hRluc-EGFP reportergeeniga. Kõigis saadud rakuliinides esines transgeen kõrge koopianumbrilise episoomina ja ekspresseeris reportervalku. Stiimulid, mis teadaolevalt reguleerivad BDNF geeni transkriptsiooni, tõstsid reportervalgu tasemeid BAC transgeensetes rakuliinides. Seetõttu saab neid rakuliine kasutada edaspidisel BDNF geeni regulatsiooni uurimisel ja selle tasemeid reguleerivate ainete otsingul.

BAC transgeensed hiire- ja rakuliinid on sobivad töövahendid edaspidisteks BDNF geeni regulatsiooni uuringuteks nii *in vitro* kui *in vivo* tingimustes.

PUBLICATION I

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

Indrek Koppel†, Tamara Aid-Pavlidis†, Kaur Jaanson, Mari Sepp, Priit Pruunsild, Kaia Palm and Tõnis Timmusk*

Address: Department of Gene Technology, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn, Estonia

Email: Indrek Koppel - indrek.koppel@ttu.ee; Tamara Aid-Pavlidis - tamara.aid@gmail.com; Kaur Jaanson - kaur.jaanson@ttu.ee; Mari Sepp - mari.sepp@ttu.ee; Priit Pruunsild - priit.pruunsild@ttu.ee; Kaia Palm - kaia@protobios.com; Tõnis Timmusk* - tonis.timmusk@ttu.ee

* Corresponding author †Equal contributors

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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].

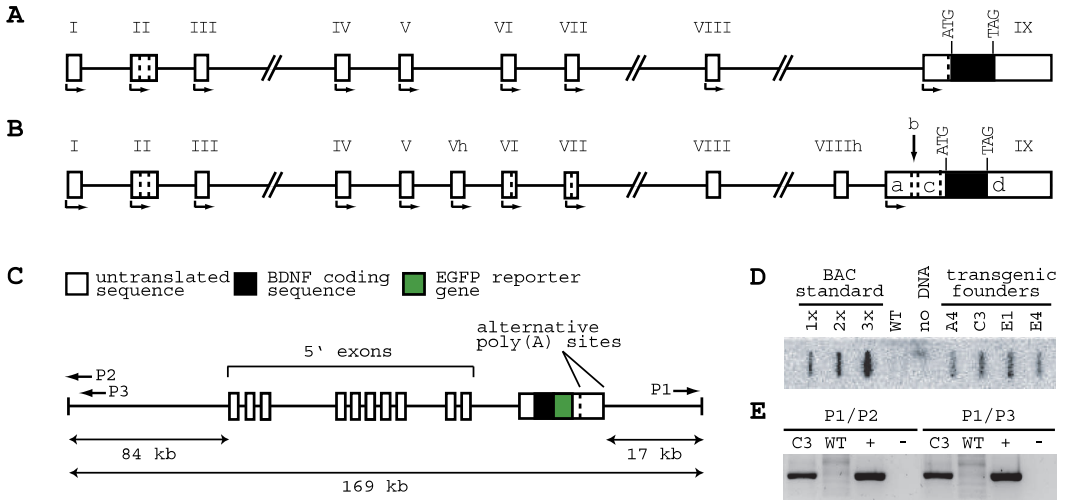

Figure 1

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

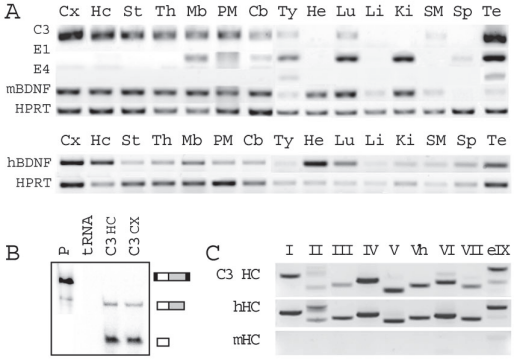

Figure 2
hBDNF-EGFP mRNA expression in tissues of three transgenic mouse lines. (**A**) RT-PCR analysis of hBDNF-EGFP mRNA expression in tissues of three transgenic BAC mouse lines – C3, E1, E4. mBDNF – mouse BDNF; hBDNF – human BDNF in human tissues; HPRT – reference gene hypoxanthine phosphoribosyltransferase. *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.

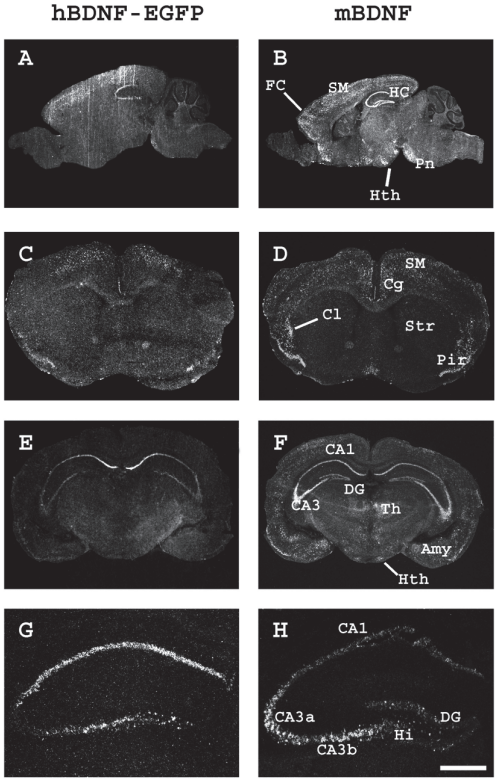

Figure 3
Overlapping patterns of BAC-driven hBDNF-EGFP and mBDNF mRNA expression in C3 mouse brain. *In situ* hybridization analysis, photoemulsion autoradiographs of 16 μm sagittal (**A**,**B**) and coronal (**C**-**H**) sections. (**C**) and (**D**) are sections taken at striatal level; (**E**) and (**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

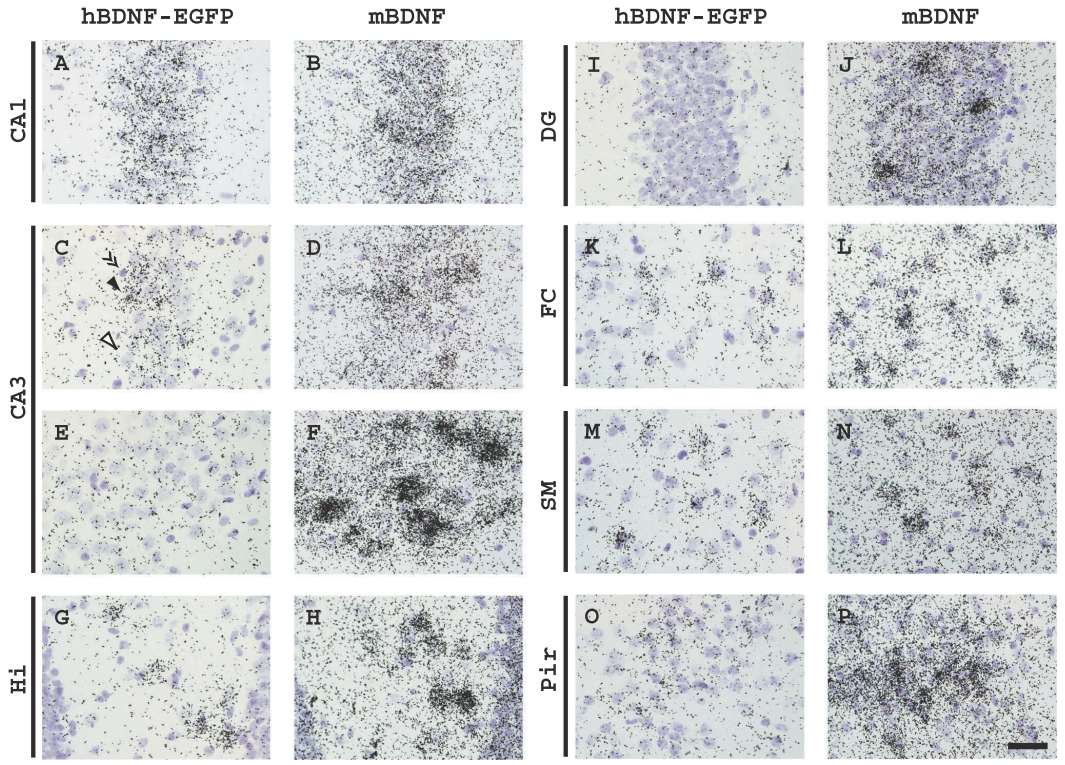

Figure 4

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

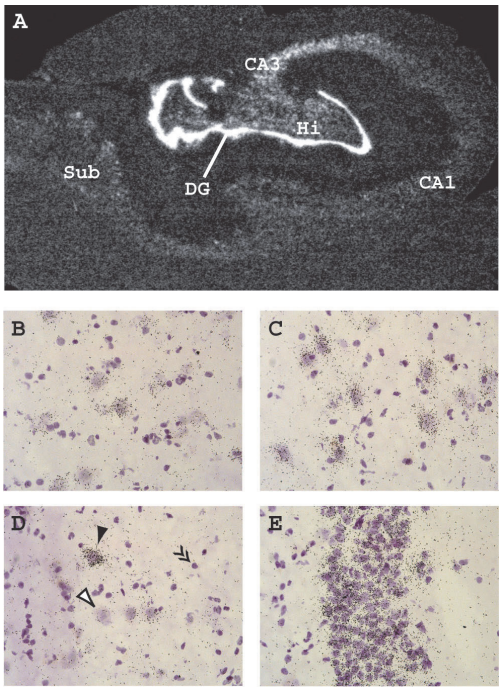

Figure 5
Expression of BDNF mRNA in the human hippocampus. (**A**) *In situ* hybridization autoradiograph of a 16 μm coronal section.*DG* – granular layer of dentate gyrus; *Hi* – hilar area of dentate gyrus; *Sub* – subiculum; *CA1*, *CA3* – hippocampal subfields. (**B-E**) High magnification bright-field 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

