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Transcriptional Mechanisms of BDNF Gene Regulation

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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 other academic degree.

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II **Koppel, I**.*, Aid-Pavlidis, T.*, Jaanson, K., Sepp, M., Palm, K., Timmusk, T. (2010). BAC transgenic mice reveal distal cis-regulatory elements governing BDNF gene expression. Genesis, 48(4), 214–19.

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INTRODUCTION

Neurotrophic factors are secreted proteins that support survival and differentiation of several neuron populations in the central and peripheral nervous system. Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family of neurotrophic factors, is highly expressed in the central nervous system. In addition to its classical neurotrophic roles as a survival and differentiation promoting factor, BDNF is involved in regulation of synaptic function in the adult nervous system. Importantly, BDNF appears to have a critical role in long term synaptic potentiation, which is believed to represent the cellular basis of learning and memory. Owing to its multitude of functions in the developing and adult nervous system, it is not surprising that altered expression or function of BDNF has been associated with a number of nervous system disorders, including major depression, bipolar disorder and other psychiatric illnesses, neurodegenerative disorders such as Alzheimer's and Parkinson's disease, epilepsy and neuropathic pain. Currently, several BDNF-related therapeutic strategies are being developed for treatment of nervous system disorders. The most straightforward of these is either systemic or topical administration of BDNF protein as a drug, although this approach suffers from the major disadvantage of poor pharmacokinetic properties of BDNF. Other strategies include conjugation of BDNF protein with different nanotechnological formulations to improve pharmacokinetics, gene therapy and modulation of endogenous BDNF production by treatments that regulate BDNF expression. The appeal of the latter approach is the possibility to use small molecules with favourable pharmacological properties and to regulate BDNF levels in cells that already produce BDNF. For such strategies to succeed, it is evident that mechanisms regulating BDNF expression have to be well understood. In this thesis, I have used two approaches to study BDNF regulation. First, BAC transgenic mice carrying large fragments of rat or human BDNF loci were used to study BDNF gene regulation *in vivo*. Second, I have investigated the ability of chromatin modifying drugs to modulate BDNF expression.

1. REVIEW OF THE LITERATURE

1.1 Introduction

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of mammalian growth factors that regulate several aspects of neuronal survival, differentiation and function in the peripheral and central nervous system. Nerve growth factor (NGF), the founding member of the neurotrophin family, was discovered in the 1950s by Rita Levi-Montalcini and Stanley Cohen as an agent stimulating neurite outgrowth from chick sensory ganglia (Levi-Montalcini, 1987). It took about three decades before the neurotrophin family expanded with the discovery of BDNF (Barde et al., 1982), followed by discoveries of neurotrophin 3 (Ernfors et al., 1990a; Maisonpierre et al., 1990b) and neurotrophin 4/5 (Berkemeier et al., 1991; Hallböök et al., 1991). In addition, two more neurotrophins (NT-6 and NT-7) have been found in bony fish (Hallböök, 1999). Recently, a functional neurotrophin was discovered in the sea mollusk *Aplysia*, showing that neurotrophin signaling is also employed by invertebrates (Kassabov et al., 2013). Neurotrophins activate two kinds of receptors - each of them binds to one or more Trk (tropomyosin receptor kinase) receptors and all of them bind to the p75 neurotrophin receptor (Reichardt, 2006).

BDNF was discovered as a non-NGF neurotrophic activity present in conditioned medium of C-6 glioma cells (Barde et al., 1978). Soon thereafter, it was shown that rat brain extracts contained a similar neurotrophic activity (Barde et al., 1980), which was later identified as a 12 kDa protein to be known as BDNF (Barde et al., 1982). Ever since, a wealth of information has accumulated suggesting important roles for BDNF in several aspects of neuronal function from neurogenesis and cell survival to synaptic function (Bibel and Barde, 2000; Park and Poo, 2013).

1.2 Functions of BDNF

1.2.1 BDNF as a survival factor - the original role

BDNF was identified as a survival factor for cultured spinal sensory neurons from chick embryos (Barde et al., 1982). In the work that followed, BDNF was found to promote survival and stimulate neurite outgrowth in sensory neurons form many different sensory ganglia (Davies et al., 1986; Lindsay et al., 1985). In addition, BDNF was found to promote survival of cultured mesencephalic

dopaminergic neurons (Hyman et al., 1991), cerebellar granule neurons (Segal et al., 1992) and retinal ganglion cells (Rodriguez-Tébar et al., 1989); promote survival and induce differentiation of septal cholinergic neurons (Alderson et al., 1990). Moreover, BDNF prevented death of spinal cord motoneurons in newborn rats following axotomy (Sendtner et al., 1992).

In order to investigate the role of BDNF in survival and differentiation of neurons *in vivo*, BDNF knockout mice were generated (Ernfors et al., 1994; Jones et al., 1994). These mice were smaller than littermates, displayed severe movement defects and died within 2-3 weeks of postnatal development. In agreement with previous findings in cultured neurons, mice lacking BDNF had substantially reduced number of neurons in several sensory ganglions such as trigeminal, nodose-petrosal and vestibular ganglia. Despite profound deficits in motor function, defects in motoneuron survival were not observed in BDNF $^{-/-}$ mice in either study. Moreover, no change in numbers of dopaminergic or other central neurons previously associated with BDNF-dependent survival was observed. Surprisingly, no gross abnormalities in the brain architecture were detected. (Ernfors et al., 1994; Jones et al., 1994). The discrepancies between *in vitro* and *in vivo* results regarding neuron types other than sensory neurons were reconciled with the possibility of redundant effects with other neurotrophins such as NT4/5 that shares the TrkB receptor with BDNF. However, degeneration of motoneurons were not observed even in NT4$^{-/-}$ BDNF $^{-/-}$ double knockout mice (Liu et al., 1995). To date, these discrepancies have not been fully resolved. Recently, it was shown that TrkA and TrkC, but not TrkB, act as dependence receptors, signaling neuronal death in ligand-unbound state (Nikoletopoulou et al., 2010). In addition, a study investigating the mechanisms determining the correct number of cortical interneurons in development showed that apoptosis of these neurons is triggered by an intrinsic, TrkB-independent mechanism. The detailed workings of these mechanisms remain to be uncovered (Southwell et al., 2012). In conclusion, current knowledge suggests that BDNF and other neurotrophins act as target derived trophic factors for the peripheral nervous system (the "classical" neurotrophic theory), whereas survival of central nervous system neurons is governed by other, largely unknown mechanisms (Dekkers and Barde, 2013).

1.2.2 Neurite growth and arborization

Early studies on NGF showed that in addition to promoting neuronal survival it also possessed neurite growth stimulating properties (Levi-Montalcini, 1987). Later, several aspects of neurite growth and arborization were shown to be regulated by neurotrophins, first in peripheral and then in central neurons. BDNF induced extensive lamellipodia formation and growth cone turning towards applied BDNF microgradient in cultured *Xenopus* spinal neurons

(Ming et al., 1997; Song et al., 1997). Tucker and co-workers showed that any one of neurotrophins NGF, BDNF, NT-3 or NT4/5 stimulated growth of sensory, but not motor neurons in mouse embryonic limb buds (Tucker et al., 2001).

In the central nervous system, the effect of BDNF on neurite growth was first demonstrated in *Xenopus* tadpoles: injection of BDNF into their optic tectum increased branching of ganglionic axon terminals, while injecting a BDNF blocking antibody had an opposite effect (Cohen-Cory and Fraser, 1995). Dendritic growth and arborization was shown to be stimulated by BDNF in organotypic slices of ferret visual cortex. Intriguingly, BDNF and NT4/5, the other TrkB ligand, produced different patterns of dendrite elaboration (McAllister et al., 1995).

Strong evidence supporting the role of BDNF in regulation of dendrite growth was provided from several studies using targeted deletion of BDNF in mice. In BDNF null mutants, retarded growth of cerebellar Purkinje neuron dendrites was observed, together with defects in layering of cerebellar neurons (Schwartz et al., 1997). Conditional deletion of BDNF in forebrain neurons during embryogenesis lead to development of a thinner neocortex, associated with smaller neuronal size and defects in maintenance of dendritic structures (Gorski et al., 2003). In addition, reduced striatal volume, shrunken somas and thinner dendrites of striatal medium spiny neurons were detected in mice with forebrain BDNF deletion (Baquet et al., 2004). Very similar striatal phenotypes were observed in mice lacking BDNF in the whole central nervous system (Rauskolb et al., 2010).

1.2.3 Synaptic activity

In addition to their roles in neuronal survival and growth, neurotrophins have rapid effects on synaptic activity. This was first demonstrated in a study showing that NT-3 and BDNF (but not NGF) potentiated synaptic activity at neuromuscular synapses in cultured cells (Lohof et al., 1993). Later, in studies with cultured rat hippocampal neurons, hippocampal slices and rat hippocampi *in vivo*, it was shown that BDNF can potentiate neurotransmission in central neurons (Kang and Schuman, 1995; Lessmann et al., 1994; Levine et al., 1995; Messaoudi et al., 1998). BDNF can elicit neuronal depolarization on a timescale comparable to the neurotransmitter glutamate (in the order of milliseconds), mediated by opening of a TrkB-associated sodium channel $Na_v1.9$ (Blum et al., 2002; Kafitz et al., 1999). Significantly reduced long term potentiation (LTP) of hippocampal Schaffer collateral-CA1 synapses was detected in BDNF knockout mice, indicating that BDNF may have a critical role in synaptic plasticity in adult CNS neurons (Korte et al., 1995). Complete reversal of the LTP deficits by viral-mediated rescue of BDNF expression provided conclusive evidence that lack of BDNF in these mice had acute

effects on synaptic activity (Korte et al., 1996; Patterson et al., 1996). Over the past decades, the role and mechanisms of BDNF action in synaptic activity and particularly LTP have been intensively studied (Bramham and Messaoudi, 2005; Panja and Bramham, 2013; Poo, 2001).

Apart from LTP, BDNF has been implicated in other forms of synaptic plasticity. For example, BDNF has been shown to regulate long term synaptic depression (LTD) in rat visual cortex slices (Akaneya et al., 1996; Huber et al., 1998), short-term synaptic plasticity at medial perforant path-dentate granule cell synapses in the hippocampus (Asztely et al., 2000) and homeostatic plasticity (compensatory adaptations to changes in synaptic function) in cultured hippocampal neurons (Jakawich et al., 2010). An overwhelming majority of studies, however, have addressed the roles of BDNF in LTP.

Evidence for the roles of BDNF in LTP has been accumulated from a large number of studies using different approaches to manipulate BDNF function such as using BDNF or TrkB germline or conditional knockout or knock-in mice, blocking BDNF-TrkB signaling with antibodies or TrkB-IgG scavengers of BDNF, or applying exogenous BDNF to the synapses where LTP is recorded (Bramham and Messaoudi, 2005). These studies have established that BDNF contributes to LTP formation in several ways. First, it participates in conditioning the synapses before LTP initiation by regulating neurotransmitter release, termed the permissive role of BDNF in LTP (Figurov et al., 1996; Pozzo-Miller et al., 1999). Second, it has become clear that BDNF-TrkB signaling contributes to the formation and maintenance of LTP - the instructive role (Bramham and Messaoudi, 2005). This was revealed by studies where prior to LTP induction hippocampal slices were treated with TrkB-IgG BDNF scavengers (Figurov et al., 1996), anti-TrkB antibodies (Kang et al., 1997) or photoactivatable anti-BDNF antibodies (Kossel et al., 2001) - all leading to decreased LTP. Maintenance of LTP is divided into two phases - early phase that is independent of new protein synthesis and the protein synthesis-dependent late phase (L-LTP). As L-LTP is being regarded as a cellular proxy for long-term memory (Mayford et al., 2012), the roles of BDNF in L-LTP are of particular interest. Indeed, BDNF has been implicated in the processes of learning, memory formation and extinction (Bekinschtein et al., 2013; Lu et al., 2008).

Although BDNF has been associated with several aspects of LTP induction and maintenance, the molecular mechanisms mediating these functions are only beginning to be elucidated. Currently, candidate mechanisms for BDNF action in LTP focus mainly on synaptic remodeling by regulation of local synaptic translation and cytoskeletal dynamics (Bramham and Panja, 2013). The hypothesis of translational control of BDNF action in LTP relies on the following findings: first, late LTP in general and BDNF-induced late LTP in particular is dependent on new protein synthesis (Abraham and Williams, 2008; Kang and Schuman, 1996). Second, BDNF stimulates translation of a subset of neuronal mRNAs (Schratt et al., 2004) via regulation of initiation and

elongation steps (Inamura et al., 2005; Leal et al., 2013; Takei et al., 2001). It is generally not well understood how altered translation of these mRNAs mediates increase in synaptic strength. In addition, it remains to be determined whether endogenous BDNF-TrkB signaling can regulate synthesis of these proteins. Relatively much, however, is known about the involvement of one BDNF-regulated synaptic protein - Arc - in synaptic plasticity. Arc is an actin-associated protein that plays a critical role in LTD and LTP via regulating AMPA receptor endocytosis and synaptic actin dynamics, respectively (Bramham et al., 2010; Shepherd and Bear, 2011). BDNF (among several other stimuli) robustly induces Arc transcription (Messaoudi et al., 2002; Ying et al., 2002), translocation of Arc mRNA to dendrites (Ying et al., 2002) and local translation at synapses (Yin et al., 2002), where it plays a critical role in LTP maintenance via regulating actin network expansion (Messaoudi et al., 2007).

1.3 Overview of BDNF molecular biology

Expression. BDNF is widely expressed in the mammalian peripheral and central nervous system. In the brain, BDNF has widespread expression, predominantly in neurons (Conner et al., 1997; Ernfors et al., 1990b; Hofer et al., 1990; Katoh-Semba et al., 1997; Maisonpierre et al., 1991, 1990a; Timmusk et al., 1994a). Expression levels of BDNF are relatively low during embryogenesis, rise in late embryonic development and peak during first postnatal weeks (Maisonpierre et al., 1990a; Timmusk et al., 1994a). Apart from the nervous system, BDNF expression has been detected in several non-neuronal tissues such as heart, lung, skeletal muscle, liver, kidney, thymus and spleen; highest expression levels have been consistently detected in heart and lung (Aid et al., 2007; Katoh-Semba et al., 1997; Maisonpierre et al., 1991, 1990a; Pruunsild et al., 2007; Yamamoto et al., 1996). The best known non-neuronal cell types that display high levels of BDNF expression are thrombocytes (Yamamoto and Gurney, 1990), vascular smooth muscle cells (Donovan et al., 1995) and endothelial cells (Donovan et al., 2000; Nakahashi et al., 2000).

Gene structure. Considering the multiple roles for BDNF in neuronal maintenance and function, its expression is expected to be tightly regulated (for details see next chapter). Indeed, even at the level of transcription BDNF expression is regulated by the use of a number of differentially regulated promoters. Each of these promoters precedes an untranslated 5' exon, joined with a common protein coding 3' exon by alternative splicing in a mutually exclusive manner - see Figure 1; (Aid et al., 2007; Pruunsild et al., 2007; Timmusk et al., 1993). Each transcript with an alternative 5' exon can contain 3'UTRs of two alternative lengths due to use of alternative polyadenylation sites (Timmusk et al., 1993). An additional layer of regulation can be exerted

by the antiBDNF gene located on the opposite strand relative to BDNF (Liu et al., 2005; Modarresi et al., 2012; Pruunsild et al., 2007). AntiBDNF transcripts have a complementary region with BDNF exon IX that is common to all BDNF transcripts (Liu et al., 2005; Modarresi et al., 2012; Pruunsild et al., 2007) and knockdown of antiBDNF mRNA leads to upregulation of BDNF expression *in vitro* and in the brain (Modarresi et al., 2012).

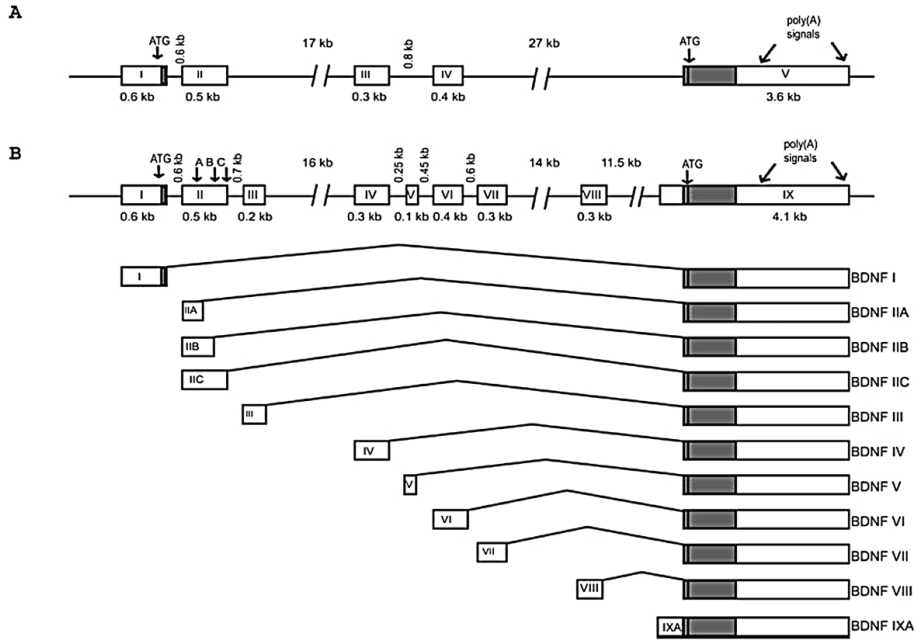

Figure 1. Structure of the rodent BDNF gene. A) Rat BDNF gene structure as described by Timmusk et al. (1993). Exons are shown as boxes and introns are shown as lines. B) Up-to-date rodent gene structure (Aid et al., 2007). The upper diagram shows the arrangement of exons and introns of mouse and rat BDNF genes, BDNF transcripts generated by alternative splicing are shown below. Protein coding regions are shown as solid boxes and untranslated regions are shown as open boxes. Alternative polyadenylation sites are indicated with arrows. For exon II, three different transcript variants, IIA, IIB, and IIC, are generated as a result of using alternative splice-donor sites in exon II. Figure is reproduced from Aid et al., 2007, with permission from John Wiley & Sons, Inc.

Subcellular localization of BDNF transcripts. BDNF mRNA and protein have been shown to localize to the cell soma and proximal dendrites in rat hippocampal neurons (Tongiorgi et al., 1997). The dendritic localization is enhanced by KCl depolarization (Tongiorgi et al., 1997) and bath application of BDNF (Righi et al., 2000). In the rat hippocampus, dendritic localization of BDNF mRNA and protein was observed after pilocarpine-induced epileptic seizures, whereas BDNF was essentially absent from the dendritic fields under basal conditions (Tongiorgi et al., 2004). After pilocarpine treatment, alternative BDNF transcripts showed differential localization - only exon II and exon VI containing transcripts were translocated to dendrites, whereas exon I and IV containing transcripts were retained in the cell soma (Chiaruttini et al., 2008). Dendritic localization of BDNF was shown to be also dependent on the longer variant of its 3'UTR. Mice with truncated BDNF 3'UTR displayed impaired dendritic localization of BDNF mRNA, defects in dendrite morphogenesis and LTP (An et al., 2008). Finally, transport of BDNF mRNA to dendrites was found to be dependent on its interaction with translin, an RNA trafficking protein, via a *cis*-element in the protein coding region (Chiaruttini et al., 2009). Considering the roles of BDNF in dendrite growth and synaptic plasticity, it is compelling to see mechanisms regulating dendritic transport and local translation of BDNF mRNA as important mediators of these outcomes. However, the notion of dendritic BDNF localization is not universally accepted. In a recent study, Dieni and co-workers showed localization of BDNF protein in presynaptic terminals and complete absence of BDNF in postsynaptic structures (Dieni et al., 2012). It is possible that dendrites contain low, but biologically relevant levels of BDNF, and perhaps detectable levels of BDNF can be found in different dendritic structures than analyzed by Dieni and co-workers. However, there is currently no data available showing endogenous BDNF protein in postsynaptic structures.