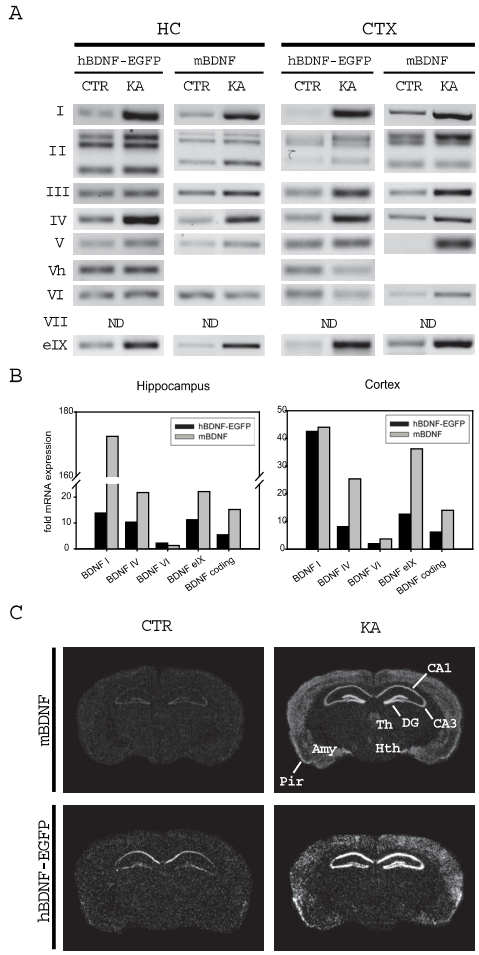

Figure 6
Kainic acid (30 mg/kg) induces transgenic hBDNF-EGFP mRNA expression in brains of C3 line transgenic mice. (**A**) Induction of alternatively spliced hBDNF-EGFP transcripts in C3 mouse hippocampus (HC) and cerebral cortex (CTX), analyzed with RT-PCR. mBDNF – mouse transcripts; ND – not determined; 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
HPRT I_s	CTTTGCTGACCTGCTGGATTAC
HPRT I_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_as I	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
rpl1_5'_as (5'end)	GGACAACAGACCCAAGGAGA
rpl1_3'_s (3'end)	GTAGGGTGTCTGGGTTGGTG
pBACe3.6_T7 (3'end)	TAATACGACTCACTATAGGG
transgene tandem integration	
rpl1_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.

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PUBLICATION II

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LETTER

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

Indrek Koppel, Tamara Aid-Pavlidis, Kaur Jaanson, Mari Sepp, Kaia Palm, and Tõnis Timmusk*
Department of Gene Technology, Tallinn University of Technology, Tallinn, Estonia

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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.

*Correspondence to: Tõnis Timmusk, Department of Gene Technology, Tallinn University of Technology, Akadeemia Tee 15, 12618 Tallinn, Estonia.
E-mail: tonis.timmusk@ttu.ee

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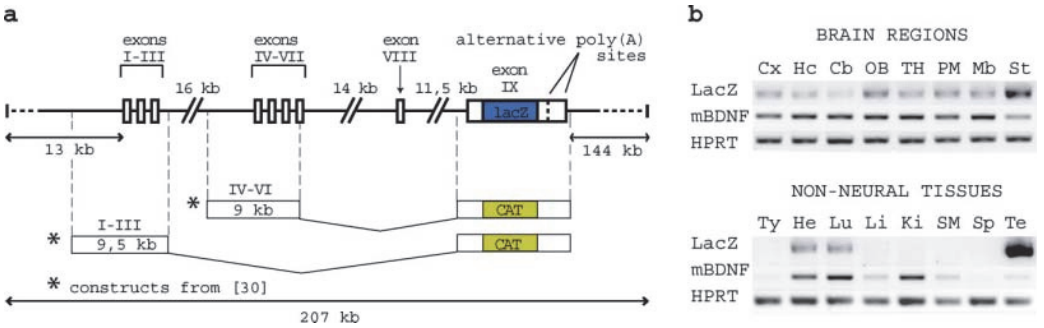

FIG. 1. (**a**) Schematic diagram of the BAC construct used for generating *rBDNF-lacZ*-BAC transgenic mice (thick lines). White boxes represent untranslated sequences and the blue filled box represents *lacZ* reporter gene that replaces the *BDNF* coding sequence. *rBDNF-CAT* constructs (I–III and IV–VI) 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

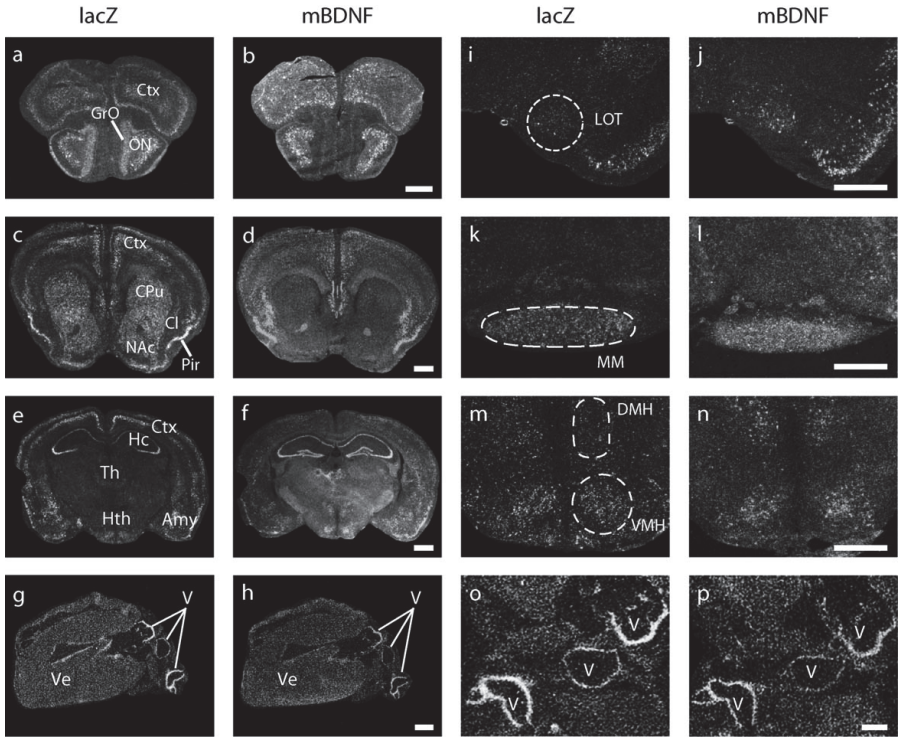

FIG. 2. In situ hybridization analysis of *rBDNF-lacZ* mRNA expression in adult *rBDNF-lacZ*-BAC transgenic mouse brain and heart. Photomicrographs of 16 μm coronal brain (**a–f; i–n**) and transverse heart sections (**g,h,o,p**) hybridized with ^{35}S-labeled *lacZ* or mouse endogenous *BDNF* (*mBDNF*) 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

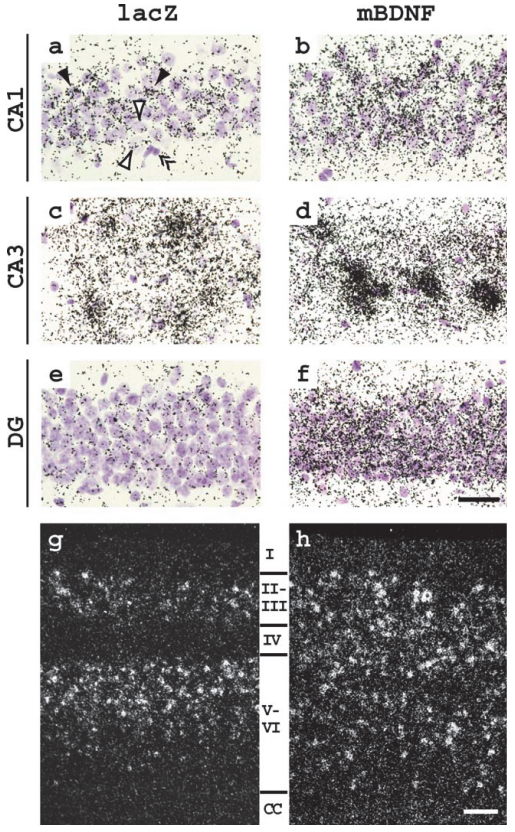

FIG. 3. Cellular expression of *rBDNF-lacZ* mRNA in adult transgenic mouse brain: in situ hybridization analysis. (**a–f**) Bright-field photomicrographs of hippocampal subfields CA1, CA3, and dentate gyrus (DG). Hybridization probes are indicated above the columns; closed arrowheads indicate neurons 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

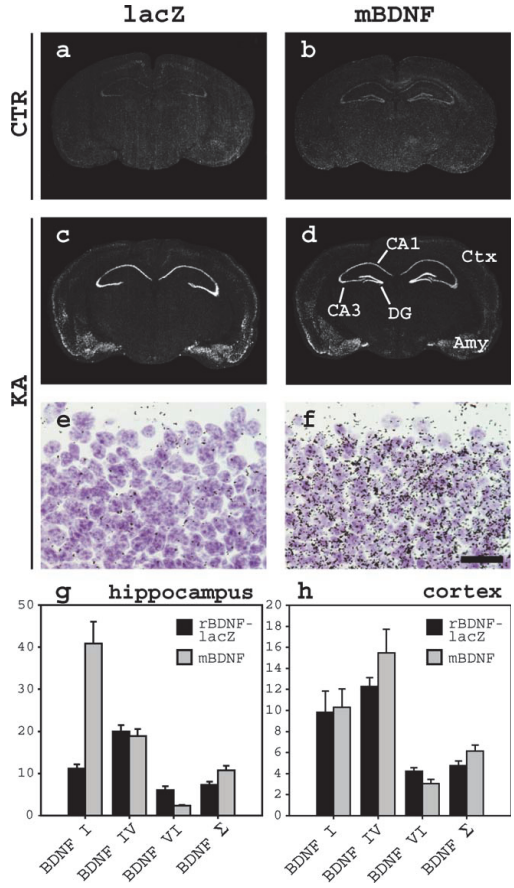

FIG. 4. Induction of *rBDNF-lacZ* mRNA in transgenic mouse brain by kainic acid treatment. (**a–f**) In situ hybridization analysis with probes for transgenic *rBDNF-lacZ* and mouse endogenous (*mBDNF*) mRNA. Autoradiographs of sections from vehicle-treated (**a**,**b**) and kainate-treated animals (**c–f**) 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.

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METHODOLOGY ARTICLE Open Access

BAC-based cellular model for screening regulators of BDNF gene transcription

Kaur Jaanson*, Mari Sepp, Tamara Aid-Pavlidis and Tõnis Timmusk

Abstract

Background: Brain derived neurotrophic factor (BDNF) belongs to a family of structurally related proteins called neurotrophins that have been shown to regulate survival and growth of neurons in the developing central and peripheral nervous system and also to take part in synaptic plasticity related processes in adulthood. Since BDNF is associated with several nervous system disorders it would be beneficial to have cellular reporter system for studying its expression regulation.

Methods: Using modified bacterial artificial chromosome (BAC), we generated several transgenic cell lines expressing humanised *Renilla* luciferase (hRluc)-EGFP fusion reporter gene under the control of rat *BDNF* gene regulatory sequences (rBDNF-hRluc-EGFP) in HeLa background. To see if the hRluc-EGFP reporter was regulated in response to known regulators of BDNF expression we treated cell lines with substances known to regulate *BDNF* and also overexpressed transcription factors known to regulate *BDNF* gene in established cell lines.

Results: rBDNF-hRluc-EGFP cell lines had high transgene copy numbers when assayed with qPCR and FISH analysis showed that transgene was maintained episomally in all cell lines. Luciferase activity in transgenic cell lines was induced in response to ionomycin-mediated rise of intracellular calcium levels, treatment with HDAC inhibitors and by over-expression of transcription factors known to increase *BDNF* expression, indicating that transcription of the transgenic reporter is regulated similarly to the endogenous *BDNF* gene.

Conclusions: Generated rBDNF-hRluc-EGFP BAC cell lines respond to known modulators of BDNF expression and could be used for screening of compounds/small molecules or transcription factors altering BDNF expression.

Keywords: BDNF, Cell line, Bacterial artificial chromosome, HDAC inhibitor

Background

Brain derived neurotrophic factor (BDNF), a nerve growth factor family member [1], has been shown to have important roles in the development and functioning of nervous system [2]. During development, BDNF supports survival and differentiation of distinct neuronal subpopulations [1,3,4]. In adulthood, BDNF has been shown to have effects in activity-dependent synaptic plasticity including learning and long-term potentiation [5], pain modulation [6], synaptogenesis [7,8] and regulation of metabolism [9].

BDNF gene has complex transcriptional regulation with different untranslated 5′ exons spliced to a common protein coding 3′ exon. Nine different promoters (I-IX) controlling transcription from nine or eleven 5′ exons, in rodents or humans respectively, and two different polyadenylation sites give rise to a range of mRNAs [10-12]. *BDNF* transcription has been shown to be regulated by a multitude of transcription factors (reviewed in [13]), for instance promoter I by cAMP response element binding protein (CREB) [14], upstream stimulatory factors (USF) [14], myocyte enhancer factor 2D (MEF2D) [15], nuclear factor kappa beta (NFκB) [16], basic helix-loop-helix (bHLH)-PAS transcription factor neuronal PAS domain protein 4 (NPAS4) and aryl hydrocarbon receptor nuclear translocator 2 (ARNT2) heterodimer (NPAS4-ARNT2) [17,18]; promoter II by repressor element-1 transcription factor (REST) [19,20]; promoter IV by CREB [21,22], calcium response factor (CaRF) [23], USF-s [18,24], methyl CpG binding protein 2 (MeCP2) [25], NFκB [26], bHLHB2 [27], and NPAS4-ARNT2 heterodimer [17,18], MEF2C [28]; promoter IX by CREB and

* Correspondence: kaur.jaanson@ttu.ee
Department of Gene Technology, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn, Estonia

NPAS4-ARNT2 heterodimer [18]. Due to the presence of many promoters and resulting large number of transcripts with the same protein coding sequence but alternating 5′ and 3′ untranslated regions, *BDNF* expression is temporally and spatially controlled in different tissues [11,12,29], developmental stages [30] and within different cell compartments [31-33]. Additionally, the *BDNF* gene locus also encompasses the *antisense BDNF* gene (*BDNFOS*) [12,34,35] with a complex splicing and expression pattern. Transcripts of the *antisense BDNF* gene have been shown to form dsRNA duplexes with *BDNF* transcripts [12] and regulate BDNF levels [35] *in vivo*.

Alterations in *BDNF* expression have been associated with several neurodegenerative disorders. *BDNF* expression has been shown to be decreased in brains of Alzheimer's [36], Parkinson's [37,38] and Huntington's disease [39] patients. Changes of BDNF levels are accompanied by several other pathologies, like neuropsychiatric disorders, obesity, impairment of learning and memory, neuropathic pain and epileptogenesis [2]. Due to BDNF involvement in nervous system disorders, it has been of great interest to use it as a therapeutic [40]. Unfortunately, direct use of recombinant BDNF protein is problematic due to its low serum half-life, poor penetration across blood brain barrier and low diffusion properties in tissues. Delivery of BDNF into the brain using viral vectors can have problems with vector toxicity, expression dosage and insertional mutagenesis. These problems have promoted screening of drug candidates that could promote expression of endogenous BDNF [41]. HDAC inhibitors are one class of drugs that have been shown to mediate their effect on memory and synaptic plasticity in models of nervous system disorders through increase in *BDNF* expression [42,43].

Bacterial artificial chromosomes (BACs) are large capacity vectors which are easy to maintain and modify using homologous recombination in *E. coli* [44]. Due to their large size, BACs can incorporate whole gene genomic loci while at the same time being easier to handle and modify than yeast artificial chromosomes (YACs). BACs have been used to create transgenic mice and cell lines for studying protein function [45], expression regulation [46-48] and for use in high-throughput screening of gene expression modulators [49].

Our group has previously created transgenic mice using BACs containing human [50] or rat [51] *BDNF* genomic sequences. Transgenes in these mice recapitulated endogenous *BDNF* expression patterns in different tissues. In the current study, we have generated transgenic cell lines expressing humanised *Renilla* luciferase (hRluc)-EGFP fusion reporter gene under the control of rat *BDNF* gene regulatory sequences. To this end we used BAC containing rat *BDNF* gene locus with BDNF protein coding region replaced with the hRluc-EGFP coding sequence. These transgenes maintain transgene episomally in high numbers and express reporter gene at high levels. Reporter gene is induced in response to rise in intracellular calcium levels, treatment with different HDAC inhibitors and overexpression of NPAS4-ARNT2 heterodimer or constitutively active CREB1 (VP16-CREB) that are known to regulate *BDNF* expression. These transgenic cell lines could be used for screening drug candidates or transcription factors that modulate *BDNF* expression.

Results

Generation of rBDNF-hRluc-EGFP HeLa stable cell lines

HeLa cell line was chosen for generation of rBDNF-hRluc-EGFP cell lines because its relatively carefree growth conditions and fast growth are good properties for transgenic cell line. Endogenous human *BDNF* gene was also expressed in HeLa cell line showing that signaling pathways regulating *BDNF* expression were active in HeLa cells (see below). The hRluc-EGFP fusion reporter was used because EGFP fluorescence was useful for initial screening and subcloning of transgenic cell lines by fluorescence microscopy and FACS. However for screening of substances or transcription factors regulating *BDNF* expression, *Renilla* luciferase luminescence detection is more sensitive and gives less background signal than fluorescence based detection methods [52]. *Renilla* luciferase also has commercial live cell substrates that allow for repeated measurements of the treated cells making it easier to assay the time dependent effect on the reporter expression while conserving reagents.

rBDNF-hRluc-EGFP BAC construct used for generating cell lines was created using BAC clone that contains rat *BDNF* locus spanning 13 kb upstream of the first exon and 144 kb downstream of the last polyadenylation signal (Figure 1A). BAC clone was modified by homologous recombination to: (i) replace BDNF coding sequence with sequence coding for humanised *Renilla* luciferase, Ala-Ala-Ala-Thr linker and EGFP fusion protein (Figure 1B) and (ii) replace *CAT* gene in BAC vector by *neo* cassette to confer resistance to G418 for positive selection during cell line generation. The final rBDNF-hRluc-EGFP BAC construct was transfected into HeLa cells by nucleofection and G418 was applied for selection.