BDNF protein synthesis and processing. BDNF is synthesized as a preproprotein into the endoplasmic reticulum. Translation of BDNF protein from alternatively spliced mRNAs eventually generates mature protein with identical primary sequence (differences in posttranslational modifications, though, cannot be ruled out). The sequences of BDNF preproprotein, too, are identical with the possible exception of protein translated from exon I containing transcripts, which have an in-frame start codon in the end of exon I (Aid et al., 2007; Pruunsild et al., 2007; Timmusk et al., 1993). In addition, human, but not rodent BDNF gene has potential start codons in exon VII and a rarely used exon VIII (Pruunsild et al., 2007). Use of these start codons would generate protein isoforms with alternative signaling peptides that may potentially affect the function of mature protein through differential intracellular targeting, secretion or posttranslational modifications (Hegde and Bernstein, 2006). The pre-region constitutes an ER signal sequence that is cleaved co-translationally and the resulting proBDNF undergoes

posttranslational processing and sorting to secretory vesicles (Lessmann et al., 2003). It is known that proBDNF undergoes N-terminal glycosylation in the pro-domain (Mowla et al., 2001) and is converted to mature BDNF by an intracellular endopeptidase - either by furin in the trans-Golgi network or by proprotein convertases PC1/2 in secretory granules (Lessmann et al., 2003). In addition, it has been suggested that proBDNF can be secreted and processed in the extracellular space by plasmin or even stay unprocessed and function independently from mature BDNF (Pang et al., 2004; Teng et al., 2005). According to this model, proBDNF binds to the p75 receptor with a higher affinity than mature BDNF and signals apoptosis, while the mature form preferentially binds the TrkB receptor and signals cell survival (Teng et al., 2005). Whereas mature BDNF is currently regarded as an important mediator (or modulator) of synaptic long term potentiation (Bramham and Messaoudi, 2005), proBDNF was shown to facilitate long term depression (decrease in synaptic efficiency)(Woo et al., 2005). This way BDNF (and other neurotrophins) could be regulated at the level of secretion and precursor processing to convey exactly opposite signals (Lu et al., 2005). It must be noted, though, that currently consensus is lacking in this matter (Barker, 2009). The heart of the matter is the question of whether the amounts of proBDNF in the extracellular space are big enough to exert physiologically relevant effects. Two studies focusing on the amounts of endogenously produced proBDNF in cultured neurons and brain tissue arrived at contradicting results (Matsumoto et al., 2008; Yang et al., 2009) and the source of this contradiction has not yet been resolved.

BDNF secretion. Proteins are secreted from cells via constitutive (non-regulated) or regulated (responsive to specific stimuli) pathways. Both pathways are used for release of neurotrophins, but individual neurotrophins have different propensities for using one or the other (Lessmann et al., 2003). NGF, for example, is more efficiently targeted to the constitutive pathway than BDNF, which is predominantly released in a regulated manner (Brigadski et al., 2005; Mowla et al., 1999). Secretion of BDNF from cultured neurons has been demonstrated following application of several stimuli such as KCl depolarization (Balkowiec and Katz, 2000; Goodman et al., 1996), electrical stimulation (Balkowiec and Katz, 2000; Hartmann et al., 2001), glutamate (Canossa et al., 2001, 1997), spontaneous synaptic activity (Kuczewski et al., 2008) and even neurotrophins themselves (Canossa et al., 1997; Krüttgen et al., 1998). Activation of AMPA and metabotropic glutamate receptors, but not NMDA receptors, triggered BDNF release from cultured hippocampal neurons (Canossa et al., 2001). Regulated secretion of BDNF depends on Ca2+ release from intracellular stores (Balkowiec and Katz, 2002; Canossa et al., 1997; Griesbeck et al., 1999; Kolarow et al., 2007) and most probably also on extracellular Ca2+ influx (Balkowiec and Katz, 2002; Goodman et al., 1996;

Kolarow et al., 2007), although the latter has been challenged (Griesbeck et al., 1999). Activation of calmodulin-dependent protein kinase II (CaMKII) and cAMP/protein kinase (PKA) have a critical role in KCl depolarization-induced BDNF secretion and neurotrophin or glutamate-induced BDNF release depends on activation of the phospholipase C gamma (PLC-γ) pathway (Canossa et al., 2001). Sorting of BDNF to the regulated secretion pathway has been shown to involve interaction of the BDNF pro-domain with a sorting receptor sortilin (Chen et al., 2005) and interaction of carboxypeptidase E (CPE) with a sequence in mature BDNF (Lou et al., 2005). A human polymorphism in proBDNF (Val66Met), associated with poorer episodic memory and abnormalities in hippocampal fMRI readings, impairs interaction of BDNF with sortilin and results in defective activity-dependent BDNF secretion in cultured neurons, underscoring the importance of BDNF function in the adult nervous system (Egan et al., 2003).

1.4 Regulation of BDNF expression

Since the cloning of the BDNF gene (Leibrock et al., 1989) and characterization of the multi-promoter containing gene structure (Timmusk et al., 1993), mechanisms of BDNF transcription regulation and, more recently, epigenetic regulation have received the biggest share of researchers' attention. Post-transcriptional regulation mechanisms such as subcellular targeting (Tongiorgi, 2008), regulation by miRNAs (Caputo et al., 2011; Lee et al., 2012; Mellios et al., 2008; Miura et al., 2012; Muiños-Gimeno et al., 2011) and natural antisense transcripts (Modarresi et al., 2012; Pruunsild et al., 2007) and regulation of BDNF translation (Autry et al., 2011; Lau et al., 2010; Timmusk et al., 1994b; Verpelli et al., 2010) have been studied much less intensively. In the overview below, I will give an overview of transcriptional regulation of the BDNF gene and cover epigenetic regulation of BDNF in more detail.

1.4.1 Transcriptional regulation of BDNF expression

Research on BDNF transcription started with a study showing that BDNF mRNA levels in cultured hippocampal neurons were induced by depolarizing concentration of K^+ in culture medium and this effect was dependent on the presence of Ca^{2+} ions in the medium (Zafra et al., 1990). In addition, a variety of neurotransmitters were tested for their ability to increase BDNF mRNA levels, of which kainic acid (a glutamate receptor subtype agonist) and, to a lesser extent, histamine and carbachol (an acetylcholine receptor agonist) significantly elevated BDNF levels in cultured neurons. Intraperitoneal injections of kainic acid to rats robustly increased BDNF mRNA levels in the

brain (Zafra et al., 1990). Subsequent studies, investigating BDNF regulation by neurotransmitters and growth factors in detail, showed that BDNF expression in the hippocampus is controlled by the balance between glutamatergic and GABAergic neurotransmission (Zafra et al., 1991), that BDNF expression in neurons is insensitive to regulation by several growth factors (neurotrophins themselves were not tested) (Zafra et al., 1992) and that pilocarpine (a muscarinic receptor agonist) effectively increased BDNF mRNA levels in the brain (da Penha Berzaghi et al., 1993). In cultured astrocytes, BDNF was shown to be regulated by dopamine and norepinephrine and not regulated by glutamate and several growth factors (Zafra et al., 1992).

Regulation of BDNF transcription by neuronal activity was demonstrated further in studies where epileptic activity was induced in the rat brain using either electrical stimulation (kindling) (Ernfors et al., 1991) or hippocampal lesions (Isackson et al., 1991). LTP induction by electrical high frequency stimulation increased BDNF mRNA levels in hippocampal slices (Patterson et al., 1992) and in freely moving rats (Castrén et al., 1993). BDNF mRNA levels in the visual cortex could be manipulated by light conditions, showing the possibility of detecting BDNF regulation by mild physiological stimuli (Castrén et al., 1992). In the past two decades, a large number of studies have reported changes in BDNF expression in relation to a variety of pharmacological, behavioural or other stimuli (at the time of writing this, query "BDNF expression" returned 6161 hits in Pubmed). Together with a few other genes such as c-fos and Arc, BDNF has become a classical model gene for studying the molecular mechanisms of neuronal activity-dependent transcription (Greer and Greenberg, 2008; Lyons et al., 2012).

Characterization of the BDNF gene revealed that four 5' untranslated exons were alternatively spliced with a common protein-coding exon and each of these 5' exons was linked with a promoter enabling separate regulation for transcription of different mRNA species. The latter was demonstrated by differential expression of alternative BDNF transcripts in tissues and variable induction of these transcripts in the rat hippocampus by kainic acid treatment (Timmusk et al., 1993). Elucidation of the BDNF gene structure lead to studies addressing molecular mechanisms of BDNF transcriptional regulation at different promoters. BDNF promoter IV has been the best studied of these promoters, owing to high expression levels of exon IV-containing transcripts in neurons and the fact that promoter IV is highly inducible by neuronal activity (Lyons et al., 2012). Three calcium response elements (CaREs) were discovered in the proximal 170 bp of the principal transcriptional start site in promoter IV, which work in concert to govern calcium-induced transcription from this promoter (Chen et al., 2003b; Shieh et al., 1998; Tao et al., 2002, 1998). Different calcium-regulated transcription factors bind to these *cis*-elements: - Ca^{2+} response factor (CaRF) binds to CaRE1 (Tao et al., 2002), upstream stimulatory factors 1/2 (USF1/2) bind to CaRE2 (Chen et al., 2003b) and cAMP/Ca^{2+}-response factor (CREB) binds to CaRE3, (also known as CRE,

the cAMP/Ca^{2+}-response element) (Shieh et al., 1998; Tao et al., 1998). The CRE element has been shown to be critically important for neuronal activity-dependent induction of BDNF promoter IV (Hong et al., 2008), but coordinated action of different *cis* elements and *trans* acting factors is needed for full control over the activity of this promoter. In addition to the CaRE-binding proteins, calcium-dependent induction of promoter IV has been shown to be regulated by MEF2 (Hong et al., 2008; Lyons et al., 2012), NPAS4 (Lin et al., 2008; Pruunsild et al., 2011), MeCP2 (Chen et al., 2003a; Martinowich et al., 2003), BHLHB2 (Jiang et al., 2008) and NFκB (Lipsky et al., 2001). BDNF promoter I is also highly induced by neuronal activity (Metsis et al., 1993; Timmusk et al., 1993) and to a large extent uses common regulation mechanisms with promoter IV (Flavell et al., 2008; Pruunsild et al., 2011; Tabuchi et al., 2002). In addition, BDNF promoters I and II are regulated by a repressor protein called the neuron-restrictive silencing factor (NRSF), binding to the neuron-restrictive silencing element (NRSE) in promoter II (Timmusk et al., 1999). Little is known about regulation of other BDNF promoters.

Transcription factors recruit additional proteins to BDNF promoters to form regulatory complexes, including several chromatin-modifying proteins such as histone acetyltransferases (HAT) and deacetylases (HDAC), histone methyltransferases (HMT) and demethylases (HDM). Importantly, neuronal activity leads to remodeling of these complexes, resulting in activated epigenetic state at promoters and a net increase in BDNF transcription (Greer and Greenberg, 2008). In the absence of neuronal activity, chromatin at BDNF promoter IV is maintained at a repressed state, partly mediated by association of promoter-bound MeCP2 with mSin3A/HDAC1 corepressor complex (Chen et al., 2003a; Martinowich et al., 2003). In addition, MEF2 that binds to promoter IV (Hong et al., 2008; Lyons et al., 2012) can recruit class II HDACs (Miska et al., 1999; Wang et al., 1999). Synaptic activity-induced calcium influx triggers phosphorylation of CREB (Sheng et al., 1990), phosphorylation of MeCP2 (Zhou et al., 2006) and dephosphorylation of MEF2 (Flavell et al., 2006), collectively leading to restructuring of repressive transcriptional complexes into activating ones, such as the complex of CREB phosphorylated at serine 133 and a histone acetyltransferase CBP (CREB-binding protein) (Chrivia et al., 1993). Dissociation of HDACs from chromatin is mediated probably by modifications of their transcription factor binding partners (Flavell et al., 2006; Zhou et al., 2006) as well as translocation of HDACs from the nucleus to the cytoplasm by neuronal activity (Chawla et al., 2003). BDNF regulation by individual HDACs - a protein family with 11 members (Haberland et al., 2009) - has not been thoroughly studied. Only HDAC2 has been shown to associate directly with BDNF promoters I, II and IV (Gräff et al., 2012; Guan et al., 2009). In addition, indirect evidence implies regulation of BDNF expression by HDAC4 (Li et al., 2012) and HDAC5 (Tsankova et al., 2006), but direct association between these proteins and BDNF promoters has not been shown.

1.4.2 Epigenetic regulation of BDNF expression

1.4.2.1 Regulation of BDNF expression by histone modifications

Histones can be covalently modified in a number of ways to affect chromatin function in transcription, replication, DNA repair and chromatin condensation. Known histone modifications include acetylation, methylation, ubiquitination and sumoylation on lysines, methylation and deimination on arginines, phosphorylation on serine, threonine and tyrosine residues, ADP-ribosylation on glutamates and arginines, proline isomerization and proteolytic trimming of histone tails. With the exception of histone tail clipping, probably all histone modifications are reversible. Histone acetylation, phosphorylation, ubiquitination and sumoylation induce structural changes in chromatin, whereas the small and chargeless methylation mark functions rather as an adapter handle for attachment of chromatin modifying complexes. Acetylation marks have been almost exclusively associated with euchromatin and facilitation of transcription. Methylation, in contrast, can signal activation or repression dependently on the position of the lysine residue in histones and even on the position of modified histones in chromatin (e.g. promoter vs gene body). Relatively little is known about the role of other modifications. (Bannister and Kouzarides, 2011; Kouzarides, 2007).

Regulation of BDNF expression was first associated with histone modifications in a study showing that pilocarpine induced status epilepticus induced histone 4 (H4) acetylation at BDNF promoter II and repressed H4 acetylation at promoter VI (then designated as promoter IV) (Huang et al., 2002). Ever since, changes in histone modifications at BDNF promoters have been shown in several studies addressing molecular mechanisms involved in memory formation, stress, addiction, epilepsy and action of several pharmacological agents (Boulle et al., 2012; Karpova, 2013; Lubin, 2011). First, such changes were reported in studies investigating epigenetic regulation of BDNF by neuronal activity. In a study by Nestler and co-workers, electroconvulsive therapy (ECT) induced H3 and H4 acetylation at BDNF promoters II and IV (designated II and III at the time of publishing) in rat hippocampi (Tsankova et al., 2004). Induction of BDNF promoter I-driven transcription by NMDA application to cultured hippocampal neurons was shown to be accompanied by increased levels of activating H3K4 methylation marks and decreased levels of repressive H3K9 methylation marks (Tian et al., 2009). Light deprivation decreased BDNF expression in the visual cortex, which was associated with increases in repressive H3K27 trimethylation at BDNF promoters in both visual cortex and hippocampus (Karpova et al., 2010). Increased histone acetylation at different BDNF promoters (including promoter IV in all studies) was observed after rats were subjected to environmental enrichment (Kuzumaki et al., 2011), communal rearing (Branchi

et al., 2011) or physical exercise (Gomez-Pinilla et al., 2011; Intlekofer et al., 2013). Stress has been shown to induce changes in BDNF epigenetic regulation in the hippocampus. This was first demonstrated in a study showing that chronic defeat stress decreased BDNF expression in the hippocampus, which was accompanied by robust increases in repressive H3K27 dimethylation at promoters III and IV (Tsankova et al., 2006). Acute immobilization stress decreased H3 acetylation at promoters I, IV and VI, suggesting that repressive epigenetic changes are induced at BDNF promoters in other stress paradigms as well (Fuchikami et al., 2009).

Learning and memory. Epigenetic BDNF regulation has been implicated in memory formation and extinction (Lubin, 2011). In the pioneering study, Barad and co-workers showed that in the fear learning paradigm, conditioning and extinction of the fear memory differentially altered histone 3 and histone 4 acetylation at BDNF promoters I and IV in the mouse prefrontal cortex (Bredy et al., 2007). Differential effects on H3 and H4 acetylation at BDNF promoters in the hippocampal CA1 region by fear memory consolidation was demonstrated in a following study (Lubin et al., 2008). Increased H3K4 trimethylation (activating modification) levels at BDNF promoter I, but not at promoter IV were observed after fear conditioning in the CA1 region (Gupta et al., 2010) and robust decreases in H3K9 dimethylation (repressing modification) were reported in the entorhinal cortex of rats after fear conditioning (Gupta-Agarwal et al., 2012).

Addiction. The first indication that BDNF epigenetic regulation is associated with drug addiction was provided in as study showing that chronic cocaine administration increased histone 3 (H3) acetylation at BDNF promoter IV in rat striatum, a central brain region in the reward circuitry implicated in addiction (Kumar et al., 2005). Recent studies have shed light on how cocaine exposure affects epigenetic BDNF regulation in other addiction-related brain regions. First, chronic cocaine administration increased H3 acetylation at BDNF promoters II and III in the shell of nucleus accumbens, a central region in the mesolimbic reward pathway (Wang et al., 2010). Induction of BDNF expression in the nucleus accumbens by cocaine has an important role in mediating the rewarding effects of cocaine (Graham et al., 2007) and the sensitization of rats to stress by cocaine (Covington et al., 2011). Second, forced cocaine abstinence in the ventral tegmental area (part of the reward pathway that projects to the nucleus accumbens) increased H3 acetylation at BDNF promoter I and expression of BDNF exon I containing transcripts (Schmidt et al., 2012). Third, chronic cocaine increased BDNF exon IV containing transcripts, histone 3 acetylation and CREB binding at BDNF promoter IV in the prefrontal cortex (PFC), another part of the mesolimbic pathway. In addition, this study found that BDNF levels in the prefrontal cortex correlated negatively with cocaine intake, suggesting that cocaine-induced

BDNF increase in this brain area may act as a compensatory adaptation (Sadri-Vakili et al., 2010). Interestingly, reduced cocaine intake, increased BDNF expression and BDNF promoter acetylation in the PFC were passed on to male, but not female offspring of cocaine-treated rats (Vassoler et al., 2013).

Antidepressants and histone deacetylase inhibitors. Antidepressants induce structural plasticity in the brain, as has been convincingly shown with experiments on the visual cortex of the rat (Castrén and Hen, 2013; Maya Vetencourt et al., 2008). The mechanisms of antidepressant-induced plasticity were addressed in a recent study showing that serotonin infusion into the visual cortex recapitulated the plasticity-inducing effects of fluoxetine and these effects were dependent on BDNF-TrkB signaling. Long-term fluoxetine treatment induced BDNF expression in the visual cortex, which was accompanied by increased acetylation at its promoters (Maya Vetencourt et al., 2011). Similar increases in BDNF promoter acetylation have been observed in the hippocampus after treatment of mice with fluoxetine (Onishchenko et al., 2008) or imipramine, another antidepressant (Tsankova et al., 2006). Changes in promoter acetylation, however, were seen only after antidepressant treatments were applied after pretreatments leading to decreased BDNF expression (Onishchenko et al., 2008; Tsankova et al., 2006). Regulation of BDNF expression by HDAC inhibitors has been explored in a number of studies (see references in publication III). In cultured neurons, different HDAC inhibitors show the ability to robustly induce BDNF expression (e.g. Aid et al., 2007; Fukuchi et al., 2009; Yasuda et al., 2009). Induction of BDNF expression and acetylation at BDNF promoters by HDAC inhibitors has also been shown *in vivo*, but it appears that these effects are best seen from the baseline of previously decreased BDNF expression (Mielcarek et al., 2011; Zeng et al., 2011). In other words, the most simplistic scenario may be that HDAC inhibitors restore BDNF expression levels by normalizing the status of histone modifications at BDNF promoters that have previously been altered by pathological processes (for comparison with similar effects with fluoxetine, see Onishchenko et al., 2008 and Tsankova et al., 2006).

1.4.2.2 Regulation of BDNF expression by DNA methylation

Methylation of cytosine at the 5th position of the base (5mC) is the predominant covalent modification of DNA (Deaton and Bird, 2011). Other known DNA modifications include sequential oxidation products of the 5mC methyl group and may represent transient intermediates of DNA demethylation (perhaps some of these may be stable and execute unknown functions) (He et al., 2011; Ito et al., 2011; Kriaucionis and Heintz, 2009; Tahiliani et al., 2009). Cytosines are methylated almost exclusively in CpG dinucleotides, overwhelming majority of which are in the methylated state in the genome. A

fraction of CpG dinucleotides found in CG rich sequences called CpG islands, however, are predominantly unmethylated and frequently colocalize with transcription start sites. CpG island methylation can be dynamic in development, hypermethylation unequivocally indicating promoter silencing. As a rule, DNA hypermethylation in promoters occurs only after the first stage of transcriptional silencing has been carried out by restrictive histone modifications (such as H3K27me3) and probably consolidates silencing in a more stable repressed state (Deaton and Bird, 2011). However, recent studies showing relatively rapid activity-dependent DNA methylation changes in postmitotic neurons suggests that this modification can also be used in a more dynamic fashion (Zovkic et al., 2013).