Following two months of G418 selection, FACS analysis of polyclonal cell population showed that 15% of cells were positive for EGFP signal. Luciferase signal measured in cell lysate was 10^4 times over the HeLa background signal (data not shown). By FACS assisted cell sorting a number of single cell clones were established displaying varying levels of transgene expression. Six cell lines were chosen for subsequent analysis: 1A4s2, 1A4s3, 2A4, 2B2s, 3E2s and 3G4s.

rBDNF-hRluc-EGFP cell lines express hRluc-EGFP reporter gene

In all six cell lines, EGFP signal was detected by fluorescence microscopy in live cells, and hRluc-EGFP fusion protein

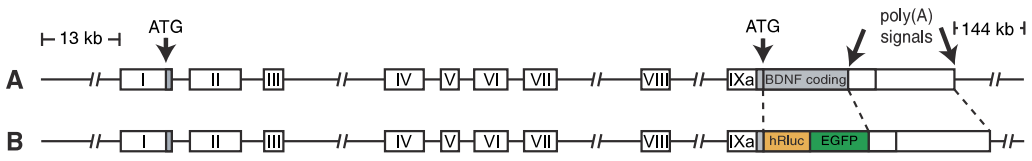

Figure 1 Schematic drawing of the rBDNF-hRluc-EGFP-BAC construct used for generation of HeLa cell-lines expressing the transgene. **(A)** The BAC used contained the rat BDNF locus spanning 13 kb upstream of the first exon and 144 kb downstream of the last polyadenylation signal. The exonic structure of rat BDNF gene is adapted from [11]. **(B)** The coding region of BDNF was replaced by homologous recombination with sequence coding for humanised *Renilla* luciferase, Ala-Ala-Ala-Thr linker and EGFP fusion protein. Protein coding regions are shown as gray (BDNF), orange (hRluc) or green (EGFP) boxes. Untranslated regions are shown as white boxes.

was distributed diffusely all over the cell (Figure 2A). Flow-cytometric analysis of the six cell lines showed that the percentage of EGFP positive cells in population varied from 95 to 59 percent (Figure 2B). Additionally there was a variance over several log units in reporter expression level within a cell line (Figure 2C). This prompted us to analyse the stability of reporter expression in time. For this we passaged the cell lines in media containing increasing concentrations of selective antibiotic G418 (0, 200, 400, 800 and 1200 μg/ml). If no antibiotic was added to the growth medium then the proportion of EGFP positive cells in different cell lines decreased 1.5 to 10 times in three weeks. At the same time the proportion did not change substantially when cells were grown in medium containing 800 or 1200 μg/ml of G418 (data not shown).

Next we measured the activity of *Renilla* luciferase in live cells using Enduren substrate and normalised it to cellular ATP levels. The signals obtained from the six cell lines were 35 to ~200 times over the background signal of the parental HeLa cells (Figure 2D). Based on reporter expression level we divided the cell lines into two groups: the group with high reporter expression includes the cell lines 1A4s2, 1A4s3 and 2A4; the low reporter expression group consists of the cell lines 2B2s, 3E2s and 3G4s. In the first group luciferase signal was ~100-200 times above background and in the second group around ~35-60 times above background.

Having detected both, *Renilla* luciferase luminescence and EGFP fluorescence, of the fusion reporter protein we decided to determine which of the alternative chimaeric rat BDNF hRluc-EGFP mRNAs are transcribed in the six cell lines. Expression of endogenous human *BDNF* transcripts in the 6 cell lines were similar to parental HeLa cells with a few exceptions: transcript I was not expressed in 3G4s cell line and transcript IXa long (corresponding to transcript IXabcd in [12]) was not expressed in 1A4s2 cell line (Figure 3A). Using RT-PCR analysis we were able to detect transgenic transcripts I, III, IV, V, VI, VIII and IXa in all cell lines (Figure 3B). Transgenic transcript I was expressed at very low levels in five out of six cell lines, its levels were elevated in 2A4 cells. Neither endogenous nor transgenic transcript II was detected in cell lines or parental HeLa cells. While overall expressions of endogenous and transgenic transcripts were similar there were some differences. First, endogenous transcript III was not expressed in any of the cell lines while transgenic transcript III was expressed in all cell lines. Second, transgenic transcript VII was not expressed in any of the cell lines although being expressed endogenously. Altogether these data show that correctly spliced transgenic rat *BDNF* hRluc-EGFP mRNAs are transcribed and functional hRluc-EGFP fusion protein is expressed in rBDNF-hRluc-EGFP cell lines.

Transgene is maintained in rBDNF-hRluc-EGFP cell lines as a high copy number episome

Since transgene integration site and copy number can influence reporter gene expression from the transgene, we aimed to determine the copy number and chromosomal state (integrated or episomal) of rBDNF-hRluc-EGFP BAC DNA in cell lines. qPCR analysis using copy number standard showed that transgene copy number varied up to five times amongst the different cell lines. Over 900 transgene copies per HeLa genome were present in 1A4s2 and 1A4s3 cell lines and ~190-300 transgene copies in 2A4, 2B2s, 3E2s, 3G4s cell lines (Figure 4A). The status of transgene DNA was analysed by fluorescent *in situ* hybridisation (FISH) with rBDNF-hRluc-EGFP BAC specific probe. As demonstrated in Figure 4B, transgenic BAC DNA was maintained episomally in all cell lines – the rBDNF-hRluc-EGFP BAC specific hybridisation signals were localised near chromosomes, but integration was not detected. Transgene copy numbers per cell also varied highly between cells of the same cell line. In conclusion, the obtained rBDNF-hRluc-EGFP cell lines contain relatively high numbers of transgenic BAC DNA per cell that replicates episomally.

Elevated intracellular calcium induces reporter gene expression in rBDNF-hRluc-EGFP cell lines

BDNF promoters contain several Ca^{2+} responsive regulatory elements [14,18,21-24,53] and BDNF levels *in vivo*

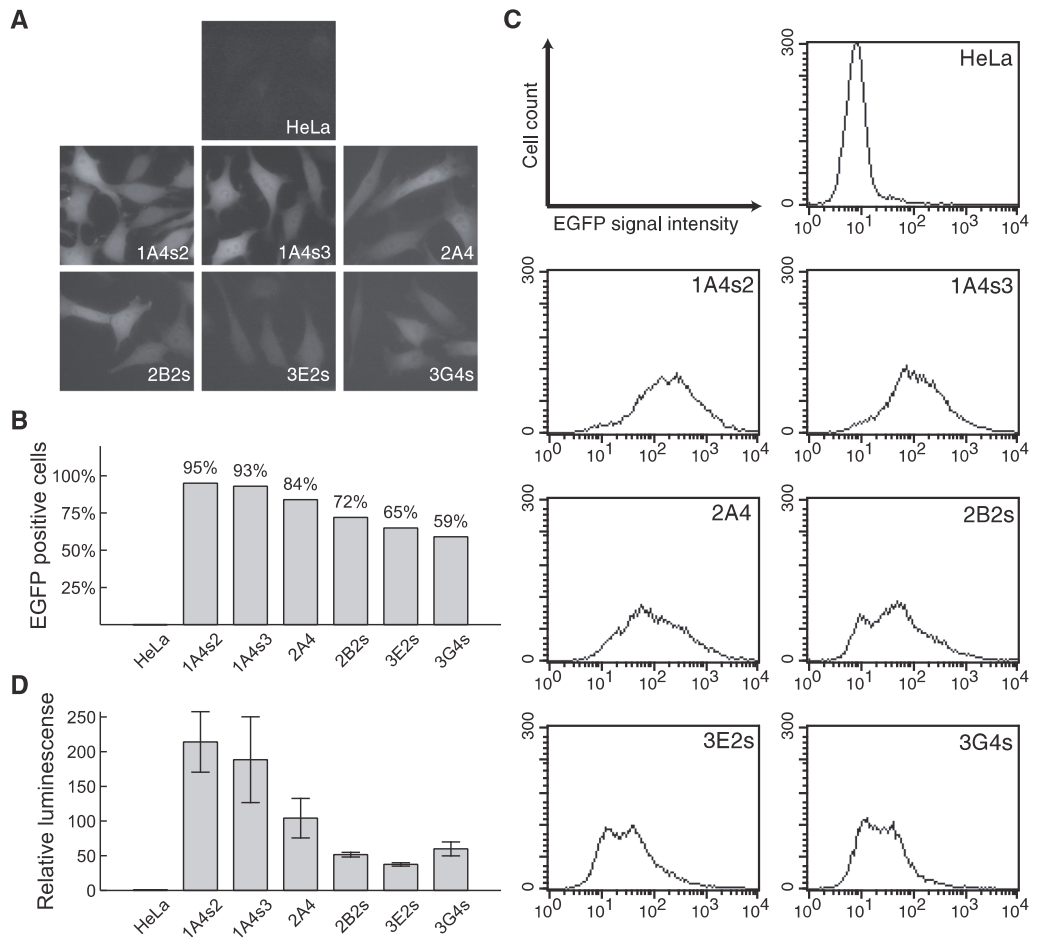

Figure 2 Detection of hRluc-EGFP reporter expression in rBDNF-hRluc-EGFP cell lines. (A) Fluorescence microscopy images of rBDNF-hRluc-EGFP cell lines. **(B)** Percentage of EGFP positive cells in different rBDNF-hRluc-EGFP BAC cell lines counted by fluorescence-activated cell sorting (FACS). **(C)** Histograms showing the variability of reporter gene expression level within a cell population of different rBDNF-hRluc-EGFP cell lines counted by FACS. **(D)** Detection of *Renilla* luciferase enzymatic activity in rBDNF-hRluc-EGFP cell lines relative to the background signal measured in parental HeLa cells. Enduren luciferase live cell substrate was added with fresh culture medium to rBDNF-hRluc-EGFP cells and HeLa cells and *Renilla* luciferase luminescence was measured after 12 hour incubation. Results are normalised to cell viability assayed by cellular ATP levels after 12 hour incubation. Error bars indicate SD of three independent experiments.

are induced by neural activity related Ca^{2+} influx into neurons [54]. To test if transgene is induced by elevated intracellular Ca^{2+} levels in different cell lines we treated cells for 12 hours with 1 μM ionomycin, a calcium ionophore known to induce *BDNF* expression in neurons [55], and monitored *Renilla* luciferase signal using Enduren live cell luciferase substrate during this period. Ionomycin treatment induced reporter gene expression in all cell lines compared to vehicle treated control. As shown in Figure 5A, the relative increase of luminescence signal was higher in cell lines with low reporter expression: 3E2s, 2B2s and 3G4s. In these three cell lines the fold change reached its peak after eight hours when it was 2.00 ($p < 0.01$); 1.95 ($p < 0.01$); and 1.86 ($p < 0.05$) respectively. In the cell lines with high reporter expression the fold change remained smaller and the maximum values for cell lines 1A4s2, 1A4s3 and 2A4 were 1.46 ($p < 0.05$); 1.56 ($p < 0.01$) and 1.43 ($p < 0.01$) respectively. To exclude the possibility that ionomycin affects cell viability we measured the cell viability levels of

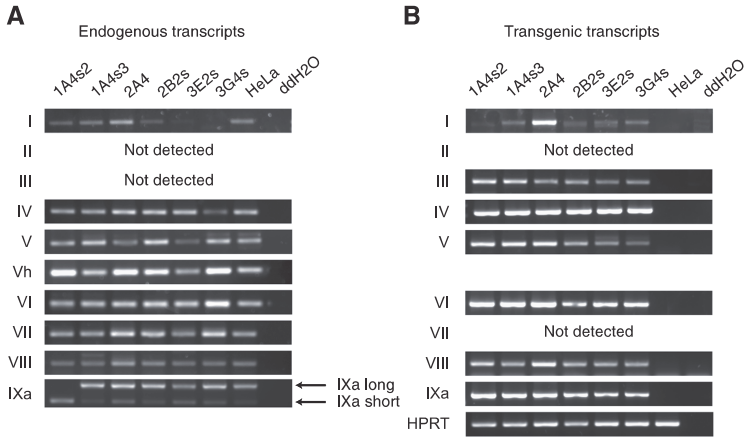

Figure 3 Expression of alternative 5′ exon-specific mRNAs transcribed from rBDNF-hRluc-EGFP cell lines and HeLa cells. Expression of alternatively spliced 5′ exon specific transcripts form **(A)** endogenous BDNF and **(B)** rBDNF-hRluc-EGFP BAC reporter construct in different rBDNF-hRluc-EGFP cell lines and parental HeLa cells. Endogenous IXa long and short transcripts correspond to human BDNF transcripts IXabcd and IXabd in [12].

control and ionomycin treated cells after 12 hours. No significant decrease in cell viability was detected in response to ionomycin treatment (Figure 5B). These results demonstrate that reporter expression in rBDNF-hRluc-EGFP cell lines is regulated by changes in intracellular calcium level.

HDAC inhibitors induced reporter gene expression in rBDNF-hRluc-EGFP cell lines

Due to interest in finding low molecular weight substances regulating *BDNF* expression we sought to establish whether our cell lines could be used for screening of substances modulating *BDNF* expression. HDAC inhibitors are a class of drugs that inhibit histone deacetylases – group of enzymes that deacetylate histones and non-histone proteins. It has been shown that certain HDAC inhibitors have antidepressant actions and regulate *BDNF* expression [56], for example valproate [42,57-59], TSA [58,60] and SAHA [61]. Since it would be of interest to use our cell lines for screening of other compounds that could epigenetically regulate *BDNF* expression, we tested response of the reporter gene in BAC cell lines to four HDAC inhibitors. Two cell lines were chosen for treatments, higher copy number cell line 1A4s2 and lower copy number cell line 3E2s. The cells were treated with 100 nM apicidin, 1 μM SAHA, 100 nM TSA and 1 mM sodium valproate for 12 hours while assaying reporter gene expression during that time using Enduren live cell *Renilla* luciferase substrate.

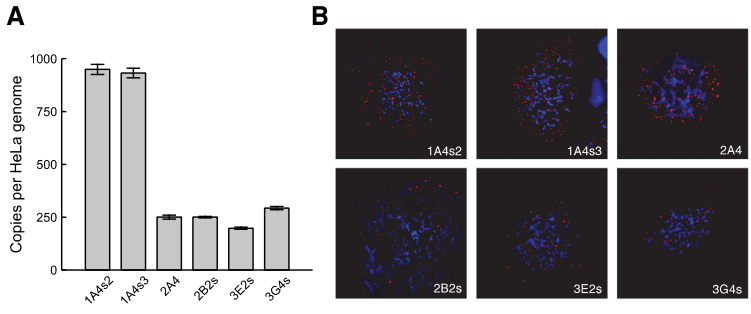

Figure 4 Analysis of transgene copy number and chromosomal state. (A) qPCR analysis of transgene copy number in different rBDNF-hRluc-EGFP cell lines. Error bars show SE of three technical replicates. **(B)** FISH analysis of reporter construct chromosomal status in different rBDNF-hRluc-EGFP cell lines. Hybridisation was performed with rBDNF-hRluc-EGFP BAC specific probe (red signal) and DNA was stained with Hoecht 33342 (blue signal).

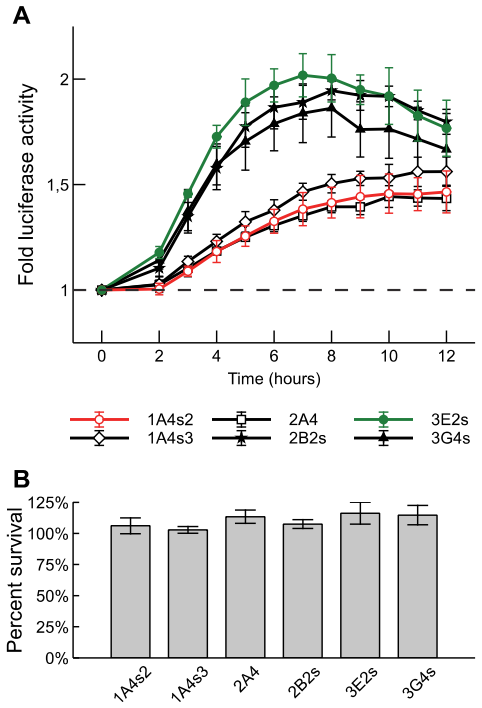

Figure 5 Treatment with ionomycin induces reporter expression in different rBDNF-hRluc-EGFP cell lines. (A) Luciferase activity in 1 μM ionomycin treated cells relative to vehicle (0.1% DMSO) treated cells (dashed line) at each time point, measured in live cells during 12 hours. *Renilla* luciferase luminescence was measured using Enduren substrate 2–12 hours after the start of the treatment at one hour intervals. Error bars indicate SD of three independent experiments. **(B)** Cell viability in ionomycin treated cells relative to vehicle treated cells after 12 hours in different cell lines. Viability was assayed by cellular ATP levels. Error bars indicate SD of three independent experiments.

HDAC inhibitors increased reporter gene expression in both cell lines compared to vehicle treated control. 100 nM apicidin increased reporter gene expression in 1A4s2 and 3E2s cell lines 1.70 and 1.58 fold at 12 hours and 11 hours of treatment, respectively (both $p < 0.01$, Figure 6A). 1 μM SAHA treatment increased reporter gene expression in 1A4s2 and 3E2s cell lines 1.65 and 1.64 fold at 12 hours and 11 hours, respectively (both $p < 0.01$ Figure 6B). 100 nM TSA treatment increased reporter gene expression in 1A4s2 and 3E2s cell lines 1.63 and 1.70 fold at 12 hours and 11 hours, respectively (both $p < 0.01$, Figure 6C). 1 mM sodium valproate increased reporter gene expression in 1A4s2 and 3E2s cell lines 1.22 and 1.24 fold at 11 hours and 10 hours, respectively ($p < 0.05$ and $p < 0.01$, Figure 6D). None of the used HDAC inhibitors showed significant effect on cellular survival after 12 hours of treatment in either cell line (Figure 6E and F). Taken together, these results show that HDAC inhibitors upregulate reporter gene expression in rBDNF-hRluc-EGFP cell lines 1A4s2 and 3E2s.