DNA methylation has been shown to contribute to long term memory formation (Zovkic et al., 2013). In seminal studies, Sweatt and colleagues showed that inhibition of DNA methyltransferases blocked induction of LTP in hippocampal slices (Levenson et al., 2006) and formation of a conditioned fear memory (Miller and Sweatt, 2007). In a following study, contextual fear learning was shown to increase BDNF expression in the rat hippocampus. This induction was accompanied by methylation changes at BDNF promoters (Lubin et al., 2008). Inhibition of the activity of endogenous DNA methyltransferases (DNMT) interfered with memory formation, decreased methylation at BDNF promoters I, II, IV and VI and increased expression of BDNF transcripts I, IV, VI and IX. Interestingly, DNMT inhibitor administration, while increasing expression of exon IV-containing BDNF transcripts, increased methylation levels at this promoter, indicating a complex regulation of BDNF expression and memory by promoter methylation. Interplay between DNA methylation and histone modifications could account for these findings as it was shown that DNMT inhibitors blocked the learning-induced increases in histone 3 acetylation at BDNF promoter IV (Lubin et al., 2008). Next, it was shown that childhood stress lead to decreased BDNF expression in the prefrontal cortex, accompanied by increased promoter methylation that persisted well into the adult age and even passed on to the next generation; this maltreatment-induced decrease in BDNF expression was reversed by treatment with a DNMT inhibitor (Roth et al., 2009). In a rat model of post-traumatic stress disorder, using exposure of rats to a cat as the traumatic experience, stress induced BDNF promoter IV hypermethylation in the hippocampus, but not in prefrontal cortex or amygdala, another two brain regions important for fear memory consolidation (Roth et al., 2011). Collectively, these results show that there is a strong basis to speculate that DNA methylation at BDNF promoters is important for acquisition of memory, or at least certain types of memory.

DNA methylation in cells, including post-mitotic cells such as neurons, can undergo plastic changes as a response to different stimuli. For that, enzymatic activities for both establishing and erasing methylation are needed. The mechanisms of generating cytosine methylation are well understood: one DNA

methyltransferase (DNMT1) is responsible for maintenance of DNA methylation, i.e. copying the methylation pattern from the parent DNA strand to the nascent strand after replication, and two proteins - DNMT3a and DNMT3b - function as *de novo* DNA methyltransferases laying down new CpG methylation marks (Klose and Bird, 2006). In contrast, definite understanding of how DNA demethylation works and the identity of protein(s) mediating this activity is still lacking. This question has been the subject of active scientific inquiry for at least three decades, during which several new candidate mechanisms and enzymes have been proposed and later refuted by other groups (Ooi and Bestor, 2008). The most recent addition to the family of candidate DNA demethylases are the TET proteins that have been reported to catalyze 5-methylcytosine conversion to 5-hydroxymethylcytosine, 5-formylcytosine and 5-carboxylcytosine (He et al., 2011; Ito et al., 2011). The following conversion of 5-carboxylcytosine to cytosine may proceed via decarboxylation (Ito et al., 2011) or via base excision repair carried out by thymine-DNA glycosylase (He et al., 2011). However, other mechanisms are under discussion as well (Wu and Zhang, 2010).

While the mechanisms of demethylation are still uncertain, it is beyond doubt that activity-dependent demethylation takes place in neurons. In the context of BDNF, it has been shown that KCl depolarization of cultured cortical neurons leads to demethylation at promoter IV (Chen et al., 2003a; Martinowich et al., 2003). In addition, NMDA receptor activation has been shown to be important for activity-dependent BDNF promoter IV demethylation in cultured hippocampal neurons (Nelson et al., 2008) and *in vivo* (Lubin et al., 2008). Finally, it has been shown that electroconvulsive treatment decreases methylation at BDNF promoter IX in the adult rat hippocampal dentate gyrus and this decrease is abolished in Gadd45b knockout mice (Ma et al., 2009).

Recently, several studies have investigated methylation at BDNF promoters in postmortem tissue or blood samples of subjects with psychiatric and neurodegenerative disorders (Ikegame et al., 2013). Increases in BDNF promoter methylation were found in postmortem brain samples of suicide victims (Keller et al., 2010), Alzheimer's disease and bipolar disorder patients (Rao et al., 2012). In the blood, increased BDNF promoter methylation has been detected in patients with major depressive disorder (D'Addario et al., 2013), bipolar disorder (D'Addario et al., 2012) and borderline personality disorder (Perroud et al., 2013). In addition to reporting increased BDNF promoter methylation, one study showed decreased binding of Gadd45b to BDNF promoter IX in cortical samples of psychotic patients, supporting the mechanistic link between Gadd45b and BDNF promoter methylation (Gavin et al., 2012).

Methylated cytosines are recognized by proteins containing the methyl-CpG binding domain that act as adaptor molecules for assembly of transcription regulation complexes (Hendrich and Tweedie, 2003). MeCP2, a member of this

family, binds to a specific methyl-CpG in BDNF promoter IV and functions as a transcriptional repressor which is released from the promoter by membrane depolarization (Chen et al., 2003a; Martinowich et al., 2003). Loss-of-function mutations in the MeCP2 gene cause a neurodevelopmental disorder called the Rett syndrome (Amir et al., 1999). Rett syndrome patients have severe mental retardation and show dendritic abnormalities in the hippocampus (Chapleau et al., 2009). Owing to its functions in regulating dendritic arborization and synaptic plasticity, BDNF is considered an important candidate for mediating the downstream effects of MeCP2 mutation in Rett syndrome (Autry and Monteggia, 2012). The functional interaction between MeCP2 and BDNF was supported by a study showing similar neuroanatomical and behavioural phenotypes in MeCP2 mice and mice with conditional deletion of BDNF in the postnatal brain; in addition, MeCP2 and BDNF co-deletion caused an earlier onset of Rett-like symptoms, while BDNF overexpression in MeCP2 knockout mice delayed the onset. (Chang et al., 2006).

1.5 Transgenic mice for studying BDNF regulation

Transgenic mice provide a valuable tool for studying gene regulation *in vivo*. Knock-in mice have been generated to dissect the function of distinct elements in the BDNF gene, such as regulatory elements in BDNF promoter IV (Hong et al., 2008) or promoter IV-driven BDNF transcription as a whole (Sakata et al., 2009). Mice carrying a polymorphism for Val66Met substitution in the pro-region of BDNF (Chen et al., 2006) have been useful for studying the molecular mechanisms underlying reduced hippocampal volume and memory impairments associated with this substitution in humans (Egan et al., 2003). Transgenic mice carrying the CAT reporter gene under the control of relatively short fragments of the BDNF locus have been used to analyze the role of different BDNF regulatory regions *in vivo* (Timmusk et al., 1995). Using these mice, it was shown that about 9 kb rat genomic fragments containing promoters I+II or IV+VI were sufficient to drive transgene expression in a pattern that largely recapitulated endogenous BDNF expression in many, but not all, brain regions and peripheral tissues. Deviations from endogenous BDNF expression can be explained with the inherent problems with plasmid-based transgenes - exclusion of distal regulatory elements and possible position effects from neighboring genomic sequences. The bacterial artificial chromosome (BAC) technology enables to generate transgenes with large genomic fragments up to hundreds of kilobases, with the possibility to insert whole mammalian genes (on average < 50kb) with large 5' and 3' flanking sequences (Heintz, 2001). Similarly to small plasmid-based vectors, BACs are easy to modify and propagate in *Escherichia coli*. An additional advantage of the BAC technology for generating transgenic mice is that BACs have been used for genome

sequencing, providing an abundance of available clones. Other high capacity vector systems can be used for transgenesis such as yeast artificial chromosomes (YAC) that can carry up to several megabases of DNA (Fabb and Ragoussis, 1995). The latter technology was used in a study where transgenic mice were generated carrying 145 kb of the human BDNF locus. In these mice, the transgene did not fully recapitulate endogenous BDNF expression, indicating the existence of distal sequence elements for BDNF regulation (Guillemot et al., 2007). Therefore, comparison of BAC/YAC BDNF transgenes carrying different lengths of sequences upstream and downstream of the BDNF gene can be useful for locating such elements.

1.6 BDNF and disorders of the nervous system

Disturbances in BDNF expression and function have been associated with a number of central nervous system diseases including neurodegenerative disorders (Nagahara and Tuszynski, 2011; Zuccato and Cattaneo, 2009), depression, drug addiction and other psychiatric disorders (Autry and Monteggia, 2012; Russo et al., 2009), epilepsy (Binder et al., 2001) and neuropathic pain (Vanelderen et al., 2010). In addition, there is strong evidence that BDNF may have an important role in central regulation of energy intake and thus be involved in pathogenesis of obesity (Rios, 2013). In most of these conditions, causal relationship between BDNF dysregulation and disease pathogenesis has not been firmly established, but the role of BDNF in some aspects of the disease is well understood. For example, despite much effort, it is not well understood how BDNF contributes to the pathogenesis of depression, but it seems clear that BDNF is essential for antidepressants to work (Autry and Monteggia, 2012; Castrén and Rantamäki, 2010).

Neurodegenerative disorders. Reduced expression of BDNF mRNA or protein has been detected in post mortem brain tissue of patients suffering from Alzheimer's (Connor et al., 1997; Hock et al., 2000; Phillips et al., 1991), Parkinson's (Howells et al., 2000; Mogi et al., 1999; Parain et al., 1999) and Huntington's (Ferrer et al., 2000; Gauthier et al., 2004) diseases. In addition, animal models have provided additional evidence for the role of BDNF in neurodegenerative disorders. In rodent and primate models of Alzheimer's disease BDNF administration showed neuroprotective effects, improved learning and memory and restored perturbations in gene expression (Nagahara et al., 2009). Depletion of BDNF in the substantia nigra by a midbrain-hindbrain specific conditional BDNF deletion (Baquet et al., 2005) or infusion of anti-BDNF oligonucleotides (Porritt et al., 2005) resulted in loss of tyrosine hydroxylase positive dopaminergic neurons in the substantia nigra and parkinsonian-like motor deficits (Baquet et al., 2005; Porritt et al., 2005). In rodent models of amyotrophic lateral sclerosis (ALS), administration of BDNF

prevented degeneration of corticospinal motor neurons (Giehl and Tetzlaff, 1996) and slowed disease progression (Mitsumoto et al., 1994). Huntington's disease is caused by expansion of polyglutamine repeats in the huntingtin protein (Ross and Tabrizi, 2011). Two mechanistic links between huntingtin mutation and BDNF dysregulation have been proposed based on cellular and animal models of Huntington's disease: first, mutant huntingtin causes repression of BDNF transcription at promoter II (Zuccato et al., 2003, 2001); second, it has been shown that mutant huntingtin impairs axonal transport of BDNF from the cortex to the striatum (Gauthier et al., 2004).

Psychiatric disorders. Major depressive disorder. The connection between BDNF and mood disorders has been established on the basis of different lines of evidence. First, BDNF conditional knockout mouse lines generated for studying BDNF function in the adult brain have displayed marked depressive or anxiety-like behaviour (Chan et al., 2006; Monteggia et al., 2007; Rauskolb et al., 2010; Rios et al., 2001). Second, decreased expression of BDNF and its receptor TrkB in the prefrontal cortex and hippocampus (Dwivedi et al., 2003; Karege et al., 2005; Pandey et al., 2008) and increased expression in the nucleus accumbens (Krishnan et al., 2007) has been observed in post mortem tissues of suicide victims. In addition, patients suffering from major depressive disorder were reported to have decreased BDNF levels in blood serum (Sen et al., 2008). Antidepressant treatment has been shown to increase BDNF levels in the brains of suicide victims (Chen et al., 2001; Karege et al., 2005) and in the serum of depressed subjects (Sen et al., 2008). Collectively, these observations suggest that BDNF may have a role in the pathophysiology of depression. A large body of research has been conducted in rodent models to investigate the contribution of BDNF dysregulation in mood disorders (Autry and Monteggia, 2012; Duman and Monteggia, 2006). It can be concluded from these studies that stress - which is considered to be a precipitating environmental factor for depression - decreases BDNF expression in the hippocampus and prefrontal cortical areas of the brain, and treatments such as medication with antidepressants or electroconvulsive therapy restore the stress-induced decreases in these brain regions (Autry and Monteggia, 2012; Duman and Monteggia, 2006). However, it is not clear how BDNF mediates vulnerability to stress and whether BDNF is a central molecular player in the process of depression pathogenesis at all. The critical role of BDNF in antidepressant action, though, appears to be unambiguously determined (Autry and Monteggia, 2012; Castrén and Rantamäki, 2010).

Bipolar disorder and schizophrenia. In contrast to depression, studies investigating levels of BDNF and TrkB in post mortem brain tissues of schizophrenia patients have found both increases and decreases of both in several brain regions without arriving at a clear conclusion. Moreover, differently from the effect of antidepressants, antipsychotic medications have

not been found to produce consistent changes in BDNF expression (for review see Autry and Monteggia, 2012). In contrast, decreased levels of BDNF protein in the serum and in post mortem hippocampal tissue have been reported in patients with bipolar disease (Cunha et al., 2006; Knable et al., 2004; Monteleone et al., 2008). Finally, Val66Met polymorphism in BDNF, known to impair activity-dependent secretion of BDNF (Egan et al., 2003), correlates with susceptibility to bipolar disorder (Lohoff et al., 2005; Neves-Pereira et al., 2002). Correlation of this polymorphism with depression and schizophrenia has also been investigated, so far without conclusive results (Autry and Monteggia, 2012).

Drug addiction. Adaptations in the brain's reward circuitry are believed to cause the behavioural outcomes associated with drug addiction (Nestler, 2005) and BDNF, being a regulator of neural circuit plasticity, may participate in the plastic changes accompanying drug addiction (Russo et al., 2009). Decreased BDNF levels have been reported in blood serum of heroin addicts (Angelucci et al., 2007) and blood plasma of alcoholics (Joe et al., 2007), whereas increased blood plasma BDNF levels have been reported in chronic methamphetamine users (Kim et al., 2005). Cocaine self-administration of rats induced BDNF expression and signaling in the nucleus accumbens, one of the brain regions central to the reward circuitry, and localized knockdown of BDNF in the nucleus accumbens decreased self-administration of cocaine (Graham et al., 2007). Cocaine's rewarding effects were reduced in BDNF heterozygous knockout mice (Hall et al., 2003) and BDNF infusions into the nucleus accumbens or ventral tegmental area (VTA - another part of the reward circuitry) potentiated the rewarding effects of cocaine (Horger et al., 1999). Intriguingly, BDNF infusion into the VTA abolished and localized BDNF knockdown in the VTA potentiated the rewarding effects of morphine, indicating that neuroplastic adaptations in addiction to stimulants and opiates rely on BDNF signaling in a completely different manner (Koo et al., 2012).

Obesity. First indications that BDNF is involved in regulation of energy intake and body weight came from studies showing decreased appetite and weight loss in rats after intracerebroventricular infusion of BDNF (Lapchak and Hefti, 1992; Pelleymounter et al., 1995). Following studies with BDNF haploinsufficient and TrkB hypomorphic mice, knock-in mice with the Val66Met substitution or mice with conditional deletion of BDNF in the brain clearly indicated that BDNF-TrkB signaling negatively regulates food intake (Rios, 2013). Hypothalamus has been identified as the critical site of action for BDNF in food intake regulation as BDNF knockdown in the hypothalamus was sufficient for induction of hyperphagia and obesity in mice (Unger et al., 2007). Moreover, it has been shown that regulation of food intake by the BDNF-TrkB signaling also involves the mesolimbic reward circuit (Cordeira et al., 2010), widely studied in relation to its role in drug addiction (Nestler, 2005). The

BDNF-obesity connection has also been well established in human genetic studies. A deletion in chromosome 11 encompassing the BDNF gene correlated strongly with obesity in patients with the WAGR syndrome (Han et al., 2008). In addition, large genome-wide studies have linked the BDNF locus to obesity (Speliotes et al., 2010; Thorleifsson et al., 2009). Finally, BDNF 66Met allele (polymorphism leading to a valine-methionine substitution in the BDNF protein) has been associated with a higher body mass index than 66Val allele (Beckers et al., 2008). This association, however, remains to be verified in larger studies.

1.7 BDNF-based therapeutic strategies

BDNF has shown striking therapeutic efficacy in animal models of several neurodegenerative disorders (reviewed in Lu et al., 2013; Nagahara and Tuszynski, 2011). However, advances in neurotrophic factor based therapies have been hampered by delivery issues. After subcutaneously administered BDNF showed clinical benefit in an early phase I/II ALS trial for amyotrophic lateral sclerosis (ALS) (Bradley et al., 1995), a phase II/III trial was quickly initiated (The BDNF Study Group (Phase III), 1999). Disappointingly, the latter trial, followed by later phase I/II and II/III trials testing intrathecal BDNF for ALS (Beck et al., 2005; Kalra et al., 2003; Ochs et al., 2000 and an unpublished phase II/III trial) failed to show any clinical benefit. In addition, no beneficial effects were observed in a trial of subcutaneous BDNF for diabetic neuropathy (Wellmer et al., 2001). These failures had a cooling effect on development of BDNF-related therapies, but the idea of testing BDNF for ALS has not been conclusively discarded - today, there is a consensus that delivery and target engagement issues had been poorly addressed in the past trials (Henriques et al., 2010; Lu et al., 2013).

With systemic administration of neurotrophic factors largely ruled out as an impractical method of delivery, an array of alternative treatment strategies are now being pursued such as intraventricular or intraparenchymal infusions of recombinant proteins, intranasal administration, prolonged release from polymer carriers, gene/cell therapy, "Trojan horse" based transcytosis, peptide and non-peptide low molecular weight mimetics of neurotrophic factors, Trk-activating antibodies, transactivators of neurotrophic factor signaling pathways and inducers of neurotrophic factor expression (Géral et al., 2013; Longo and Massa, 2013; Lu et al., 2013; Nagahara and Tuszynski, 2011).

Although direct delivery of recombinant proteins to target tissue via intraparenchymal, intracerebroventricular and intrathecal routes has shown some encouraging results in preclinical studies and clinical trials (Géral et al., 2013), the invasiveness and complexity of these procedures render them infeasible for large-scale use, especially for chronic diseases requiring repeated dosing. Intranasal delivery is a promising strategy that has the benefits of

simplicity of administration, minimal systemic exposure and non-invasiveness. Intranasal administration resulted in effective delivery of BDNF to brain parenchyma, accompanied by increased Akt phosphorylation in the cortex (Alcala-Barraza et al., 2010) and reduced immobility time in the forced swimming test, a behavioural test used to assess antidepressant-like activity (Vaka et al., 2012).

Gene and cell therapy applications using virus vectors for local expression of BDNF in brain tissue or transplantation of BDNF expressing cells are being developed for treatment of several neurodegenerative disorders, as well as spinal cord injury and epilepsy (Géral et al., 2013). The advantages of these systems are long-lasting expression and the ability to achieve high concentrations of therapeutic proteins in specific brain regions. On the other hand, sustained expression that is not controllable after surgery carries the disadvantage of not being able to switch off the possible side affects associated with therapy. Therefore, it is desirable to design systems with regulated gene expression as has been demonstrated with a doxycycline-regulated promoter system for lentiviral delivery of NGF (Blesch et al., 2005). An elegant miRNA-based BDNF autoregulatory system has been demonstrated, allowing control of food intake by fine-tuned expression of BDNF in the hypothalamus. In this system, the viral BDNF gene transfer cassette includes a BDNF-targeting miRNA which is expressed under the control of a promoter activated by excessive weight loss (Cao et al., 2009). Disadvantages of gene and cell therapy include potential problems with toxicity, risk of tumor development and inflammatory responses (Géral et al., 2013). Nevertheless, gene therapy holds great promise for growth factor based treatments. Several clinical trials have been performed already, testing gene delivery applications for neurodegenerative disorders (Nagahara and Tuszynski, 2011).

The Trojan horse technology makes use of carrier mediated transport (CMT) to carry large molecular weight therapeutic cargo through the blood brain barrier. A Trojan horse agent is a fusion protein consisting of an antibody targeting a CMT receptor (e.g. transferrin or insulin receptor) and therapeutic protein of interest. Upon sequestering the Trojan horse from the blood, the CMT receptors expressed by endothelial cells are endocytosed and release their cargo to the opposite (brain) side of the endothelium (Gabathuler, 2010). Therapeutic BDNF concentrations in the rat brain have been achieved using BDNF conjugated with a human insulin receptor binding antibody (Boado et al., 2007). Limitations to this technology include poor tissue specificity and possible toxicity from blocking endogenous functions of these receptors (Gabathuler, 2010).