VP16-CREB and NPAS4-ARNT2 transcription factors increased transgene expression in BAC transgenic cell lines

BDNF is regulated by 9 different promoters containing binding sites for different transcription factors regulating expression of different *BDNF* transcripts. Of these transcription factors CREB and bHLH transcription factor heterodimer NPAS4-ARNT2 have been shown to regulate *BDNF* expression from various promoters [18,21,22]. To test if the transgenic cell lines could be used for screening of transcription factors that induce *BDNF* transcription we transfected cell lines 1A4s2 and 3E2s with constructs expressing VP16-CREB (constitutively active form of CREB1, fused with viral transactivation domain) [62] or NPAS4 and ARNT2, or with empty pRC vector for comparison.

24 hours after transfection VP16-CREB transcription factor increased transgene expression in 1A4s2 and 3E2s cell lines 1.55 ($p < 0.01$) fold. Transcription factor NPAS4 together with ARNT2 increased reporter expression in 1A4s2 and 3E2s cell lines 1.89 ($p < 0.01$) and 1.35 fold, respectively (Figure 7A and B). These results show that VP16-CREB and NPAS4-ARNT2 heterodimer increase transgene expression in 1A4s2 and 3E2s rBDNF-hRluc-EGFP cell lines.

Discussion

In the current study, we have developed rBDNF-hRluc-EGFP reporter cell lines in HeLa background, using bacterial artificial chromosome (BAC) containing rat *BDNF* genomic locus with protein coding region replaced with hRluc-EGFP fusion reporter gene, for studying the regulation of the *BDNF* gene and for analysis of the effect of different compounds and transcription factors on *BDNF* expression. Generation of transgenic mice for studying *BDNF* regulation using large transgenic constructs by us [50,51] and others [63] has shown that use of BAC (or YAC) transgenic constructs helps to better recapitulate endogenous *BDNF* expression. It has also been demonstrated that using BAC constructs for generating cell lines helps to avoid transgene integration specific effects and provides levels and timing of transgene expression that mimic that of the endogenous gene [64]. The large rat *BDNF* genomic locus contained in the BAC construct used in this work should include regulatory elements positioned further away from *BDNF* gene and help to better emulate endogenous *BDNF* expression.

Out results show that rBDNF-hRluc-EGFP reporter construct was maintained extrachromosomally in high copy numbers in all established cell lines. Previously it

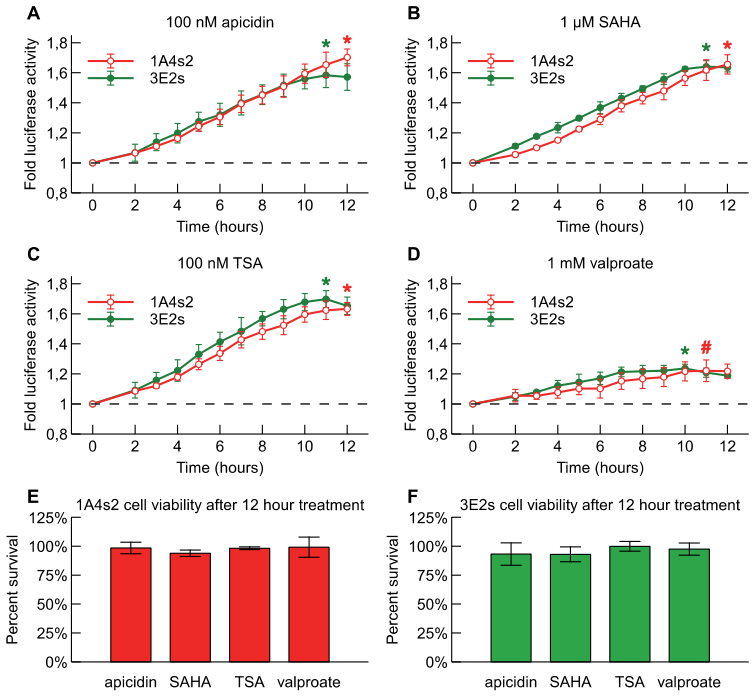

Figure 6 Treatment with HDAC inhibitors induces reporter expression in 1A4s2 and 3E2s rBDNF-hRluc-EGFP cell lines. (A, B, C, D) Fold luciferase activity in HDAC inhibitor treated cells relative to vehicle treated cells (dashed line) measured in live cells during 12 hours. 1A4s2 and 3E2s cells were treated with HDAC inhibitors apicidin (100 nM) **(A)**, SAHA (1 μM) **(B)**, TSA (100 nM) **(C)**, sodium valproate (1 mM) **(D)** and vehicle control (0.1% DMSO or water) together with Enduren live cell substrate. Error bars indicate SD of three independent experiments (* $p < 0.01$, # $p < 0.05$, relative to vehicle treated control). **(E, F)** Cell viability in HDAC inhibitor treated cells relative to vehicle treated cells after 12 hours of treatment in 1A4s2 cell line **(E)** and 3E2s cell line **(F)**. Viability was assayed by cellular ATP levels. Error bars indicate SD of three independent experiments.

has been shown that plasmids containing matrix attachment regions (MARs) are maintained in double minute extrachromosomal elements in HeLa cells [65]. These are autonomously replicating extrachromosomal elements that are up to a few Mb in size [66], have been known to be associated with active histones [67] and can be purified by histone immunoprecipitation [68]. MARs and scaffold attachment regions (SARs), together named as S/MARs, are regions on the DNA which attach to nuclear matrix and have been associated with functions such as anchoring of DNA, maintenance of nuclear architecture, regulation of transcription and replication. It has been estimated that S/MARs are spaced on average 70 kb from each other in mammalian genomes [69]. Given the large size of *BDNF* locus contained in BAC construct used in this study (207 kb) it is probable that it contains S/MAR elements enabling extrachromosomal maintenance in HeLa cells. We used SMARTest tool [70] to predict the existence of 6 candidate sites in the genomic locus included in rBDNF-hRluc-EGFP BAC which exhibit S/MAR like characteristics (data not shown). It would be of interest to study which regions of the *BDNF* BAC used in this study are responsible for its extrachromosomal maintenance.

We detected expression of almost all 5′ exon-specific BDNF mRNAs in the rBDNF-hRluc-EGFP cell lines showing that the entire *BDNF* gene is maintained in inserted transgene. We also observed stable and copy number dependent expression of hRluc-EGFP fusion reporter protein by fluorescence and luminescence based methods. Previously it has been shown that protein expression in BAC transgenic cell lines is proportional to transgene copy numbers [71]. BAC-derived *BDNF* gene is not highly expressed, as shown by transgenic animals previously produced in our lab where one or two copies of transgenic *BDNF* BAC construct were inserted in the genome. In contrast, the high copy numbers of transgene in the cell lines developed in this study directed high levels of reporter gene expression.

We used hRluc-EGFP fusion protein, analogous to the Rluc-GFP reporter used previously by [72], as a reporter

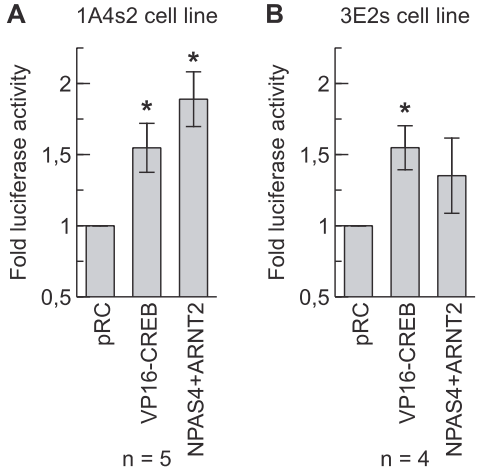

Figure 7 Overexpression of VP16-CREB and NPAS4 + ARNT2 induces reporter expression in 1A4s2 and 3E2s rBDNF-hRluc-EGFP cell lines. **(A, B)** 1A4s2 **(A)** and 3E2s **(B)** cells were transfected with constructs expressing VP16-CREB, NPAS4 and ARNT2 (at 1:1 ratio) transcription factors or empty vector control pRC. *Renilla* luciferase luminescence was assayed 24 hours after transfection and fold luciferase activity relative to empty vector control (pRC) was calculated. Error bars indicate SD. (n – number of independent experiments, * $p < 0.01$, relative to empty vector control).

in the BAC construct. The use of hRluc-EGFP protein makes it possible to apply both fluorescence and more sensitive luminescence based methods for reporter detection. While EGFP fluorescence could be used for measuring reporter expression via flow cytometry, use of live cell substrates allow for sensitive detection of *Renilla* luciferase signal which could be advantageous for high-throughput screening procedures. The half-life of *Renilla* luciferase is 3–4 hours and the half-life of EGFP is about 26 hours [73] in mammalian cells. However, stability of the hRluc-EGFP fusion protein is not known. It is probable that the induction of measurable reporter activity in response to different treatments underestimates the true effect on transcriptional activity due to slow turnover of the hRluc-EGFP fusion protein, suggesting that it would be important to develop a less stable hRluc-EGFP fusion protein in future studies.

To assess the suitability of the cell lines generated by us for use in studying *BDNF* gene regulation, the cells were treated with stimuli known to induce *BDNF* expression. Ionomycin induces *BDNF* expression through Ca^{2+} mediated signalling pathways [54], HDAC inhibitors through increasing histone acetylation leading to transcriptionally active chromatin around subset of genes, including *BDNF* [56,74]. Transcription factor CREB and NPAS4-ARNT2 heterodimer have been shown to promote transcription from several *BDNF* promoters [18,21,22]. As expected, treatment with different modulators of *BDNF* expression induced expression of hRluc-EGFP fusion reporter protein. The effect of treatment with ionomycin or HDAC inhibitors was different when comparing high and low transgene copy number cell lines. Ionomycin induced reporter expression to a higher extent in lower copy number cell lines than in higher copy number cell lines. However, treatment of high and low transgene copy number cell lines with different HDAC inhibitors induced transgene expression similarly in both cell lines regardless of the used inhibitor. The apparent copy number dependent effect on reporter induction by ionomycin might be explained by higher number of transcription factor response elements in high copy number cell lines competing for limited supply of Ca^{2+} dependent transcription factors. In contrast, the effect of HDAC inhibitors on gene expression is more general, regulating expression of large number of genes, and they may act by inducing transgene expression independent of copy number.

Since BDNF has been primarily studied as a neuronal gene, the non-neuronal nature of the generated transgenic cell lines sets certain limitations to their use in studying neuron-specific regulation of BDNF expression. For example, BDNF mRNAs have been known to be transported to dendrites [75,76] and their translation there to be regulated in response to local synaptic signalling [77], regulatory steps that are not recapitulated in our cell lines. Also, neuronal stimuli known to regulate BDNF expression, for example depolarisation by potassium [54] or glutamate [78], do not recapitulate in HeLa background. Therefore, modulators that have been found to regulate transgene expression in these transgenic cell lines should be also verified in neuronal background. However, the active transcription of endogenous BDNF mRNAs in HeLa cells and the robust nature of the HeLa cells make these cell lines convenient tools for screening of factors regulating BDNF expression.

Conclusions

In conclusion, we have generated a rBDNF-hRluc-EGFP BAC cellular reporter model for use in studying *BDNF* regulation. Transgene is maintained in cell lines extrachromosomally as high copy number episome. High transgene copy number makes it possible to reliably detect reporter expression. Transgene expression is induced in response to known modulators of *BDNF* expression making these cell lines useful for further studies of *BDNF* regulation.

Methods

Constructs

BAC clone CH230-106 M15 containing rat BDNF gene was purchased from Chori BACPAC Resources. Clone

CH230-106 M15 contains rat genomic DNA region spanning the *BDNF* gene locus cloned into the EcoRI site of pTARBAC2.1 vector. The vector carried chloramphenicol resistance and resided in *E. coli* host strain DH10B (recA$^-$, recBC$^+$). Sequence of the BAC clone CH230-106 M15 was obtained from NCBI GeneBank [GenBank:AC108236]. Vectors pCDNA3.1-NPAS4, pCDNA3.1-ARNT2 and pACT-CREB1 (containing VP16 viral transcription activation domain) have been described previously [18].

Homologous recombination

BAC modifications using Red/ET homologous recombination were performed according to the BAC Modification Counter-Selection System protocol (Gene Bridges GmbH). For amplification of inserts, 75-mer oligonucleotides were synthesised (Proligo). The 5′-end of each oligonucleotide contained 50 nucleotides of homology region shared by the target BAC and a linear insert followed by a 25 nucleotide primer for PCR amplification of the linear insert from the template. Where necessary, linker sequence was added between homology arm and primer sequences. Inserts for homologous recombination were amplified by PCR using Expand Long Template PCR system (Roche) or Hot GyroPol PCR system (Solis BioDyne). The synthetic humanised version of *Renilla* luciferase reporter gene (hRluc), the red-shifted variant of wild-type *Aequorea* green fluorescent protein (EGFP) reporter gene and SV40-Neo^r-polyA cassette were amplified from pTK-hRluc (Promega), pEGFP-N1 (Clontech) and pEGFP-C1 vectors (Clontech), respectively. Following PCR primers were used for insert synthesis – hRLuc: sense 5′-CCT GTT CTG TGT CTG TCT CTG CTC CTT CCC ACA GTT CCA CCA GGT GAG AAG AGT GAT GGC TTC CAA GGT GTA CGA CCC CG-3′, antisense 5′-ATA CAA ATA GAT AAT TTT TGT CTC AAT ATA ATC TAT ACA ACA TAA ATC CAT TAC TGC TCG TTC TTC AGC ACG CGC T-3′; EGFP: sense 5′-TGG GTA AGT ACA TCA AGA GCT TCG TGG AGC GCG TGC TGA AGA ACG AGC AGG CCG CCG CCG CCA CCA TGG TGA GCA AGG GCG AGG AGC TG-3′, antisense 5′-ATA CAA ATA GAT AAT TTT TGT CTC AAT ATA ATC TAT ACA ACA TAA ATC CAT TAC TTG TAC AGC TCG TCC ATG CCG A-3′; SV40- Neo^r -polyA cassette: sense 5′-CAC CAT AAT GAA ATA AGA TCA CTA CCG GGC GTA TTT TTT GAG TTA TCG AGA TTT TCA GGA GCT AAG GAA GCT AAA TTC AAA TAT GTA TCC GCT CAT GAG A-3′, antisense 5′-ATT CAT CCG CTT ATT ATC ACT TAT TCA GGC GTA GCA ACC AGG CGT TTA AGG GCA CCA ATA ACT GCC TTT TTT ATT CTG TCT TTT TAT TGC CGT C-3′. BAC was modified by first replacing BDNF protein coding region with hRluc coding sequence, then inserting Ala-Ala-Ala-Thr linker and EGFP coding sequences to the end of hRluc sequence and finally by replacing the *CAT* gene in BAC vector with SV40-Neo^r-polyA cassette. The modified BAC containing colonies were screened by colony PCR and colony hybridisation and further verified by restriction analysis and sequencing.

Cell culture and transfection

HeLa (DSMZ) human cervical cancer cells were propagated in DMEM (Dulbecco's Modified Eagle Medium; PAA) supplemented with 10% fetal bovine serum (PAA), penicillin (PAA) and streptomycin (PAA) at 37°C in 5% CO2. For BAC DNA transfection 1 μg of BAC DNA purified with Large-Construct Kit (Qiagen) was mixed with 5×10^6 HeLa cells and transfection was performed using Amaxa Nucleofector program A-28 and Cell Line Nucleofector Kit R (Amaxa). 72 hours later 400 μg/ml G418 (Sigma) was added to the growth medium to select for BAC containing cells. After two months of selection, single EGFP-positive clones were isolated with FACSAria cell sorting system (Becton-Dickinson). The cell lines were routinely grown in medium containing 800 μg/ml G418. For transfection of plasmid constructs cells were seeded into white 96-well clear bottom microtiter plate (Greiner Bio-One) at 10 000 cells per well in a volume of 200 μl of G418-containing culture medium. The next day medium was replaced with 100 μl of medium without G418 and cells were transfected with GenJet Hela transfection reagent (SignaGen Laboratories) using 100 ng of DNA per well at 1:3 DNA to lipid ratio. NPAS4 and ARNT2 constructs were cotransfected at 1:1 ratio. Five hours post-transfection medium was replaced with 200 μl of G418-containing medium.

Drug treatments

One day before treatments, cells were seeded into white 96-well clear bottom microtiter plates (Greiner Bio-One) at 10 000 cells per well. The next day medium was replaced with 75 μl of fresh medium containing ionomycin, apicidin, sodium valproate (all Sigma Aldrich), SAHA or TSA (both Cayman Chemical). All drugs except sodium valproate were dissolved in DMSO and added to cells at a final DMSO concentration of 0.1%. Sodium valproate was dissolved in MilliQ grade water. Appropriate vehicle controls (DMSO or water) were included in all experiments.

Reporter assays

EGFP was detected by fluorescence microscopy (Axiovert 200 M, Zeiss) and flow cytometry (FACSCalibur, Becton-Dickinson). In flow-cytometric analysis no compensation was used and markers for positive EGFP-signals were set on FL1 vs FL2 dot blot using the autofluorescence diagonal of parental HeLa cells. EGFP-positive cells were identified by divergence from the autofluorescence diagonal towards higher FL1 fluorescence. For monitoring hRluc

enzymatic activity in live cells, 30 μM Enduren substrate (Promega) was added to cells at the beginning of drug treatment and luminescence was measured once per hour at 2–12 hour time points. For measuring endpoint hRluc activity, cells were lysed in 20 μl Passive Lysis Buffer (Promega) 24 hours after transfection and subjected to Renilla-Glo Luciferase Assay System (Promega) according to the manufacturer's instructions. Relative luminescence was measured with GENios Pro plate reader (TECAN). For normalisation and monitoring cell viability ViaLight Plus Cell Proliferation And Cytotoxicity BioAssay Kit (Lonza, USA) were used. For drug treatments, three independent experiments were performed, each in triplicate. Luciferase signal in response to drug treatments was normalised to vehicle control for each time point, means and standard deviations were calculated and t-tests for analysis of statistical significance for indicated time points were performed. For transcription factor transfections, four or five independent experiments were performed, each in triplicate. Luciferase signal in response to expression of transcription factor(s) was normalised to signal in pRC empty vector transfected cells, means and standard deviations were calculated and t-tests for analysis of statistical significance were performed.