In addition to using the full-length BDNF protein, TrkB signaling can be targeted using low molecular weight (LMW) compounds such as peptide mimetics of BDNF, other LMW TrkB agonists, activators of BDNF intracellular signaling and agents modulating BDNF expression. BDNF peptidomimetics have been shown to enhance survival of cultured embryonic

chick dorsal root ganglion sensory neurons (Fletcher and Hughes, 2006; O'Leary and Hughes, 2003). However, the ability of these compounds to activate TrkB receptor has not been shown. In contrast, several non-peptide LMW TrkB agonists with TrkB activating and neurotrophic properties have been reported (Jang et al., 2010a, 2010b, 2010c, 2009; Massa et al., 2010), but it remains unknown whether these compounds act as TrkB agonists docking to the BDNF site at the receptor or rather as trans-activators binding elsewhere (Lu et al., 2013).

Finally, it is possible to enhance BDNF-TrkB signaling through stimulation of endogenous BDNF synthesis. This strategy, using BDNF activators benefits from target specificity - BDNF function is reinforced in cells where it is expressed already. The caveats of this approach, on the other hand, are non-specific activation of BDNF expression, e.g. in non-neuronal tissues, and side effects from off-target reactivity. A major advantage of this approach is that a variety of different chemical entities can be considered as drug candidates, sometimes compounds that are already in clinical use and therefore tested for safety. Since the characterization of BDNF regulation by glutamatergic, GABAergic and cholinergic neurotransmission in a series of seminal studies (da Penha Berzaghi et al., 1993; Zafra et al., 1990, 1991), a large number of pharmacological agents have been reported that alter BDNF expression levels *in vitro* or *in vivo*. Among these, antidepressants have received particular interest as a substantial body of evidence indicates that these compounds not only increase BDNF expression *in vivo,* but BDNF has a clear and critical role in antidepressant action (Castrén and Rantamäki, 2010). AMPA receptor modulators or ampakines are another class of perspective BDNF activators. Ampakines robustly increased BDNF expression in hippocampal slices (Lauterborn et al., 2000) and in adult hippocampi (Rex et al., 2006); ampakine treatment also rescued functional deficits in mouse models of Huntington's disease (Simmons et al., 2009) and Rett syndrome (Ogier et al., 2007). Recently, it was discovered that a mechanism of BDNF translational derepression plays a critical role in mediating rapid antidepressant effects of ketamine and other NMDA receptor antagonists (Autry et al., 2011). As rapid antidepressant effects of ketamine have been reported in several clinical studies (Berman et al., 2000; Price et al., 2009; Zarate et al., 2006), NMDA receptor targeted antidepressant strategies are the subject of growing research attention. If the translational regulatory mechanism proposed by Autry and colleagues can be confirmed in future studies, this discovery may suggest novel molecular targets for antidepressant development.

Mood stabilizers lithium and valproate have been shown to increase BDNF expression in cultured cortical and dopaminergic neurons (Chen et al., 2006; Fukuchi et al., 2009; Hashimoto et al., 2002; Yasuda et al., 2009) and also in the rat brain (Calabrese et al., 2012; Einat et al., 2003; Fukumoto et al., 2001). Valproate is an inhibitor of histone deacetylases (Göttlicher et al., 2001; Phiel et al., 2001). Several other histone deacetylase (HDAC) inhibitors of different

structure classes have been shown to induce BDNF expression in cultured neurons or in the brain (Chen et al., 2006; Fukuchi et al., 2009; Lin et al., 2012; Mielcarek et al., 2011; Sui et al., 2012; Zeng et al., 2011; Tian et al., 2009). In addition to HDACs, inhibition of other chromatin modifying enzymes such as DNA methyltransferases (DNMT) has been shown to regulate BDNF expression in cultured neurons (Nelson et al., 2008) and *in vivo* (Lubin et al., 2008; Roth et al., 2009). HDAC inhibitors are now being considered a perspective class of drugs in developing therapeutics for neurodegenerative and psychiatric disorders, although better understanding of the biological roles of individual HDAC proteins (totaling 11) and improved isoform selectivity of HDAC inhibitors is needed (Fischer et al., 2011; Kazantsev and Thompson, 2008). DNMT inhibitors may also hold potential for treatment of neurological and psychiatric disorders through normalization of altered methylation changes at gene promoters (Day and Sweatt, 2012).

2. AIMS OF THE STUDY

The aims of this study were as follows:

1) to generate and characterize BAC transgenic mouse lines that carry human and rat BDNF loci as tools for studying BDNF gene regulation

2) to study regulation of the BDNF gene by drugs that target chromatin modifying enzymes

3. MATERIALS AND METHODS

Following methods were used in this study:

- semiquantitative RT-PCR analysis (publications I and II)
- PCR genotyping (publications I and II)
- *in situ* hybridization (publications I and II)
- quantitative real-time RT-PCR analysis (publication III)
- molecular cloning and mutagenesis (publication III)
- cell culture (rat primary neurons and heart endothelial cells, HEK cells) and transfections (publication III and unpublished - see Appendix 2)
- Western blot (publication III)
- luciferase reporter assays (publication III)
- chromatin immunoprecipitation (publication III)
- DNA methylation analysis by bisulfite sequencing (unpublished - see Appendix 2)

4. RESULTS AND DISCUSSION

4.1 BAC transgenic mice for studying BDNF gene regulation (**publications I and II**)

The purpose of generating BDNF-BAC transgenic mice was twofold. First, we asked a fundamental biological question - are large fragments of the BDNF locus containing the full BDNF gene and long 3' and 5' flanking sequences (13-144 kb) sufficient to drive BDNF expression similarly to endogenous BDNF, i.e. in the same cells and in an activity-dependent manner. Second, reporter genes were introduced as a BDNF-reporter fusion (publication I) or substitution of BDNF coding sequence (publication II) to facilitate analysis of transgene expression. In principle, provided that the transgene recapitulates expression of the gene of interest and reporter proteins are expressed at sufficient levels, such transgenic mice could be used for screening compounds that modulate BDNF expression.

We generated transgenic mouse lines using two modified BAC constructs: 1) BAC carrying the human BDNF (hBDNF) gene with 84 kb 5' and 17 kb 3' flanking sequences, where the BDNF coding sequence was substituted with BDNF-EGFP fusion protein sequence (publication I); 2) BAC carrying the rat BDNF gene with 13 kb 5' and 144 kb 3' flanking sequences, where the BDNF coding sequence was substituted with the LacZ reporter (publication II). Among three human BDNF-BAC mouse lines obtained, transgenic human BDNF-EGFP expression in one line (C3) largely recapitulated endogenous mouse BDNF (mBDNF) expression in different brain regions and peripheral tissues. All 9 analyzed alternative BDNF transcripts expressed in the human hippocampus were expressed in the C3 mouse line. In C3 mice, hBDNF transcripts were induced by kainic acid injections in a similar pattern to endogenous mBDNF transcripts, indicating that elements for activity-dependent BDNF regulation were included in the transgenic construct. Importantly, this was the first study to show regulation of the human BDNF gene by neuronal activity. However, hBDNF expression was not detected from some brain regions expressing endogenous BDNF, e.g. dentate granule cells of the hippocampus. In addition, none of the hBDNF-BAC mouse lines expressed the transgene in the heart, where BDNF is highly expressed (Aid et al., 2007; Pruunsild et al., 2007; Timmusk et al., 1993). The only rat BDNF-LacZ transgenic mouse line that was obtained recapitulated BDNF expression in the heart, lung and largely in the brain; activity-dependent induction of BDNF alternative transcripts was also recapitulated. Notably, transgenic rat BDNF-BAC mRNA was not expressed in dentate granule cells of the hippocampus

and ectopic expression was observed in the striatum where endogenous rat BDNF is not expressed (Timmusk et al., 1994b).

Transgenic mice carrying reporter genes under the control of BDNF regulatory regions have been generated previously. In a study by Timmusk et al. (1995), transgenic mice carrying the CAT reporter gene under the control of 2-9 kb of the rat BDNF promoter recapitulated BDNF expression in several brain regions and peripheral tissues. However, in these mice absent or very low transgene expression was detected in hippocampal dentate granule cells and in the heart. In addition, ectopic expression was detected in the striatum (Timmusk et al., 1995). In another study, BDNF-YAC mice were generated carrying the whole human BDNF gene (with part of the coding sequence replaced with EGFP), 45kb of 5' and 33 kb of 3' flanking sequences. These mice showed transgenic BDNF expression in a number of brain regions and peripheral tissues. Importantly, in these mice transgenic BDNF mRNA was detected in the heart, where transgene expression was absent in human BDNF-BAC mice (publication I) and rat BDNF-CAT mice (Timmusk et al., 1995). Collectively, on the basis of the four available BDNF transgene studies (publications I &II; Guillemot et al., 2007; Timmusk et al., 1995) it can be predicted that the distal regulatory element needed for BDNF expression in the heart lies 3' of the BDNF gene, within 17-33 kb for the human gene. It is more difficult to predict the localization of dentate granule cell-specific regulatory elements. Impaired transgenic BDNF mRNA expression in these cells were observed in BDNF-CAT mice (Timmusk et al., 1995) and transgene expression was completely undetectable in the rat BDNF-BAC mice in the present study, even if expression was stimulated by kainic acid (publication II). In the human BDNF-BAC transgenic mice of the present study, transgene expression in dentate granule cells was detected only after kainic acid treatments (publication I). In contrast, EGFP fluorescence was readily detected in scattered dentate granule cells in human BDNF-YAC mice (Guillemot et al., 2007). Comparing the genomic sequences used in these transgenes does not allow any clear conclusion to be drawn about the putative localization of the dentate granule cell specific enhancer. It is possible that such an enhancer was included in some transgenes where expression was not detected, but its activity was occluded by the activity of neighbouring mouse genomic sequences. Further transgenic studies are needed to settle this question.

Unfortunately, reporter proteins were not expressed in any of the four lines at detectable levels, measured either by reporter activity - EGFP-fluorescence or LacZ enzymatic activity - or by direct quantification of the reporter proteins by Western blot. A possible explanation for this is that the transgenes were expressed at levels that escaped detection. Transgene mRNA expression levels in the human BDNF-BAC mouse line C3, showing highest levels of transgene mRNA expression among three hBDNF mouse lines, were directly compared with endogenous BDNF mRNA levels and found to be about tenfold lower. Considering that endogenous BDNF is expressed at relatively low levels in the

brain, it is plausible that low expression accounted for the failure to detect the EGFP reporter in these mice. It is possible that the LacZ reporter was not detected for the same reasons, although we have not analyzed BDNF-LacZ mRNA expression levels in comparison with endogenous BDNF mRNA.

In conclusion, these BDNF-BAC transgenic mice carrying rat and human BDNF loci recapitulate endogenous BDNF expression to a large extent and can be used as valuable tools for studying BDNF regulation *in vivo*.

4.2 Chromatin modifying drugs as modulators of BDNF expression (**publication III** and unpublished results)

Chromatin modifying enzymes are currently being regarded as promising drug targets for combating neurodegenerative and psychiatric disorders (Fischer et al., 2010; Kazantsev and Thompson, 2008). A number of studies have indicated that BDNF may be a mediator of the beneficial effects of chromatin modifying drugs (Chen et al., 2006; Chiu et al., 2011; Zeng et al., 2011; Yasuda et al., 2009). We aimed to systematically analyze the effects of chromatin modifying drugs of different classes on BDNF expression. First, we performed a small-scale screening in rat primary cortical neurons analyzing the effect of chromatin-modifying drugs on BDNF mRNA levels by quantitative RT-PCR. Cortical neurons isolated from prenatal rats (embryonic day 21) were treated with different concentrations of chromatin modifying compounds at 6 days *in vitro* – a widely used experimental system for studying BDNF transcriptional regulation (Pruunsild et al., 2011; Tao et al., 1998). Inhibitors of the following enzyme classes were tested: histone deacetylases (HDAC), histone acetyltransferases (HAT), DNA methyltransferases (DNMT), and one inhibitor of histone demethylase (HDM) LSD1 - tranylcypromine hydrochloride. In these experiments, drugs dissolved in DMSO were applied to neuronal cultures for 12h in concentrations spanning 3-4 orders of magnitude, centering on effective concentrations reported in the literature. Examples of collected data are shown in Figure 2, see Appendix 1 for a detailed dose response analysis for other tested compounds.

Our analysis showed that among the compounds tested, HDAC inhibitors proved to be most effective in stimulating BDNF expression in cultured neurons (Figure 2 and Appendix 1). Short chain fatty acid HDAC inhibitors (sodium butyrate, sodium valproate, and sodium 4-phenylbutyrate) were effective in increasing BDNF mRNA in low millimolar concentration range (Figure 2A), which is in agreement with previously published data (Fukuchi et al., 2009). Several HDAC inhibitors of the hydroxamic acid structure class (TSA, SAHA, HA-7) and other structure classes (MS-275, apicidin, psammaplin A, depudecin) effectively increased BDNF mRNA levels in nanomolar (Figure 2B) or low micromolar concentrations (Appendix 1). A few

tested hydroxamic acid compounds, however, were ineffective in increasing BDNF mRNA in all tested concentrations (scriptaid, CAY10433) or effective only at a 100 µM concentration (HDAC8-selective inhibitor PCI-34051) (Appendix 1). Splitomicin and EX527, inhibitors of sirtuins (unrelated lysine deacetylases sometimes called class III HDACs), were not effective in inducing BDNF expression at three lower concentrations tested and showed effects in opposing directions at highest tested concentrations, indicating possible off-target mechanisms (Appendix 1).

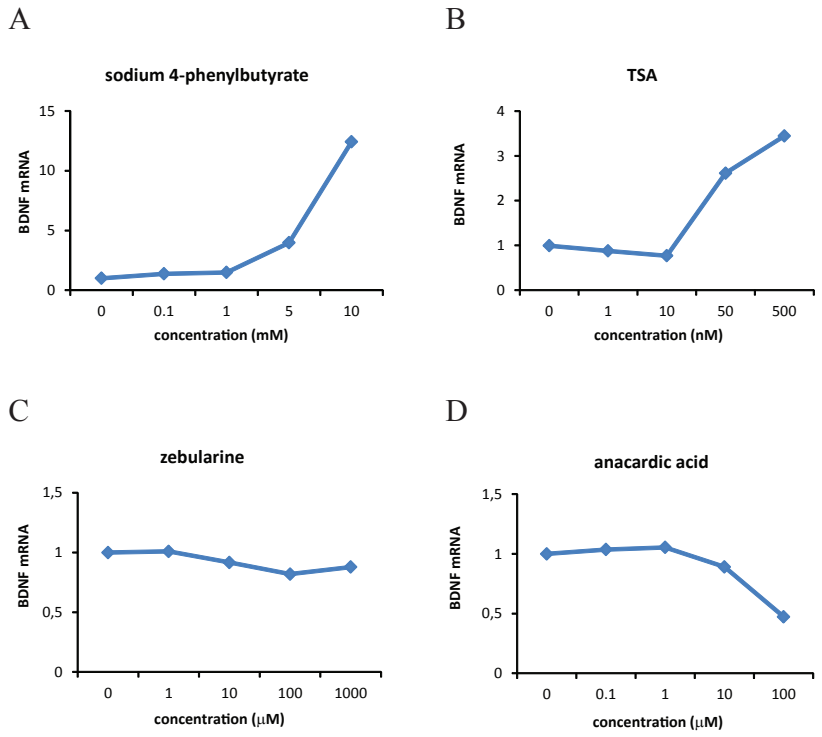

Figure 2. Example dose response curves for the effects of chromatin modifying drugs on BDNF mRNA levels. Rat primary cortical neurons were treated at 6 days *in vitro* with indicated compounds or 0,1% DMSO (concentration 0) and BDNF mRNA was analyzed by qRT-PCR (n=1, these data represent means of qPCR triplicates; normalized to RNA amount used for cDNA synthesis). **A)** inhibitor of class I histone deacetylases: sodium 4-phenylbutyrate; **B)** inhibitor of class I/IIb histone deacetylases: trichostatin A; **C)** DNA methyltransferase inhibitor zebularine; **D)** inhibitor of histone acetyltransferase p300: anacardic acid

DNA methyltransferase (DNMT) inhibitors have previously been shown to increase BDNF mRNA expression in the brain (Lubin et al., 2008; Roth et al., 2009) and in primary mouse hippocampal neurons (Nelson et al., 2008). However, in primary rat cortical neurons used in this study, three different DNMT inhibitors proved ineffective in inducting BDNF expression at all tested

concentrations, except for 100 μM of 5-azacytidine (Figure 2C and Appendix 1), which is markedly higher than the effective concentration (2.5 μM) previously reported for this compound (Nelson et al., 2008). It is possible that this discrepancy may be caused by different DNA methylation status of BDNF promoters in different neuronal culture preparations. For example, low basal methylation levels may prevent DNMT inhibitors from having effect on BDNF expression. Indeed, in the neuronal cultures used in this study, BDNF promoter IV was essentially unmethylated at all examined positions except for CpG at position -148 (Figure 3). As a reference, BDNF promoter IV was substantially more highly methylated in mouse primary cortical neurons in a previous study (Martinowich et al., 2003). Moreover, different growth conditions have been shown to affect BDNF promoter IV methylation status (Chen et al., 2003a).

Two inhibitors of histone acetyltransferases - butyrolactone 3, an inhibitor of Gcn5 (Biel et al., 2004), and anacardic acid, an inhibitor of PCAF (Balasubramanyam et al., 2003) were tested. Butyrolactone was ineffective in changing BDNF mRNA levels, while anacardic acid decreased BDNF mRNA about twofold at 100 μM (Figure 2D and Appendix 1). This is in accordance with the findings that HDAC inhibitors, which have an opposite effect on gene expression, induced BDNF expression in the same experimental system. In addition, phosphorylated CREB binding to BDNF promoters I and IV is known to recruit p300/CBP cofactors that have histone acetyltransferase activity (Lyons and West, 2011). Finally, induction of BDNF expression *in vivo* by systemic administration of a p300/CBP activator has been reported (Chatterjee et al., 2013).

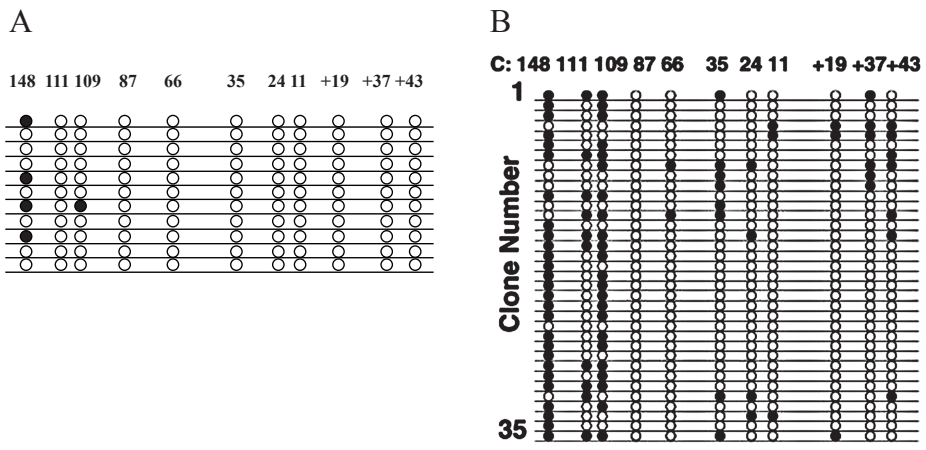

Figure 3. **A)** BDNF promoter IV methylation in rat primary cortical neurons used in this study, at 6 days *in vitro.* **B)** BDNF promoter IV methylation in mouse cortical neurons at 3 days *in vitro* (from Martinowich et al., 2003, reprinted with permission from AAAS). Numbers indicate CpG positions 5' or 3' (+) relative to promoter IV transcription start site, filled circles designate methylated CpG-s and open circles designate unmethylated CpG-s. Circles aligned to a line correspond to one analyzed DNA clone.

4.2.1 Differential regulation of BDNF expression in cortical neurons by class-selective histone deacetylase inhibitors (**publication III**)

Analysis of BDNF regulation by different classes of chromatin modifying drugs showed that among the analyzed drugs HDAC inhibitors produced the most robust and consistent induction of BDNF mRNA expression. Therefore, we focused on BDNF regulation by HDAC inhibitors. The ability of HDAC inhibitors to stimulate BDNF expression has been shown in a number of studies (Calabrese et al., 2012; Chen et al., 2006; Fukuchi et al., 2009; Lin et al., 2012; Lv et al., 2012; Mielcarek et al., 2011; Sui et al., 2012; Zeng et al., 2011; Tian et al., 2009; Yasuda et al., 2009). Not much, however, is known about how individual HDAC isoforms contribute to BDNF regulation. Only recently has it been shown that HDAC2 binds to BDNF promoters I, II and IV (Gräff et al., 2012; Guan et al., 2009).

In order to gather insight about the roles of different HDAC isoforms in BDNF gene regulation, we compared the effects of HDAC inhibitors of different selectivity – SAHA, MS-275, MC1568 and tubacin - on BDNF mRNA levels in cultured rat cortical neurons. Designing isoform-selective HDAC inhibitors is complicated by the fact that 11 HDAC proteins share a high degree of similarity (Kazantsev and Thompson, 2008). However, several HDAC inhibitors are available that display class or isoform selectivity: MS-275, apicidin and short chain fatty acids such as valproic acid/sodium valproate, sodium butyrate and sodium 4-phenylbutyrate target only class I HDACs, MC1568 targets class II HDACs, tubacin targets HDAC6 and PCI-34051 targets HDAC8. SAHA and other hydroxamic acid HDAC inhibitors have previously been regarded as pan-HDAC inhibitors almost equipotently inhibiting all HDACs (Khan et al., 2008). This view has been challenged by a study showing that SAHA and several other hydroxamic acids show very weak inhibitory activity on class IIa HDACs (Bradner et al., 2010). The latter study likely offers a more accurate account on selectivity of HDAC inhibitors, because the accuracy of older assays - e.g. in Khan et al., 2008 - has been questioned by the following findings. First, class II HDACs demonstrated very low activity on conventional acetyl-lysine substrates in a study by Lahm and co-workers (Lahm et al., 2007). Second, immunopurification of class II HDACs from mammalian cells has been shown to co-purify class I enzymes, which can interfere with the HDAC assays using the presumed “pan-HDAC” (in fact, class I HDAC) substrates (Jones et al., 2008). Nevertheless, the ability of SAHA, trichostatin A and many other hydroxamic acid HDAC inhibitors to inhibit HDAC6, a class IIb enzyme, is beyond doubt as it can be easily demonstrated by their effect on acetyl tubulin (a natural HDAC6 substrate) levels in cells. Therefore, on the basis of current understanding SAHA and similar HDAC inhibitors should be regarded as class I/IIb selective inhibitors.

MC1568 treatment has been shown to increase acetyl histone 3 (AcH3) levels in breast cancer cells at 20 μM, indicating that it can act as a class I HDAC inhibitor at higher concentrations (Duong et al., 2008). Therefore, using AcH3 and acetyl-tubulin Western blot assays, we first established that at 5 μM MC1568 inhibits class II, but not class I HDACs. We then performed dose-response curves of SAHA, MS-275 and MC1568 determining their effects on BDNF mRNA levels in cultured neurons. We determined 5 μM as the saturating concentration for the effects of all three inhibitors on BDNF mRNA expression and proceeded to analyze the dynamics of BDNF induction by these compounds. This analysis led to the key finding in this study (publication III) – class II HDAC inhibitor MC1568 rapidly increased BDNF mRNA levels, whereas class I inhibitor MS-275 produced a delayed inhibition, manifested only at 6-24h of treatment. Treatment with SAHA, a class I/IIb inhibitor, produced an intermediate time course of BDNF induction, suggesting that class IIb members may contribute to its effects. Tubacin, inhibitor of a class IIb member HDAC6, indeed induced BDNF – albeit weakly – at 1-3h of treatment. Altogether, our results suggest that class II HDACs participate in regulation of BDNF expression and probably do so in a more direct manner than class I proteins. Inhibition of class I HDACs may affect BDNF mRNA levels through modulating expression of other genes that affect BDNF transcription rather than directly change acetylation levels at BDNF promoters. This idea found support in experiments showing rapid induction of histone acetylation at BDNF promoters I and IV after SAHA treatment, contrasting with relatively slow accumulation of BDNF transcripts. In addition, BDNF mRNA induction by HDAC inhibitors was sensitive to inhibition of *de novo* protein synthesis at 24h, but not at 3h, indicating the contribution of indirect regulation mechanisms in the late - class I HDAC-dependent - phase of BDNF induction.

It has been shown that HDACs 2, 3, 4, 5 and 11 are highly expressed in the rat brain (Broide et al., 2007). Of these isoforms, class II HDACs 4 and 5 serve as potential targets for MC1568. Next, we investigated the effect of HDAC4 and HDAC5 overexpression on BDNF promoter IV in cultured neurons. We restricted our analysis to promoter IV because it is highly expressed in cultured neurons and its regulation mechanisms have been thoroughly studied enabling comparison with mechanisms governing its regulation by neuronal activity (Lyons and West, 2011). Both HDAC4 and HDAC5 repressed and MC1568 treatment increased luciferase activity under the control of ~0,5kb of rat promoter IV sequence containing all previously described proximal regulatory elements (Lyons and West, 2011), suggesting that class II HDACs indeed regulate promoter IV activity. BDNF regulation by class II HDACs has been implicated in a recent study showing that nuclear accumulation of HDAC4 observed in cerebellar neurons of *Atm*$^{-/-}$ (ataxia telangiectasia mutated) mice was accompanied by reduced binding of MEF2 to BDNF promoter IV and reduced binding of CREB to BDNF promoters I and IV (Li et al., 2012). Class II HDACs associate with and repress the activity of MEF2 (Lu et al., 2000b;

Miska et al., 1999) and CREB (Li et al., 2012). To the best of our knowledge, our study is the first to directly show BDNF regulation by class II HDACs.

Next, we asked which *cis*-regulatory elements are necessary for mediating regulation of BDNF promoter IV activation by HDAC inhibitors. CRE (cAMP/Ca^{2+} response element) is known to play a central role in mediating neuronal activity-dependent regulation of promoter IV (Hong et al., 2008; Shieh et al., 1998; Tao et al., 1998). In addition, CRE-dependent transcriptional activity has been shown to be stimulated by HDAC inhibitors (Canettieri et al., 2003; Fass et al., 2013, 2003). The mechanism of CRE-dependent transcription regulation by class I histone deacetylases was proposed by Canettieri and co-workers: HDAC1 associates with CREB and recruits protein phosphatase PP1 to dephosphorylate Ser133 of CREB, as a result decreasing CRE-driven transcriptional activity (Canettieri et al., 2003). Here, using luciferase assays we showed that the CRE element has a critical role in BDNF promoter IV induction by both class I and class II HDAC inhibitors. It has been reported that deletion of a BDNF promoter IV fragment containing the CRE element does not affect promoter IV induction by HDAC inhibitor valproic acid (Yasuda et al., 2009). However, in our experimental conditions CRE mutation effectively abolished promoter IV induction by valproate. While it is possible that differences in experimental conditions (transfections and drug treatments, age of cultures) can account for the different outcomes, it must be noted that in Yasuda et al., 2009 data showing the effect of *CaRE1-3* deletion on promoter IV induction by valproic acid was inconclusive as they did not compare the latter with induction of wild type promotor IV. In addition to CRE, we tested the possibility that BDNF promoter IV regulation by HDAC inhibitors is mediated by the CaRE1 element that binds MEF2 (Lyons et al., 2012). MEF2 is a transcription factor, the activity of which is regulated by interaction with class II HDACs (Lu et al., 2000a; Miska et al., 1999). However, mutating CaRE1 did not affect promoter IV induction by SAHA, indicating that MEF2-dependent regulation is not involved in this mechanism. Surprisingly, SAHA treatment (as well as treatment with MS-275 and MC1568 – unpublished observations) significantly decreased luciferase activity under the control of three consensus MEF2 response elements (3xMEF2-luc). 3xMEF2-luc has been shown to be downregulated by co-expressed HDAC4 in myocytes (Backs et al., 2011) and inhibition of either HDAC catalytic activity or HDAC/MEF2 binding was expected to derepress MEF2-dependent transcriptional activity. However, it has been shown that the MEF2-HDAC interaction can be stabilized by class II selective HDAC inhibitor MC1568 (Nebbioso et al., 2009) and thus the observed downregulation of 3xMEF2-luc activity by HDAC inhibitors may have been caused by increased recruitment of repressive HDAC complexes to the promoter. Alternatively, indirect effects mediated by altered expression of unknown transcriptional regulators may have been responsible for repression of 3xMEF2-luc activity.

Finally, we studied if the differential regulation of gene expression by HDAC inhibitors of different selectivity can be seen with other synaptic plasticity related genes such as Arc/Arg3.1 and c-fos. Interestingly, we saw robust rapid induction of both Arc and c-fos mRNAs by class II HDAC inhibitor MC1568. Induction of Arc and c-fos by SAHA and MS-275 was more moderate and, similarly to the effects on BDNF, delayed. Rapid induction of BDNF, Arc, and c-fos by MC1568 indicate that the response to this inhibitor may be mediated by similar molecular mechanisms. These three genes share regulation by MEF2 and CREB transcription factors (Lyons and West, 2011), which are known to interact with class II HDACs (Li et al., 2012; Lu et al., 2000a; Miska et al., 1999). Recently, expression of Arc has been shown to be regulated by HDAC4 in neurons (Schlumm et al., 2013). In addition, overexpression of a HDAC9 mutant that is retained in the nucleus has been shown to downregulate c-fos expression (Sugo et al., 2010). The exact mechanisms of BDNF, Arc and c-fos regulation by class II HDACs needs to be addressed in future studies, the importance of which has been underscored in a recent report elucidating the role of HDAC4 in governing the global transcriptional program of synaptic plasticity-related genes (Sando et al., 2012).

4.2.2 Regulation of BDNF expression by HDAC inhibitors in heart endothelial cells (unpublished)

In parallel with analyzing the effects of HDAC inhibitors on BDNF mRNA levels in neurons, we decided to test their effects on BDNF in a non-neural cell type. In addition to the nervous system, BDNF is expressed in several non-neural tissues such as heart and lung (Ernfors et al., 1990c; Koppel et al., 2009, 2010; Yamamoto et al., 1996). In the heart, BDNF is expressed in endothelial cells of arteries and capillaries, and arterial smooth muscle cells (Donovan et al., 2000; Scarisbrick et al., 1993). BDNF has been shown to act as a survival factor for endothelial cells, helping to maintain blood vessel stability (Donovan et al., 2000).

Cardiac microvascular endothelial cells (CMEC) can be isolated from neonatal hearts using a simple technique based on their rapid adherence to cell culture plastic (Kasten, 1972; Lee and Wu, 1999). Using this method, we isolated endothelial cells from rat heart ventricles on postnatal day 3 (P3) and cultured for 5 days in DMEM + 10% fetal bovine serum. Cells were fixed directly to the plastic surface of cell culture dishes and stained with an endothelial cell marker isolectin B4 (IB4) from *Bandeiraea simplicifolia* (Figure 4A). Fibroblasts isolated from the skin of P3 rats displayed weak staining with IB4, showing specificity of the signal (Figure 4B). At 4-5 days *in vitro*, cells were treated with SAHA, MS-275 or MC1568 for 3 or 24h and BDNF mRNA levels were analyzed by qRT-PCR (Figure 4C). Our results

showed that the effects of these compounds on BDNF expression in endothelial cells were remarkably similar to the effects observed in cortical neurons (publication III), suggesting that the regulation mechanisms involved are not neuron-specific, but rather of a more general nature. Specifically, class I selective HDAC inhibitor MS-275 decreased BDNF mRNA at 3h (not statistically significant), class II HDAC inhibitor MC1568 significantly induced BDNF mRNA already at 3h and class I/IIb inhibitor SAHA produced an effect that was intermediate to these of MS-275 and MC1568.

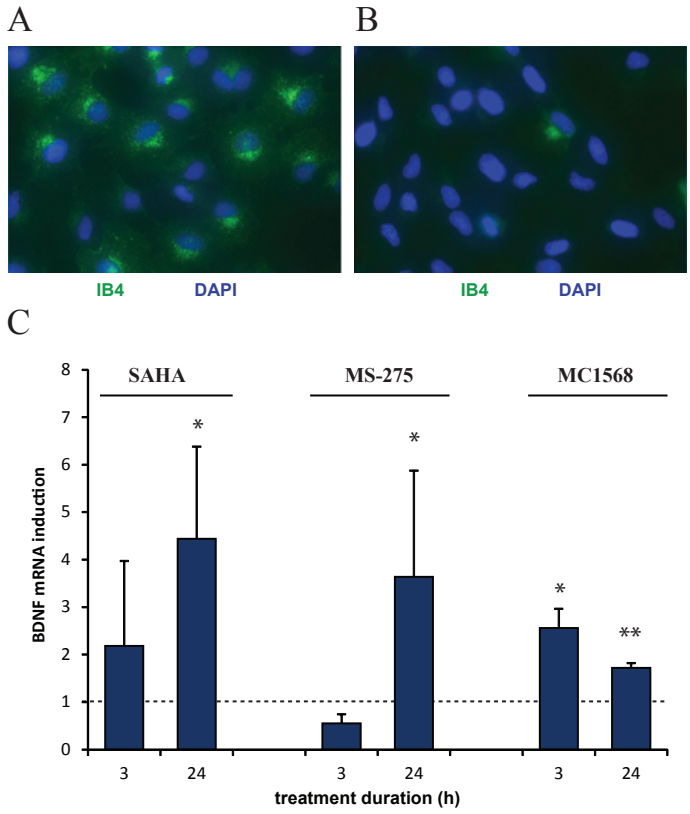

Figure 4. **A)** IB4 staining (green) of primary endothelial cells isolated from neonate (postnatal day 3) rat heart, cultured for 5 days *in vitro*. Cell nuclei are stained with DAPI (blue). **B)** IB4 staining of fibroblasts isolated from neonate (P3) rat skin. **C)** Induction of BDNF mRNA in primary heart endothelial cells by treatment with HDAC inhibitors for 3 or 24 h. BDNF mRNA levels were quantified by qRT-PCR, normalized to cycB levels and are expressed here as induction relative to DMSO-treated cells (indicated with a dotted line) . * $p < 0.05$, ** $p < 0.01$ (t-test). n=3, error bars - SEM

4.2.3 Atypical BDNF expression response to a hydroxamic acid HDAC inhibitor HA-7 (unpublished)

Among HDAC inhibitors, one hydroxamic acid compound (working name HA-7) showed an atypical dose response curve with maximum induction of BDNF mRNA achieved at 1 μM (Figure 5A). When compared to BDNF induction by other hydroxamic acid HDAC inhibitors, it appears that treatment of neurons with 10-100 μM HA-7 results in comparable increases in BDNF mRNA levels (Figure 5A). However, at 1 μM HA-7 robustly induced BDNF mRNA (Figure 5A), but did not affect histone acetylation levels (Figure 5C), suggesting involvement of a different molecular target, perhaps a non-HDAC metalloenzyme known to be targeted by hydroxamic acid compounds (Bantscheff et al., 2011; Chen et al., 1999). The HDAC inhibitory activity of hydroxamic acids drops dramatically if the hydroxamic acid moiety is converted to a carboxylic acid (Hildmann, 2004; Jung et al, 1999). We had at hand a methyl ester synthesis intermediate of HA-7, which could also be expected to be inactive as a HDAC inhibitor (Figure 5D). This compound dose-dependently increased BDNF mRNA levels in neurons (Figure 5E) and this effect was independent of histone acetylation (Figure 5F). Interestingly, induction of BDNF transcription by HA-7 showed different transcript-selectivity compared with SAHA, another hydroxamic acid HDAC inhibitor. We compared the effects of HA-7 and SAHA on exon I and exon IV containing BDNF mRNAs, transcripts that are highly induced by neuronal activity (Metsis et al., 1993; Pruunsild et al., 2011). HA-7 (1 μM) robustly induced BNDF exon IV-containing transcripts at 24h, while exon I-containing transcripts showed weaker induction (Figure 5G). Other major BDNF transcripts – exon II- and VI-containing transcripts – were only weakly induced by 1 μM HA-7 treatment (Figure 5H). This suggests a rather selective activation of BDNF promoter IV as exon I BDNF mRNAs are generally more highly induced than exon IV mRNAs regardless of the stimulus (e.g. the effects of SAHA (Figure 5G), depolarization by high KCl (Pruunsild et al., 2011) and NMDA treatment (Tian et al., 2009)). The non-HDAC molecular target and mechanisms underlying robust and promoter IV-selective activation of BDNF transcription by HA-7 remain to be determined in future studies. Currently, we have no clue regarding the molecular target except for the fact that induction of BDNF mRNA by 1 μM HA-7 can be abolished by inhibition of the MAPK/ERK pathway with MEK1/2 inhibitor U0126 (Figure 5I). Nevertheless, our results suggest that this compound can be used as a starting point for development of a potent BDNF promoter IV activator.

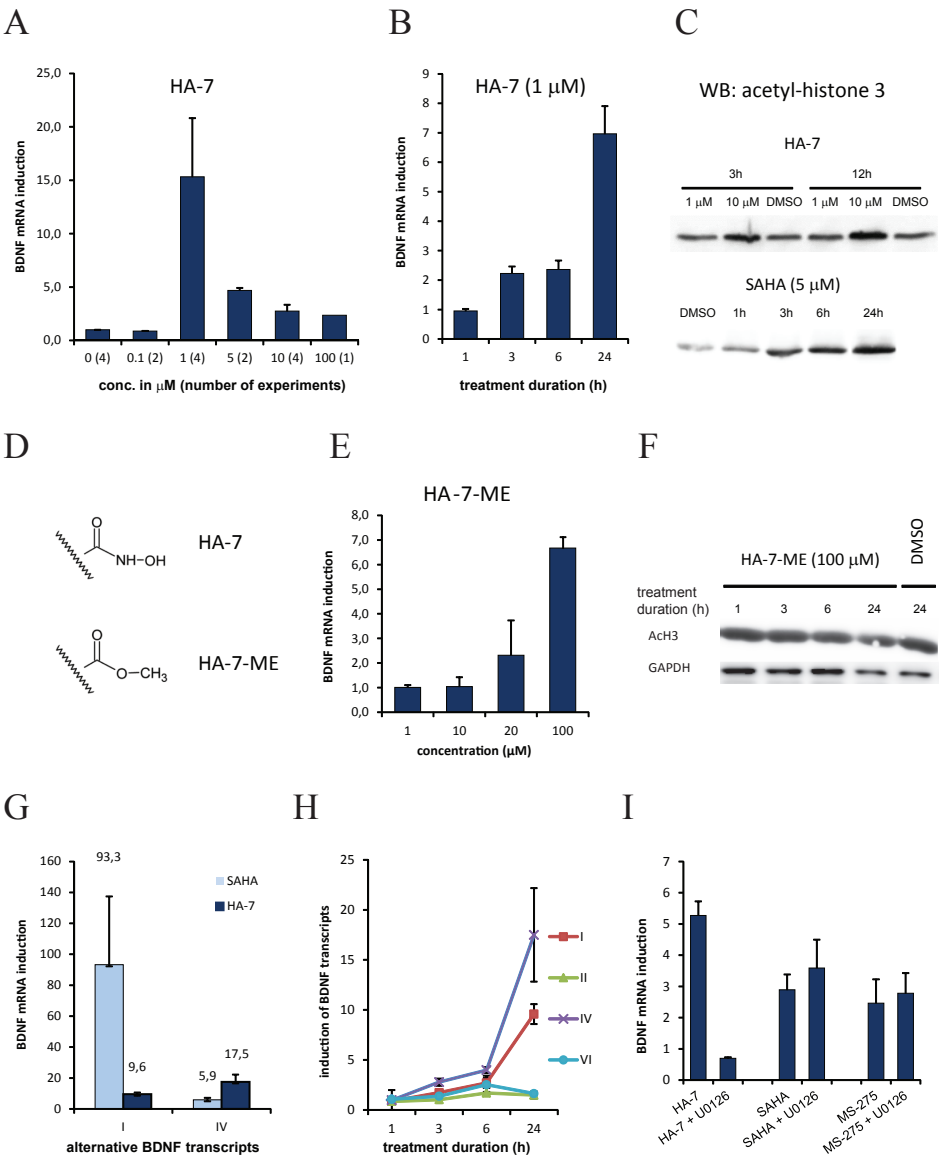

Figure 5. Histone acetylation-dependent and acetylation-independent effects of a hydroxamic acid HDAC inhibitor HA-7 (working name, structure undisclosed) on BDNF mRNA expression in rat primary cortical neurons (treated at 6 days *in vitro*). **A)** A bell-shaped dose-response curve of BDNF mRNA induction in neurons upon treatment for 12h with 0,1-100 μM of HA-7 or 0,1% DMSO (concentration 0). BDNF mRNA levels were quantified by qRT-PCR, normalized to cycB levels and are expressed here as induction relative to DMSO-treated cells. Number of individual experiments is indicated in brackets after concentrations. **B)** Time course of BDNF mRNA induction by 1 μM HA-7 treatment (quantified as in A; three independent experiments). **C)** Treatment of cortical neurons with 10 μM, but not 1 μM HA-7 increased histone 3 acetylation levels at 3 and 12h. Induction of histone 3 acetylation by 5 μM SAHA is shown for comparison (Western blot analysis). **D)** Substitution of hydroxamic acid moiety in HA-7 with methyl ester moiety in HA-7-ME. **E)** Effects of increasing concentrations of HA-7-ME on BDNF mRNA

levels in cortical neurons. **F)** HA-7-ME at 100 μM did not increase histone 3 acetylation in neurons. **G)** 1 μM HA-7 treatment (24h) induced BDNF exon IV-containing mRNAs to a higher extent than exon I-containing mRNAs, while 5 μM SAHA treatment produced a relatively higher induction of exon I-mRNAs. Shown are BDNF mRNA levels relative to DMSO treated controls (n=3). **H)** induction of four major BDNF transcripts in cortical neurons by 1 μM HA-7 treatment (n=3). **I)** The effect of 1 μM HA-7 on BDNF mRNA expression at 24h was abolished when cultures were co-treated with MEK1/2 inhibitor U0126 (10 μM). In contrast, U0126 did not affect BDNF mRNA induction by SAHA or MS-275 (MS) treatment (both at 5 μM). BDNF mRNA levels are expressed as relative to levels in DMSO-treated controls (HDAC inhibitors alone) or U0126-treated controls (HDACi + U0126); n=3. Error bars – SEM.