Fluorescence in situ hybridisation

Mitotic blocking was performed by treating cells with 50 ng/ml colcemid (Sigma) for 4 hours. The cells were harvested by shakeoff and subjected to hypotonic treatment with 0.075 M KCl for 15 min. The cells were fixed in methanol:acetic acid (3:1) and used for chromosome slide preparation. Slides were chemically aged and denatured as described [79]. Prior to denaturation and hybridisation, chromosome preparations were treated with RNase A (100 μg/ml in 2 × SSC), pepsin (50 μg/ml in 0.01 N HCl) and 1% formaldehyde (in PBS containing 50 mM $MgCl_2$). rBDNF-hRluc-EGFP BAC specific probe was labeled with digoxigenin-11-dUTP (Roche) by nick translation. For each hybridisation 45 ng of the labeled probe was used together with 25 μg of salmon sperm DNA. Hybridisation was carried out by incubating slides in 50% deionised formamide, 2 × SSC, 0.1 M phosphate buffer, 10% dextran sulfate overnight at 37°C in humid chamber. Hybridised probe was detected by affinity reaction with mouse anti-digoxygenin primary antibody (Roche) followed by Alexa 546 conjugated anti-mouse secondary antibody (Life Technologies, USA) and chromosomes were counterstained with Hoecht 33342. Slides were mounted in ProLong Gold anti-fade reagent (Life Technologies, USA) and imaged with Zeiss LSM DUO microscope.

RNA extraction and RT-PCR

Total RNA from cells was purified with RNeasy Micro kit (Quiagen) as recommended by the manufacturer and treated with DNase I using DNA-free kit (Ambion). First-strand cDNA was synthesised from 5 μg of total RNA with Superscript III reverse transcriptase (Life Sciences) according to manufacturer's recommendations. PCR reactions were performed with HotFire polymerase (Solis Biodyne) in a volume of 10 μl containing 1/80 of reverse transcription reaction as a template. Human *BDNF* 5′ exons' specific primers have been described previously [12]. Rat *BDNF* 5′ exons' specific primers have been described previously [11] and were used in combination with *hRluc* specific antisense primer 5′-GTA CTT GTA GTG ATC CAG GAG GCG AT-3′.

Genomic DNA extraction and quantitative PCR

Genomic DNA was extracted from cells by proteinase K digestion and phenol:chloroform extraction followed by ethanol precipitation and resuspension overnight in TE (pH 8.0). Genomic DNA concentration was quantified with UV spectrophotometer (NanoDrop) and diluted to 16 ng/μl for qPCR. For standard curve, a series of mixtures in which the number of pEGFP-C1 (Promega) plasmid molecules ranged from 128 to 1024 copies per HeLa genome were prepared using HeLa genomic DNA. 32 ng of genomic DNA from different cell lines or copy number standards were subjected to quantitative PCR. Quantitative PCR was performed on Roche LightCycler 2.0 using qPCR Core kit for SYBR® Green I No ROX (Eurogentec). qPCR reactions with copy number standards were performed in duplicate. qPCR reactions with cell line genomic DNAs were performed in triplicate. Melting curve analysis was carried out at the end of cycling to confirm amplification of a single PCR product. Following *EGFP* and human *TRKB* (genomic control) specific PCR primer sets were used: *EGFP* sense 5′- CAG AAG AAC GGC ATC AAG GTG-3′, antisense 5′- TGG GTG CTC AGG TAG TGG TTG -3′; *TRKB* sense 5′- CAC AGG GCT CCT TAA GGA TAA C -3′, antisense 5′- GCA CAG TGA GGT TGA CAG AAT C-3′. Copy number estimates were calculated with qBASEPlus 2.6 software (Biogazelle) using *EGFP* as target and *TRKB* as reference.

Abbreviations

BAC: Bacterial artificial chromosome; BDNF: Brain derived neurotrophic factor; FISH: Fluorescence in situ hybridisation; HDAC: Histone deacetylase; *hRluc*: Humanised *Renilla* luciferase; MAR: Matrix attachment region; SAR: Scaffold attachment region; YAC: Yeast artificial chromosome.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KJ performed reporter expression analysis, qPCR analysis of transgene copy number, fluorescent in situ hybridisation, drug treatments, transcription factor transfection, data analysis of the results and drafting of the manuscript. MS carried out BAC transfection, established cell lines, performed FACS sorting and analysis, fluorescence microscopy, ionomycin treatments and contributed to the initial design of the study. TAP prepared the

rBDNF-hRluc-EGFP BAC construct and contributed to the initial design of the study. TT conceived and coordinated the study. All authors contributed to the preparation of the manuscript. All authors read and approved the final manuscript.

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CURRICULUM VITAE

Name: Kaur Jaanson
Date and place of birth: 1.02.1985, Tartu
Citizenship: Estonian

Contact information

Department of Gene Technology, Tallinn University of Technology,
Akadeemia tee 15, 12618 Tallinn
Tel.: +372 56689445
Email: kjaanson@gmail.com, kaur.jaanson@ttu.ee

Education

2009-2015	Department of Gene Technology, Tallinn University of Technology (PhD student)
2007-2009	Department of Gene Technology, Tallinn University of Technology (MSc)
2004-2007	Department of Gene Technology, Tallinn University of Technology (BSc)
2004-2005	Estonian IT College, IT Systems Developer (unfinished)
2000-2003	Ülenurme Gymnasium

Language competence

Estonian	native
English	

Special courses

2011	Mouse Phenotyping, EMBO Practical Course, Zurich
2010	Principles of Flourescence Techniques, Madrid

Honours and awards

2009	Second prize in the national competition of research for masters students in the field of biotechnology and environmental sciences

Employment

2008-… Department of Gene Technology, Tallinn University of Technology, engineer
2009-2015 Competence Centre for Cancer Research, researcher

Supervised dissertations

Angela Pärn, MSc, 2014, supervised by Kaur Jaanson and Tõnis Timmusk
Development of bacterial artificial chromosome constructs for studying BDNF gene regulation

Angela Pärn, BSc, 2011, supervised by Kaur Jaanson and Tõnis Timmusk
Transfection of rat primary neurons with bacterial artificial chromosome

Publications

1. **Jaanson, K.**, Sepp, M., Aid-Pavlidis, T., Timmusk, T., (2014). BAC-based cellular model for screening regulators of BDNF gene transcription.
   BMC Neuroscience 15, 75. doi:10.1186/1471-2202-15-75

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

Inventions

1. Transgenic mouse and cell models and their uses for identification of drugs targeting brain-derived neurotrophic factor; Owner: Tallinn University of Technology; Authors: Tõnis Timmusk, Indrek Koppel, Mari Sepp, **Kaur Jaanson**, Tamara Aid, Priit Pruunsild, Kaia Palm; Priority number: US61/168,319; Priority date: 10.04.2009

ELULOOKIRJELDUS

Nimi: Kaur Jaanson
Sünniaeg ja -koht: 1.02.1985, Tartu
Kodakondsus: Eesti

Kontaktandmed

Geenitehnoloogia Instituut, Tallinna Tehnikaülikool, Akadeemia tee 15, 12618 Tallinn
Tel.: +372 56689445
Email: kjaanson@gmail.com, kaur.jaanson@ttu.ee

Hariduskäik

2009-2015	Geenitehnoloogia Instituut, Tallinna Tehnikaülikool, doktoriõpe
2007-2009	Geenitehnoloogia Instituut, Tallinna Tehnikaülikool, magistrikraad
2004-2007	Geenitehnoloogia Instituut, Tallinna Tehnikaülikool, bakalaureusekraad
2004-2005	Eesti Infotehnoloogia Kolledž, IT Süsteemide arendaja (lõpetamata)
2000-2003	Ülenurme Gümnaasium

Keeled

Eesti	emakeel
Inglise	

Kursused

2011	Mouse Phenotyping, EMBO Practical Course, Zurich
2010	Principles of Flourescence Techniques, Madrid

Teaduspreemiad ja -tunnustused

2009	Üliõpilaste teadustööde riiklikul konkursil bio- ja keskkonnateaduste valdkonnas magistriõppe üliõpilaste astmes II preemia

Teenistuskäik

2008-…	Geenitehnoloogia Instituut, Tallinna Tehnikaülikool, Insener
2009-2015	Vähiuuringute Tehnoloogia Arenduskeskus AS, Teadur

Juhendatud lõputööd

Angela Pärn, magistritöö, 2014, juhendajad Kaur Jaanson ja Tõnis Timmusk
Bakteriaalse kunstliku kromosoomi konstruktide arendamine BDNF geeni regulatsiooni uurimiseks

Angela Pärn, bakalaureusetöö, 2011, juhendajad Kaur Jaanson ja Tõnis Timmusk
Roti primaarsete neuronite transfekteerimine bakteriaalse kunstliku kromosoomiga

Publikatsioonid

1. **Jaanson, K.**, Sepp, M., Aid-Pavlidis, T., Timmusk, T., (2014). BAC-based cellular model for screening regulators of BDNF gene transcription.
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Patentsed leiutised

1. Transgenic mouse and cell models and their uses for identification of drugs targeting brain-derived neurotrophic factor; Owner: Tallinn University of Technology; Authors: Tõnis Timmusk, Indrek Koppel, Mari Sepp, **Kaur Jaanson**, Tamara Aid, Priit Pruunsild, Kaia Palm; Priority number: US61/168,319; Priority date: 10.04.2009

DISSERTATIONS DEFENDED AT
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