CONCLUSIONS

I) BDNF-BAC transgenic mice

- BAC transgenic mice carrying 168 kb of the rat BDNF locus or 207 kb of the human BDNF locus recapitulated endogenous BDNF expression in the brain and non-neuronal tissues to a large extent. Moreover, similarly to endogenous BDNF, transgenic BDNF expression was regulated by neuronal activity.

- Analysis of transgene expression in this and two earlier studies using BDNF transgenic mice allows to predict that a regulatory element necessary for BDNF expression in the heart lies downstream of the BDNF gene.

II) regulation of BDNF expression by chromatin modifying drugs

- Among different classes of chromatin modifying drugs tested, histone deacetylase (HDAC) inhibitors consistently increased BDNF expression in cultured rat cortical neurons.

- HDAC inhibitors with different isoform selectivity were effective in increasing BDNF mRNA levels in neurons.

- Different time courses of BDNF induction in neurons by class I and class II selective HDAC inhibitors indicate that distinct mechanisms operate mediating these effects.

- Class II members HDAC4 and HDAC5 may act as direct transcriptional regulators of BDNF expression.

- Increased BDNF expression by class I HDAC inhibition probably results from indirect effects on BDNF transcription, e.g. by altering expression levels of direct regulators of BDNF transcription.

- Regardless of class selectivity, induction of BDNF promoter IV activity by HDAC inhibitors is critically dependent on the CRE element in the promoter.

- Similar time courses of BDNF mRNA induction by class-selective HDAC inhibitors were observed in neurons and endothelial cells, suggesting that the mechanisms involved are not neuron-specific.

- A HDAC inhibitor was identified that increases BDNF expression via a HDAC-independent mechanism, with selectivity towards promoter IV.

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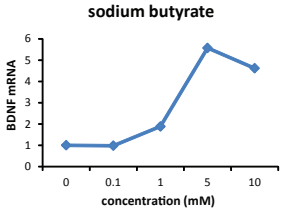
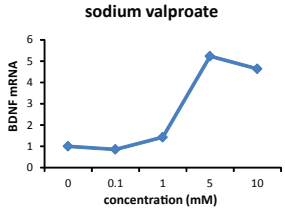
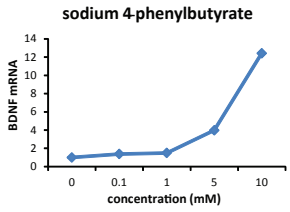
APPENDIX 1

I. histone deacetylase inhibitors

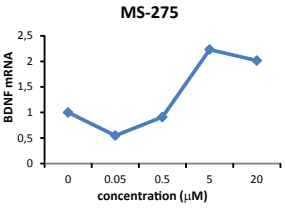
Short chain fatty acids (class I selective)



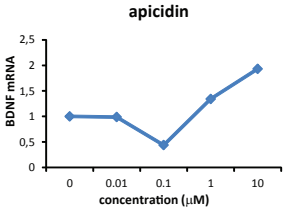




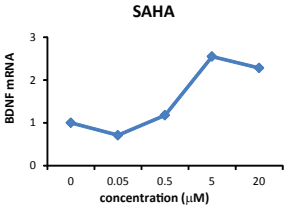
Benzamides (class I selective)

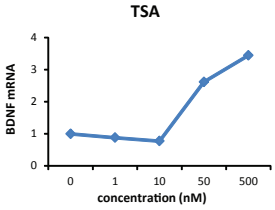


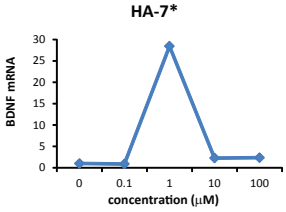
AODA (class I selective)

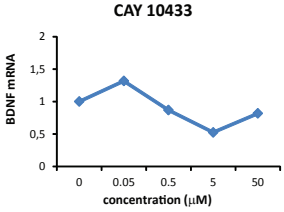


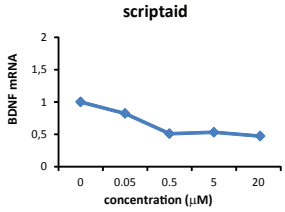
Hydroxamic acids (class I/IIb selective)



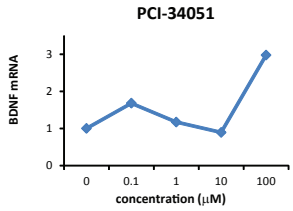




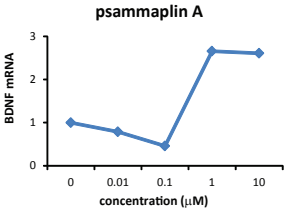




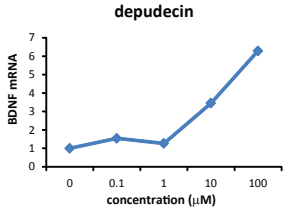
Hydroxamic acids (HDAC8 selective)



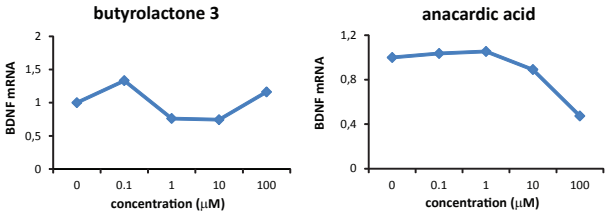
Disulfides (selectivity?)



Epoxides (selectivity?)

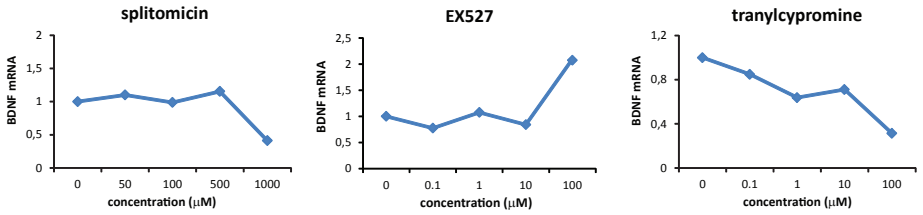


II. histone acetyltransferase inhibitors

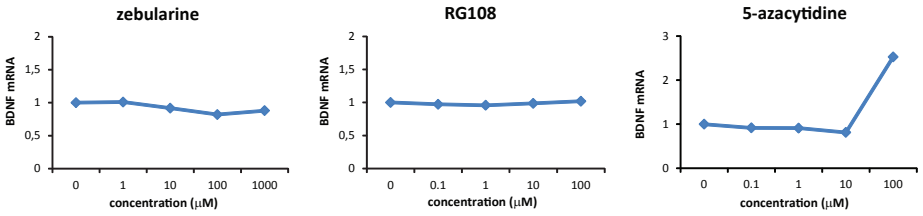


III. sirtuin inhibitors

IV. histone demethylase inhibitors



V. DNA methyltransferase inhibitors



Appendix 1. Effects of chromatin modifying drugs on BDNF mRNA expression in cultured rat primary neurons. Rat primary cortical neurons were treated at 6 days *in vitro* with indicated compounds or 0,1% DMSO (concentration 0) and BDNF mRNA was analyzed by qRT-PCR (n=1, these data represent means of qPCR triplicates; normalized to RNA amount used for cDNA synthesis). * working name for the compound, structure undisclosed.

APPENDIX 2

Supplementary methods

1. Endothelial cell culture

Cardiac microvascular endothelial cells (CMEC) were isolated from neonatal hearts using a method based on their adherence to the surface of cell culture dishes (Kasten, 1972; Lee and Wu, 1999). Sprague-Dawley rats were killed on postnatal day 3 by decapitation and heart ventricles were dissected. 6-8 ventricles were minced into ~1-2 mm^3 pieces and dissociated in four successive 10 min incubation steps with 2 ml trypsin (0,25% trypsin, 1 mM EDTA) at 37°C. The supernatant of the first fraction was discarded, three following fractions were combined and cells were pelleted by 1 min centrifugation at 200g. Cells were suspended in DMEM (PAA laboratories) and allowed to adhere to a 100 mm cell culture dish for 1,5h. The culture medium was removed and washed with warm HBSS buffer to remove non-adherent cells. Cells were grown in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin (PAA) and 0,1 mg/ml streptomycin (PAA) and split 1:3 when 70-80% confluent. For immunocytochemistry, confluent cells were fixed directly in the wells of 48-well cell culture dish (15 min with 4% formaldehyde at room temperature), blocked with 2% BSA and stained with biotinylated *Bandeiraea simplicifolia* isolectin B4 (1:100, Vector Labs) and streptavidin-conjugated AlexaFluor488 (Life Technologies).

2. DNA methylation analysis

Genomic DNA was isolated from rat cortical neurons grown in 6-well dishes at 6 DIV with DNeasy Blood & Tissue kit (Qiagen) and eluted from purification columns with 200 µl AE buffer. Bisulfite treatment of genomic DNA was performed to convert unmethylated cytosines to uracils using Epitect Bisulfite Kit (Qiagen) following manufacturer's instructions. 300 ng of DNA was used for one reaction. After purification, bisulfite-treated DNA was eluted with 20 µl buffer EB. BDNF promoter IV sequence was amplified from bisulfite-converted DNA using a two-step PCR using following conditions: PCR I: reaction volume 20 ul, 2,5 mM $MgCl_2$, 2,5 µM primer F1 5'- AGTTT GTTAGGATTGGAAGTGGAAATGT-3', 2,5 µM primer R1 5'- AATAACC AATATATACTCCTATTCTTCAAC-3', 2,5 U FirePol *Taq* polymerase in matching buffer (Solis Biodyne, Estonia); cycling conditions: 95°C for 3 min, 40 cycles of 95°C for 30 s, 50°C for 45 sec, 72°C for 45 sec, followed by final elongation at 72 °C for 10 min. 1 µl of PCR I reaction product was used as a

template in PCR II: reaction volume 20 ul, 3,0 mM $MgCl_2$, 2,5 μM primer F2 5'- F2 GAAATGTTTATAAAGTATGTAATGTT TTGG -3', 2,5 μM primer R2 5'- TTACATAACAAAAATAATACTCAC ACACC -3' and 2,5 U FirePol *Taq* polymerase in matching reaction buffer (Solis Biodyne, Estonia); cycling conditions: 95°C for 3 min, 25 cycles of 95°C for 30 s, 55°C for 45 sec, 72°C for 45 sec, followed by final elongation at 72 °C for 10 min. PCR products were purified from agarose gel using UltraClean 15 DNA Purification Kit (Mobio), cloned into pTZ57R/T vector and analyzed by sequencing. Cytosines in CpG dinucleotides that where resistant to C →U conversion were identified as methylated nucleotides. Conversion efficiency at non-CpG sites was >95%.

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

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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].

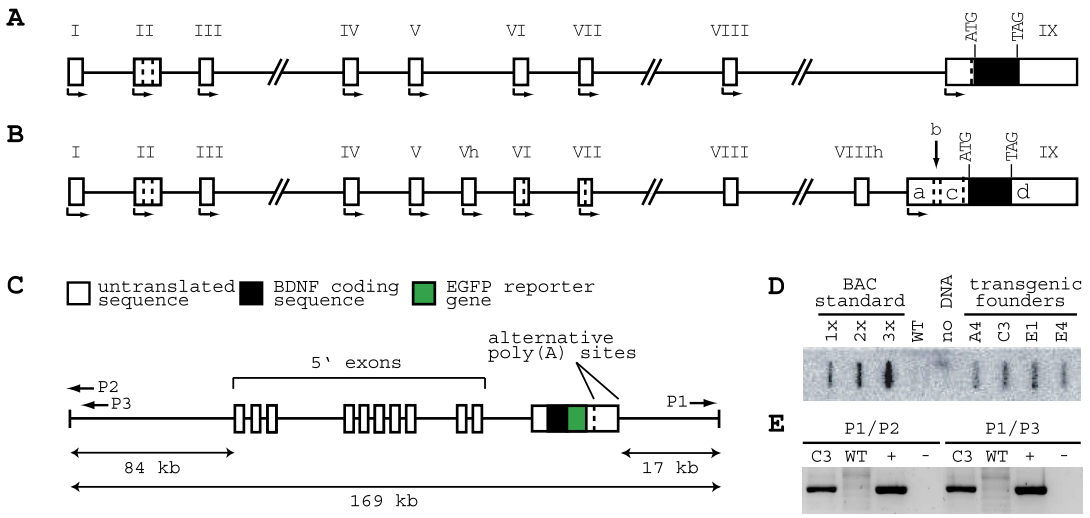

Figure 1

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

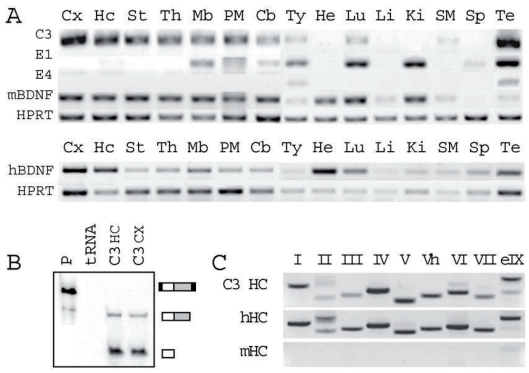

Figure 2
hBDNF-EGFP mRNA expression in tissues of three transgenic mouse lines. (**A**) RT-PCR analysis of hBDNF-EGFP mRNA expression in tissues of three transgenic BAC mouse lines – C3, E1, E4. mBDNF – mouse BDNF; hBDNF – human BDNF in human tissues; HPRT – reference gene hypoxanthine phosphoribosyltransferase.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

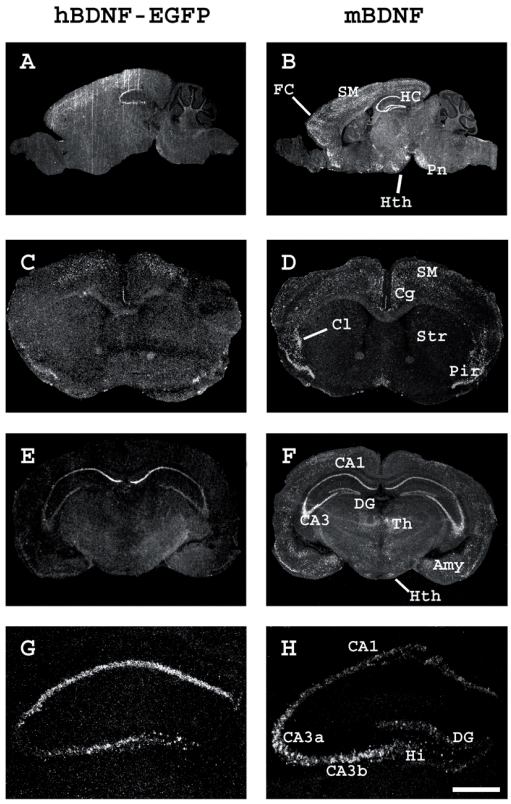

Figure 3
Overlapping patterns of BAC-driven hBDNF-EGFP and mBDNF mRNA expression in C3 mouse brain. *In situ* hybridization analysis, photoemulsion autoradiographs of 16 μm sagittal (**A**,**B**) and coronal (**C**-**H**) sections. (**C**) and (**D**) are sections taken at striatal level; (**E**) and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

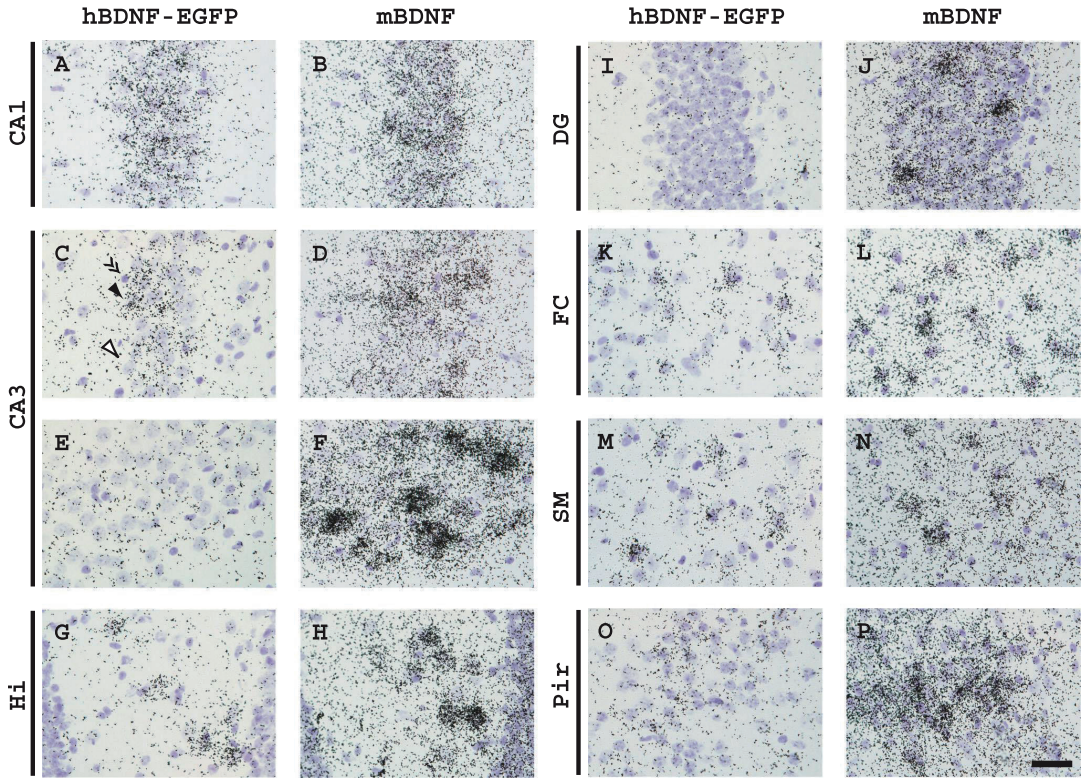

Figure 4
Cellular expression of hBDNF-EGFP mRNA in adult C3 mouse brain. *In situ* hybridization analysis, shown are bright-field autoradiographs of emulsion-dipped sections. Hybridization probes are indicated above the columns. Filled arrowhead indicates a neuron with strong labeling, empty arrowhead 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

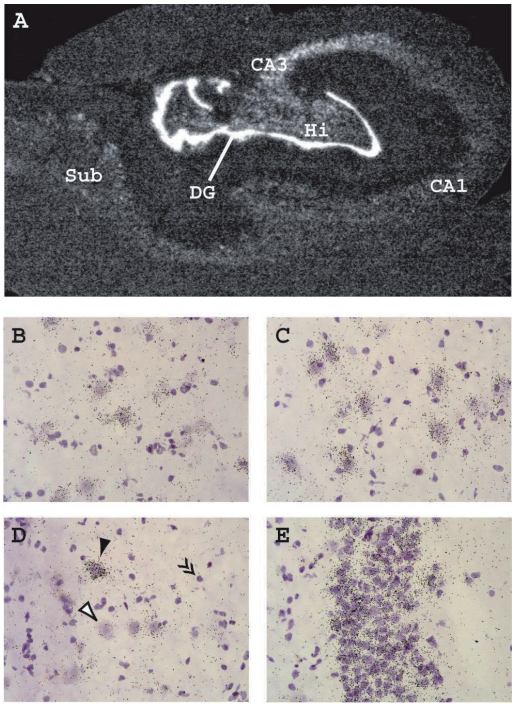

Figure 5
Expression of BDNF mRNA in the human hippocampus. (**A**) *In situ* hybridization autoradiograph of a 16 μm coronal section.*DG* – granular layer of dentate gyrus; *Hi* – hilar area of dentate gyrus; *Sub* – subiculum; *CA1*, *CA3* – hippocampal subfields. (**B-E**) High magnification bright-field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

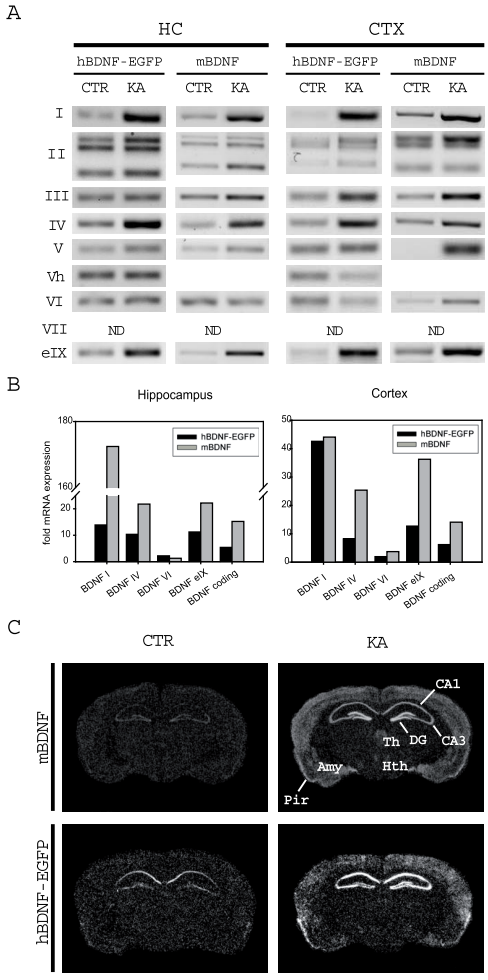

Figure 6
Kainic acid (30 mg/kg) induces transgenic hBDNF-EGFP mRNA expression in brains of C3 line transgenic mice. (**A**) Induction of alternatively spliced hBDNF-EGFP transcripts in C3 mouse hippocampus (HC) and cerebral cortex (CTX), analyzed with RT-PCR. mBDNF – mouse transcripts; ND – not determined; 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
HPRT1_s	CTTTGCTGACCTGCTGGATTAC
HPRT1_as	GTCCTTTTCACCAGCAAGCTTG
hBDNF_I_s	GATGCCAGTTGCTTTGTCTTCTGTAG
hBDNF_II_s	GGGCGATAGGAGTCCATTCAGCACC
hBDNF_III_s	AGTTTCGGGCGCTGGCTTAGAG
hBDNF_IV_s	GCTGCAGAACAGAAGGAGTACA
hBDNF_V_s	TCGCGTTCGCAAGCTCCGTAGTG
hBDNF_Vh_s	GGCTGGAACACCCCTCGAA
hBDNF_VI_s	GGCTTTAATGAGACACCCACCGC
hBDNF_VII_s	GAACTGAAAGGGTCTGCGACACTCT
hBDNF_IXb_s	GCTGCTAAAGTGGGAAGAAGG
hBDNF_IX_as1	GTCCTCATCCAACAGCTCTTCTATC
hBDNF_IX_as2 (with VII_s)	GAAGTGTACAAGTCCGCGTCCTTA
expression analyis (qPCR)	
EGFPq_s	CAGAAGAACGGCATCAAGGTG

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

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

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

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

Conclusion

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

Methods

Generation of transgenic mice

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

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

agreement with the local Ethical Committee of Animal Experimentation.

Cell culture, antibodies and animal experiments

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

RT-PCR

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

Ribonuclease protection assay

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

In situ hybridization

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

Authors' contributions

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

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LETTER

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

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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,

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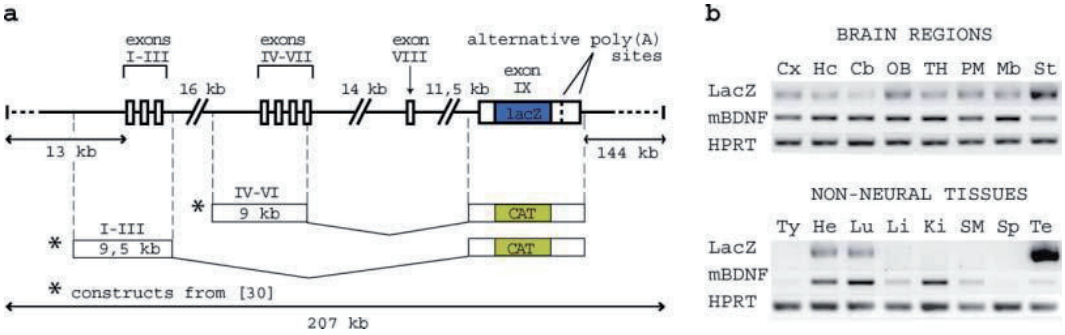

FIG. 1. (**a**) Schematic diagram of the BAC construct used for generating *rBDNF-lacZ*-BAC transgenic mice (thick lines). White boxes represent untranslated sequences and the blue filled box represents *lacZ* reporter gene that replaces the *BDNF* coding sequence. *rBDNF-CAT* constructs (I–III and IV–VI)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

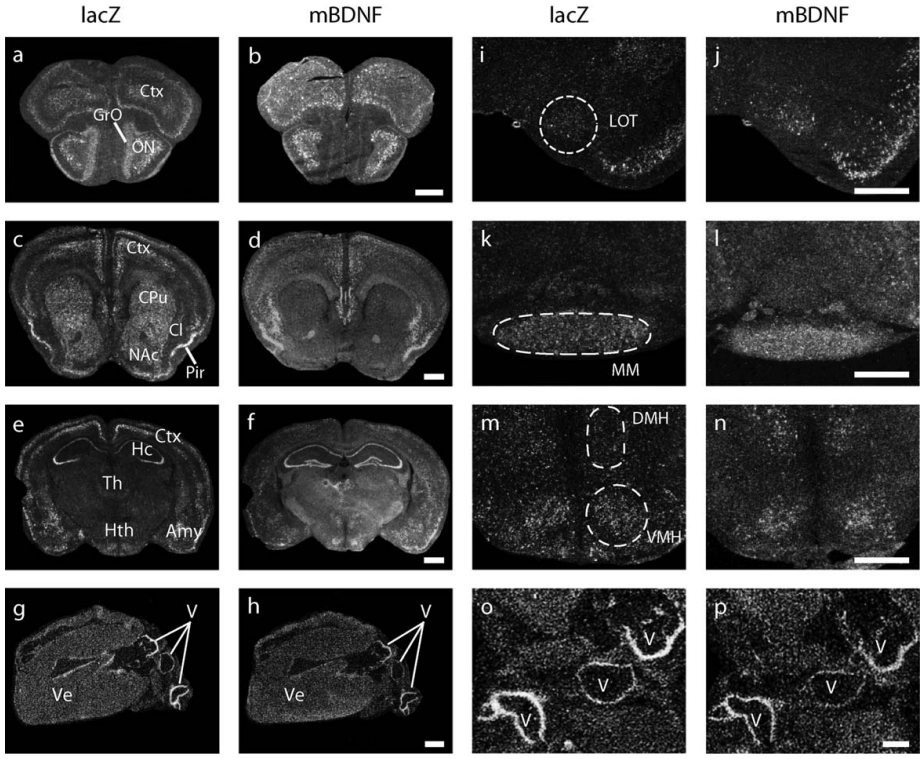

FIG. 2. In situ hybridization analysis of *rBDNF-lacZ* mRNA expression in adult *rBDNF-lacZ*-BAC transgenic mouse brain and heart. Photomicrographs of 16 μm coronal brain (**a–f; i–n**) and transverse heart sections (**g,h,o,p**) hybridized with ^{35}S-labeled *lacZ* or mouse endogenous *BDNF* (*mBDNF*)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

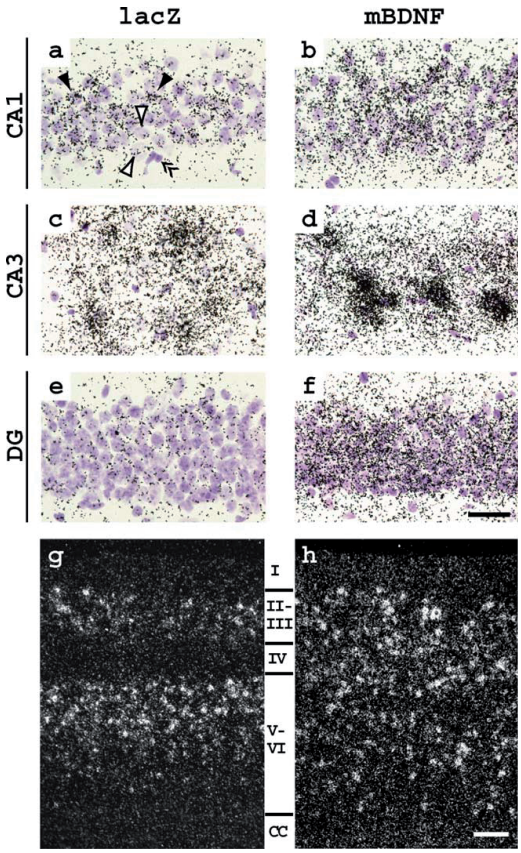

FIG. 3. Cellular expression of *rBDNF-lacZ* mRNA in adult transgenic mouse brain: in situ hybridization analysis. (**a–f**) Bright-field photomicrographs of hippocampal subfields CA1, CA3, and dentate gyrus (DG). Hybridization probes are indicated above the columns; closed arrowheads indicate neurons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

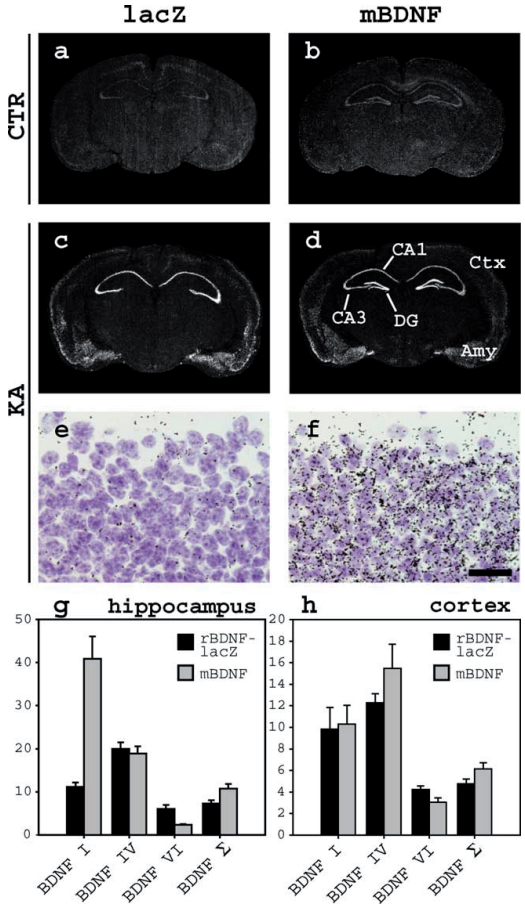

FIG. 4. Induction of *rBDNF-lacZ* mRNA in transgenic mouse brain by kainic acid treatment. (**a–f**) In situ hybridization analysis with probes for transgenic *rBDNF-lacZ* and mouse endogenous (*mBDNF*) mRNA. Autoradiographs of sections from vehicle-treated (**a**,**b**) and kainate-treated animals (**c–f**) 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® 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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Differential regulation of *Bdnf* expression in cortical neurons by class-selective histone deacetylase inhibitors

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ABSTRACT

Histone deactylase (HDAC) inhibitors show promise as therapeutics for neurodegenerative and psychiatric diseases. Increased expression of brain-derived neurotrophic factor (BDNF) has been associated with memory-enhancing and neuroprotective properties of these drugs, but the mechanism of BDNF induction is not well understood. Here, we compared the effects of a class I/IIb selective HDAC inhibitor SAHA, a class I selective inhibitor MS-275, a class II selective inhibitor MC1568 and a HDAC6 selective inhibitor tubacin on *Bdnf* mRNA expression in rat primary neurons. We show that inhibition of class II HDACs resulted in rapid upregulation of *Bdnf* mRNA levels, whereas class I HDAC inhibition produced a markedly delayed *Bdnf* induction. In contrast to relatively slow upregulation of *Bdnf* transcripts, histone acetylation at BDNF promoters I and IV was rapidly induced by SAHA. *Bdnf* induction by SAHA and MS-275 at 24 h was sensitive to protein synthesis inhibition, suggesting that delayed *Bdnf* induction by HDAC inhibitors is secondary to changed expression of its regulators. HDAC4 and HDAC5 repressed *Bdnf* promoter IV activity, supporting the role of class II HDACs in regulation of *Bdnf* expression. In addition, we show a critical role for the cAMP/Ca^{2+} response element (*CRE*) in induction of *Bdnf* promoter IV by MS-275, MC1568, SAHA and sodium valproate. In contrast, MEF2-binding *CaRE1* element was not necessary for promoter IV induction by HDAC inhibition. Finally, we show that similarly to *Bdnf*, the studied HDAC inhibitors differentially induced expression of neuronal activity-regulated genes *c-fos* and *Arc*. Together, our findings implicate class II HDACs in transcriptional regulation of *Bdnf* and indicate that class II selective HDAC inhibitors may have potential as therapeutics for nervous system disorders.

1. Introduction

Histone deacetylase inhibitors have recently received much attention as perspective drug candidates for neurodegenerative and psychiatric diseases, owing to their neuroprotective, neurorestorative and cognition-enhancing properties (Fischer et al., 2010; Kazantsev and Thompson, 2008). Neurotrophic factors such as glial-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) have been considered as possible mediators of the beneficial effects of HDAC inhibitors in these disorders (Chen et al., 2006; Chiu et al., 2011; Zeng et al., 2011; Yasuda et al., 2009) and therefore understanding the mechanisms of *Bdnf* regulation by these treatments is of high importance. The *Bdnf* gene contains at least nine differentially regulated promoters and multiple untranslated 5′ exons alternatively spliced to one protein-coding 3′ exon (Aid et al., 2007; Pruunsild et al., 2007). A number of *cis*-regulatory elements have been identified in *Bdnf* promoters, of which the best characterized are elements mediating activation of promoter IV by neuronal activity (Lyons and West, 2011). Among these, the cAMP/Ca^{2+} response element (*CRE*) has been found to play a central role as knock-in mice with mutated *CRE* element lack activity-dependent transcription of exon IV-containing mRNA (Hong et al., 2008; Shieh et al., 1998; Tao et al., 1998). *CRE*-dependent transcription is regulated also by HDAC inhibitors (Canettieri et al., 2003; Fass et al., 2013, 2003), which have been shown to enhance memory and synaptic plasticity (Vecsey et al., 2007).

The family of 11 Zn-dependent mammalian histone deacetylases is divided into classes I, II, and IV on the basis of sequence homology. Class III contains a family of NAD-dependent deacetylases now called sirtuins, which are structurally and functionally distinct from “classical” Zn-dependent HDACs. Major differences have been found between HDACs in terms of tissue specific expression, subcellular localization and physiological roles. Class I HDACs 1, 2, 3 and 8 are ubiquitously expressed and localize predominantly in the nucleus. In contrast, HDACs 4, 5, 7 and 9 (class

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IIa), HDACs 6 and 10 (class IIb), and HDAC 11 (class IV) display more restricted expression patterns and are cytoplasmic (HDAC6) or shuttle between nucleus and cytoplasm (HDACs 4, 5, 7 and 9–11) (Fischer et al., 2010; Haberland et al., 2009). In adult rat brain, a study mapping expression of all 11 HDACs reported highest expression levels for HDACs 2, 3, 4, 5 and 11 (Broide et al., 2007). Targeting individual HDACs with specific inhibitors is an important aim in developing HDAC-related therapeutic strategies, but this has proved challenging due to the highly conserved active site of these enzymes (Kazantsev and Thompson, 2008).

Several studies have explored the connection between BDNF and HDACs in nervous system disorders, using class I-selective inhibitors such as valproic acid/sodium valproate (Calabrese et al., 2012; Chen et al., 2006; Fukuchi et al., 2009; Lv et al., 2012; Yasuda et al., 2009), sodium butyrate (Sui et al., 2012; Zeng et al., 2011), MS-275 (Lin et al., 2012) or class I/II inhibitors trichostatin A (Fukuchi et al., 2009; Tian et al., 2009) and SAHA (Mielcarek et al., 2011). However, little is known about the contribution of individual HDAC isoforms to *Bdnf* transcriptional regulation, with the exception of HDAC2 that has been shown to bind to *Bdnf* promoters I, II and IV (Gräff et al., 2012; Guan et al., 2009). In this study, we compared the effects of HDAC inhibitors with different selectivity on *Bdnf* expression in cultured rat neurons. SAHA (also known as vorinostat), once regarded as a pan-HDAC inhibitor, has been recently shown to be incapable of inhibiting the catalytic activity of class IIa HDACs and therefore should be considered a class I/IIb-selective inhibitor (Bradner et al., 2010). MS-275 is a class I inhibitor with selectivity for HDACs 1, 2 and 3 over HDAC8 and some selectivity for HDAC1 over HDACs 2 and 3 (Hu et al., 2003; Khan et al., 2008). MC1568 is a class II HDAC inhibitor (Mai et al., 2005; Nebbioso et al., 2009) and tubacin is a HDAC6-selective inhibitor (Haggarty et al., 2003).

2. Materials and methods

2.1. Primary neuronal cultures

All animal procedures were performed in compliance with the local ethics committee. Primary cortical neuronal cultures were generated using Sprague Dawley rat embryos (day 21) as described previously (Pruunsild et al., 2011), with minor modifications. Briefly, cortices were dissected and dissociated into single cell suspension with 0.25% trypsin (Life Technologies) and 0.5 mg/ml DNase I (Roche) digestion, followed by gentle trituration. Cells were plated on poly-L-lysine (0.1 mg/ml) coated dishes at a density of $2 \times 10^5/cm^2$ in Neurobasal A medium (Life Technologies), supplemented with B27 (Life Technologies), 1 mM L-glutamine (PAA Laboratories) and penicillin/streptomycin (PAA Laboratories). At day 2 in vitro (DIV), 5-fluoro-2-deoxyuridine (Sigma) was added to the medium (10 μM) to inhibit growth of glial cells. Transfections and drug treatments were carried out at 6 DIV.

2.2. Drug treatments

SAHA was purchased from Cayman Chemical, MS-275 was purchased from Axxora, sodium valproate was purchased from Sigma-Aldrich, MC1568, tubacin and cycloheximide were purchased from Tocris Bioscience. HDAC inhibitors were dissolved in DMSO and added to neuronal cultures at a final DMSO concentration of 0.1%. Cycloheximide was dissolved in culture medium.

2.3. RNA extraction and quantitative RT-PCR

RNA was isolated using RNeasy Mini or Micro kits (Qiagen) and cDNA was synthesized from 500 to 1000 ng of RNA with Superscript III reverse transcriptase (Life Technologies) using oligo(dT) primers. Quantitative PCR was performed on a LightCycler 480 instrument using LightCycler 480 SYBR Green I Master (Roche). All qPCR reactions were performed in triplicate and target expression was normalized to levels of cyclophilin B (*Ppib*) mRNA. Following primers were used in this study: BDNF-total F, 5′-GGCCCAACGAAGAAAAC CAT-3′; BDNF-total R, 5′-AGCATCACCCGGGAAGTGT-3′; BDNF-I F, 5′-AACAAGACACATTACCTTCCAGCAT-3′; BDNF-I R, 5′-CTCTTCT CACCTGGTGGAACATT-3′; BDNF-II F, 5′-TGGTATACTGGGTTAACTTT GGGAAA-3′; BDNF-II R, 5′-CACTCTTCTCACCTGGTGGAACTT-3′; BDNF-IV F, 5′-GCTGCCTTGA TGTTTACTTTGA-3′; BDNF-IV R, 5′-GCAACCGAAGTATGAAATAACC-3′; BDNF-VI F, 5′-GAACAAACGATTGCTGAAAATG-3′; BDNF-VI R, 5′-TTCTCACCT GGTGGAACTTTATG-3′; c-fos F, 5′-GCTGACAGATACGCTCCAAG-3′; c-fos R, 5′-CATTGGGGATCTTGCAGGCAGGTCG-3′; Arc F, 5′-TGAGTCCTCACCCCCAGCTGA-3′; Arc R, 5′-GTGTCTTGGAACCTCTGTGCAGG-3′; cycB F, 5′-AGATCGAAGT GGAGAAACCCTTTG-3′; cycB R, 5′-TAAAAATCAGGCCTGTGGAATGTG-3′. Cyclophilin B primer sequences were taken from Yasuda et al. (2009).

2.4. DNA constructs and site-directed mutagenesis

BDNF promoter IV construct pIV wt was generated by amplifying the pIV sequence from rat genomic DNA using the Expand High Fidelity PCR System (Roche) and cloning into pGL4.15[luc2P/Hygro] (Promega) in front of firefly luciferase coding sequence. Site-directed mutagenesis was performed as previously described (Pruunsild et al., 2011) using Phusion DNA polymerase (Finnzymes) and following primers: BDNF pIV *CRE* mutation: sense 5′-AGGAGGTATCATATGACAGCCAGCTGC AAGGCAGCGTGGAGCCCTCTCG-3′, antisense 5′-CGAGAGGGCTCCACGCTGCCTTG CAGCTGGCTGTCATATGATACCT CCT-3′; BDNF pIV *CaRE1* mutation: sense 5′-TGCACTAGAGTGTCTTCCGCCAGGCAGAGGAGGTA-3′, antisense 5′-TACCTCCTCTG CCTGGCGGAAGACACTCTAGTGCA-3′. The pIV wt and mCRE constructs were generated by Priit Pruunsild. pIV 4,5K construct was generated by ligating HindIII-NdeI fragment from a previously described BDNFIII-CAT reporter construct (Timmusk et al., 1993) into KpnI-NdeI fragment of pIV wt-pGL4.15 vector. HDAC4 and HDAC5 expression plasmids were generated by amplifying the respective coding regions from HDAC4-FLAG and HDAC5-FLAG expression vectors (kindly provided by H. Bading, University of Heidelberg, Germany) and cloning into the pcDNA3.1 vector (Life Technologies). 3xMEF2-luc (Lu et al., 2000b) was kindly provided by R. Bassel-Duby and E. Olson (University of Texas, Southwestern Medical Center) and 3xCRE-luc (pGL4.29) was obtained from Promega.

2.5. Transfections and luciferase assays

HEK293 cells were transfected with LipoD293 reagent (Signagen) according to the manufacturer's instructions. For luciferase assays, neurons grown in 48-well dishes were transfected at 6 DIV with luciferase reporter plasmids using Lipofectamine 2000 (Life Technologies) according to manufacturer's instructions. 0.5 μg of DNA and 1 μl of Lipofectamine was used per one well of a 48-well plate. For cotransfections of luciferase and HDAC4/5 expression constructs, a 1:1 plasmid ratio was used. Cells were incubated in the transfection medium (Neurobasal A) for 3–4 h with gentle agitation, after which the medium was replaced with conditioned growth medium. For 24/48 h treatments with HDAC inhibitors or DMSO, these drugs were added with the last medium change. 24–48 h after transfection, luciferase activity was measured using Dual-Glo Luciferase Assay System (Promega). Assays were performed in triplicate and averaged to represent one biological replicate. We could not use Renilla luciferase for normalization as all promoters we tested for this purpose responded to treatments with one or more HDAC inhibitors. Use of agitation in the transfection procedure and triplicate measurements allowed to minimize errors related to uneven transfection efficiencies.

2.6. Western blot

Neurons or HEK293 cells grown in 12-well dishes were lysed in 200 μl Laemmli buffer, boiled for 5 min and sonicated briefly. Proteins (20 μl of lysate) were separated by 12% PAGE and transferred to PVDF membrane (Amersham). 5% skimmed milk in TBST buffer was used for blocking membranes and 2% milk-TBST was used for incubations with primary and secondary antibodies. Membranes were incubated for 1 h at room temperature with acetyl-histone H3 antibody (06-599, Millipore) at 1:1000 dilution, acetyl-tubulin antibody (6-11B-1, T7451 Sigma) at 1:5000 dilution and tubulin beta antibody (E7-s, DSHB) at 1:1000 dilution. Incubations with HDAC4 (sc-11418, Santa Cruz) and HDAC5 (sc-11419, Santa Cruz) antibodies (both at 1:1000 dilution) were carried out overnight at 4 °C. HRP-conjugated secondary antibodies (Thermo Scientific) were used at 1:5000 dilution. Bands were visualized by SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific) and quantified using ImageQuant LAS 4000 imager and ImageQuant TL software (GE Healthcare).

2.7. Chromatin immunoprecipitation

Neurons grown on 6-well dishes were crosslinked with 1% formaldehyde for 10 min at room temperature, followed by quenching the reaction with 0.125 M glycine. Samples were transferred to ice and washed with 1 ml PBS. 200 μl lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8.0, protease inhibitors (Complete, Roche)) was added to cells, incubated on ice for 10 min and then lysate from three wells was combined into one sample. The lysate was sonicated to generate 200–1000 bp chromatin fragments. Protein concentration was measured by BCA assay (Thermo Scientific Pierce) and 200 μg protein was used for one immunoprecipitation reaction. Lysate was diluted 1:10 with dilution buffer (1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 20 mM Tris–HCl, pH 8.0, protease inhibitors), 3 μg of anti-AcH3 antibody (06-599, Millipore), 3 μl of anti-AcH4 antiserum (06-866, Millipore) or 3 μl of preimmune rabbit serum (Millipore) was added and samples were rotated overnight at 4 °C. 50 μl of 50% Protein A sepharose beads (GE Healthcare) were used for one reaction. Beads were preabsorbed with 200 μg/ml BSA and 10 μg/ml sheared salmon sperm DNA by rotating overnight at 4 °C in dilution buffer. Beads were added to

lysates, rotated for 2 h at 4 °C, washed 3 times with wash buffer1 (1% Triton X-100, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 20 mM Tris–HCl, pH 8.0, protease inhibitors) and once with wash buffer 2 (1% Triton X-100, 0.1% SDS, 500 mM NaCl, 2 mM EDTA, 20 mM Tris–HCl, pH 8.0, protease inhibitors). Chromatin complexes were eluted with 2 × 100 µl elution buffer (1% SDS and 100 mM NaHCO3) and eluates were combined. Cross-links were reversed by adding 8 µl 5 M NaCl and incubating at 65 °C overnight. DNA was purified with a QIAquick PCR Purification Kit (Qiagen) and analyzed by qPCR using following primers: BDNF_promI_F, 5′-ACGTCCGCTGGAGACCCTTAGT-3′; BDNF_promI_R, 5′-GGCAGCCTCTCTGAGCCAGTTA-3′; BDNF_promIV_F, 5′-ATGCAATGCCCTGGAACGGAA-3′; BDNF_promIV_R, 5′-CGGTGAATGGGAAAGTGGGTGG-3′.

2.8. Data analysis

All data were log transformed before statistical analysis (performed using GraphPad InStat and Microsof Excel) and back-transformed for graphical representation. Two-tailed *t*-tests were used when comparing two groups and ANOVA with Tukey's post hoc tests when comparing more than two groups.

3. Results

3.1. Effect of MC1568 on class I HDAC and HDAC6 specific substrates

MC1568 is a hydroxamic acid HDAC inhibitor which has been proposed to be selective for class II HDACs (Mai et al., 2005; Nebbioso et al., 2009). However, increased histone acetylation has been reported in a breast cancer cell line treated with MC1568 (Duong et al., 2008), which must be attributed to class I HDAC inhibition as class IIa HDACs have only very modest activity on acetylated histones (Lahm et al., 2007). To test the inhibitory activity of MC1568 on class I and class II HDACs, we treated rat primary cortical neurons with 5 µM (concentration used in Nebbioso et al. (2009) MC1568 for 1-24 h and analyzed the levels of acetylated histone 3 (a class I-specific substrate) and acetylated tubulin (a substrate specific to the class II member HDAC6) by Western blot (Fig. 1A,B). Our analysis revealed no significant increase in either histone or tubulin acetylation at any tested time point. As positive controls, we treated neurons for 24 h with SAHA, MS-275 and tubacin (all at 5 µM) (Fig. 1C,D). As expected, class I/IIb inhibitor SAHA significantly increased both histone 3 and tubulin acetylation, class I-selective inhibitor MS-275 increased histone 3, but not tubulin acetylation and HDAC6-selective inhibitor tubacin increased tubulin, but not histone 3 acetylation. In HEK293 cells (Fig. 1E,F), the effects of different HDAC inhibitors were similar to those seen in neurons, except for the higher induction of tubulin acetylation by SAHA and tubacin, most probably due to lower baseline acetyl-tubulin levels in HEK293 cells compared with neurons. Notably, MC1568 showed a weak but consistent ability to increase tubulin acetylation in HEK293, whereas no effect on histone acetylation was noticed. In conclusion, our results showed that MC1568 at 5 µM has weak inhibitory activity for HDAC6 and no inhibitory activity for class I HDACs.

3.2. Bdnf mRNA is upregulated in cultured neurons by both class I and class II HDAC inhibitors

To study the effect of HDAC inhibitors on *Bdnf* expression, we treated primary cortical neurons with SAHA, MS-275, MC1568 and tubacin and analyzed *Bdnf* mRNA levels by quantitative RT-PCR. Treatments were applied at 6 DIV as cultures of similar age have been used for most studies on *Bdnf* transcriptional regulation in primary neurons (e.g. Pruunsild et al., 2011; Tao et al., 1998). Dose response curves showed that *Bdnf* mRNA was induced by SAHA, MS-275 and MC1568 treatment and established 5 µM for a saturating concentration for increasing *Bdnf* mRNA by all three inhibitors (Fig. 2A) and for increasing acetylated histone 3 levels by SAHA and MS-275 (Fig. 2B,C). Neurons were treated for 24 h with SAHA and MS-275 and 3 h with MC1568 based on preliminary data

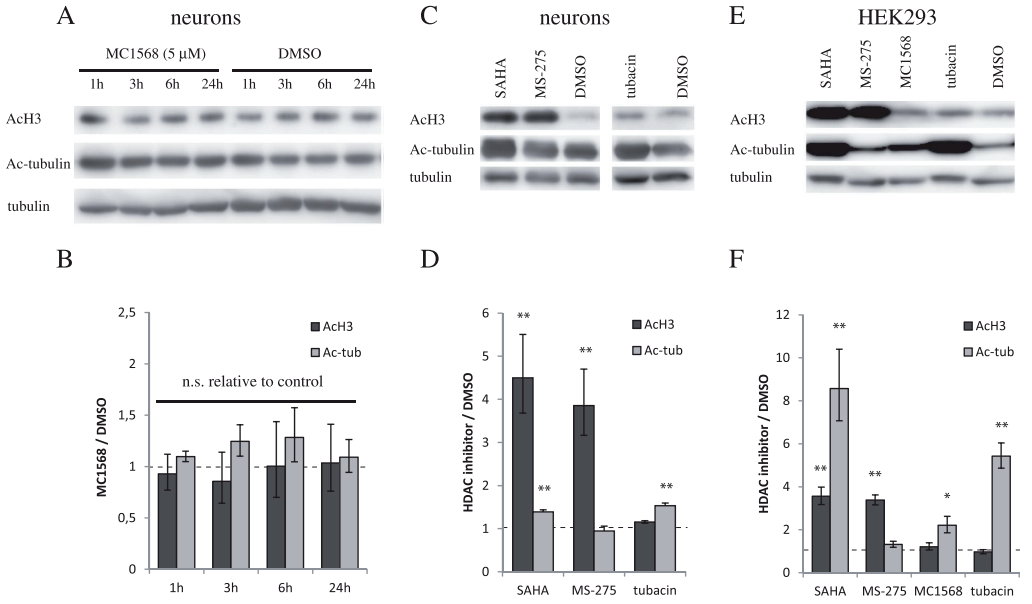

Fig. 1. Effects of MC1568 treatment on histone 3 and tubulin acetylation in cultured neurons and HEK293 cells. **(A,B)** Primary rat cortical neurons were treated at 6 DIV with MC1568 (5 µM) or 0.1% DMSO for indicated times and acetylated histone 3 (AcH3) and acetylated tubulin (Ac-tubulin) levels were analyzed by Western blot. Total tubulin was used as loading control. For quantification (three independent experiments), AcH3 and Ac-tubulin levels were normalized with total tubulin levels and are expressed here as ratios relative to DMSO-treated controls (indicated with a dotted line). **(C,D)** Western blot analysis of primary neurons treated for 24 h with SAHA, MS-275, tubacin (all 5 µM) or DMSO; ($n = 3$) **(E,F)** Western blot analysis of HEK293 cells treated for 24 h with indicated inhibitors (all 5 µM) or DMSO; ($n = 3$) Statistically significant effects relative to DMSO-treated controls are denoted with asterisks $^{*}p < 0.05$, $^{**}p < 0.01$, n.s. – not significant (*t*-test) Error bars – SEM.

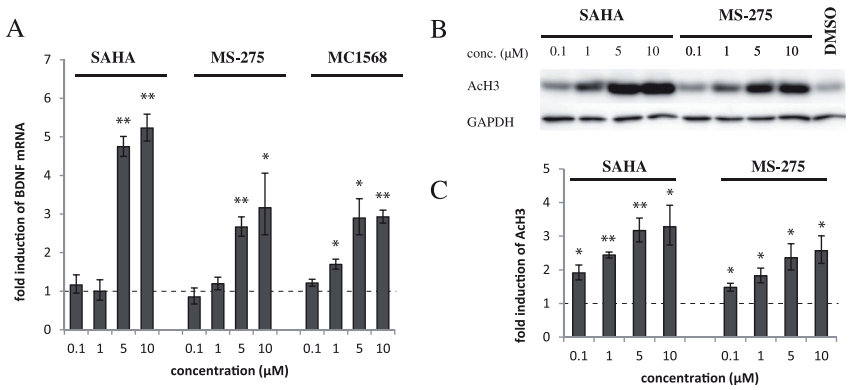

Fig. 2. Inhibitors of both class I and II HDACs induce *Bdnf* mRNA expression in cultured neurons. **(A)** Primary rat cortical neurons were treated at 6 DIV with indicated concentrations of HDAC inhibitors for 24 h (SAHA, MS-275) or 3 h (MC1568) and *Bdnf* mRNA levels were analyzed with quantitative RT-PCR. qRT-PCR data (three independent experiments) were normalized to cyclophilin B expression and are expressed here as fold induction relative to vehicle (DMSO) treated controls (dotted line). **(B)** Primary neurons were treated with SAHA and MS-275 for 24 h and acetylated histone 3 levels were analyzed by Western blot using GAPDH as loading control. **(C)** Quantification of acetyl-H3 levels relative to DMSO-treated controls (dotted line) in three independent neuronal cultures treated with indicated concentrations of HDAC inhibitors. $^{*}p < 0.05$, $^{**}p < 0.01$ relative to DMSO-treated controls (*t*-test); error bars indicate SEM.

on time courses. Time course analysis (Fig. 3A) revealed that class I/IIb HDAC inhibitor SAHA significantly increased *Bdnf* mRNA levels 3–24 h after treatment, whereas the effect of class I-selective inhibitor MS-275 was apparent only after 6 h of treatment. In fact, at 3 h of MS-275 treatment we observed a statistically significant decrease in *Bdnf* mRNA relative to DMSO-treated neurons. In contrast, class II-selective inhibitor MC1568 produced an immediate, robust increase in *Bdnf* mRNA levels already at the 1 h time point followed by gradual decrease up to 24 h, the last time point analyzed. HDAC6-selective inhibitor tubacin produced only a slight increase in *Bdnf* mRNA at earlier time points. Next, we analyzed the induction time courses of four major *Bdnf* mRNAs transcripts containing exons I, II, IV and VI (Aid et al., 2007; Timmusk et al., 1993) in neurons treated with SAHA, MS-275 and MC1568 (Fig. 3B). Tubacin was excluded in this experiment as it was a weak inducer of total *Bdnf* mRNA (Fig. 2A). Our analysis showed that similarly to total *Bdnf* mRNA, all four transcripts were significantly induced within 1–3 h treatment by SAHA and MC1568, but not by MS-275, which significantly increased all transcripts at 6 h and transcripts I and IV at 24 h. Exon I and IV-containing mRNAs were more highly induced than exon II- and VI-containing mRNAs by all three HDAC inhibitors studied. Exon I mRNAs showed a markedly delayed and robust induction by all three HDAC inhibitors, peaking at 24 h.

3.3. *Bdnf promoters I and IV are rapidly hyperacetylated after SAHA treatment*

Next, we asked if the dynamics of exon I and exon IV *Bdnf* mRNA induction by HDAC inhibition correlates with histone acetylation at respective promoters. After validation of *Bdnf* promotor I and IV immunoprecipitation with AcH3 and AcH4 antibodies in cultured neurons (Fig. 4A), we analyzed AcH3 and AcH4 levels at these promoters after treating neurons with SAHA, HDAC inhibitor that most potently induced *Bdnf* mRNA levels (Figs. 2A and 3A) and global histone acetylation (Fig. 2B,C). Surprisingly, levels of acetylated H3 and acetylated H4 at both promoter I and promoter IV were robustly induced by 1 h of SAHA treatment, followed by a gradual decrease within 1–24 h (Fig. 4B,C). This was in sharp contrast with relatively slow induction of *Bdnf* mRNA by SAHA, especially exon I transcripts, which showed steady accumulation in the analyzed time window (Fig. 3).

3.4. *Late phase of Bdnf induction by HDAC inhibitors is sensitive to protein synthesis inhibition*

Analysis of *Bdnf* induction time courses suggested that distinct mechanisms are used to activate *Bdnf* transcription by class I and class II HDAC inhibitors. To test whether *Bdnf* induction by these compounds requires *de novo* protein synthesis, we treated cortical neurons with SAHA, MS-275 and MC1568 in combination with protein synthesis inhibitor cycloheximide (Fig. 5). Cycloheximide treatment completely blocked the increase of *Bdnf* mRNA by SAHA at 24 h, but did not alter the induction amplitude after 3 h SAHA treatment. *Bdnf* induction by 3 h MC1568 treatment was not significantly inhibited by cycloheximide; *Bdnf* induction by 24 h MS275 treatment was inhibited by cycloheximide, but not fully abolished as with 24 h SAHA treatment.

3.5. *Bdnf promoter IV activity is repressed by HDAC4 and HDAC5 and activated by MC1568*

A class II-selective inhibitor, MC1568 is expected to target HDACs 4–7 and 9–10, of which HDAC4 and HDAC5 are highly expressed in neurons (Broide et al., 2007). We next investigated the role of HDAC4 and HDAC5 as regulators of *Bdnf* expression, co-transfecting cortical neurons with respective expression constructs and luciferase reporter construct carrying 0.5 kb of rat *Bdnf* promoter IV sequence in front of firefly luciferase coding sequence (Fig. 6). We focused on promoter IV as its regulation mechanisms have been thoroughly studied, exon IV containing transcripts are highly expressed in neurons (Lyons and West, 2011; Timmusk et al., 1993) and exon IV-containing *Bdnf* mRNAs were also robustly induced by MC1568 treatment (Fig. 3B). Our analysis showed that promoter IV activity was significantly repressed by both HDAC4 and HDAC5 (Fig. 6C) and induced by MC1568 treatment (Fig. 6D).

3.6. *Induction of Bdnf promoter IV by SAHA is CRE-dependent*

We next sought to investigate the putative sequence elements responsible for activation of *Bdnf* promoter IV by HDAC inhibitors. To that end, we used the same luciferase construct as in 3.5 (in Fig. 7A designated as pIV wt) that comprises all known proximal regulatory elements identified in this promoter (Lyons and West,

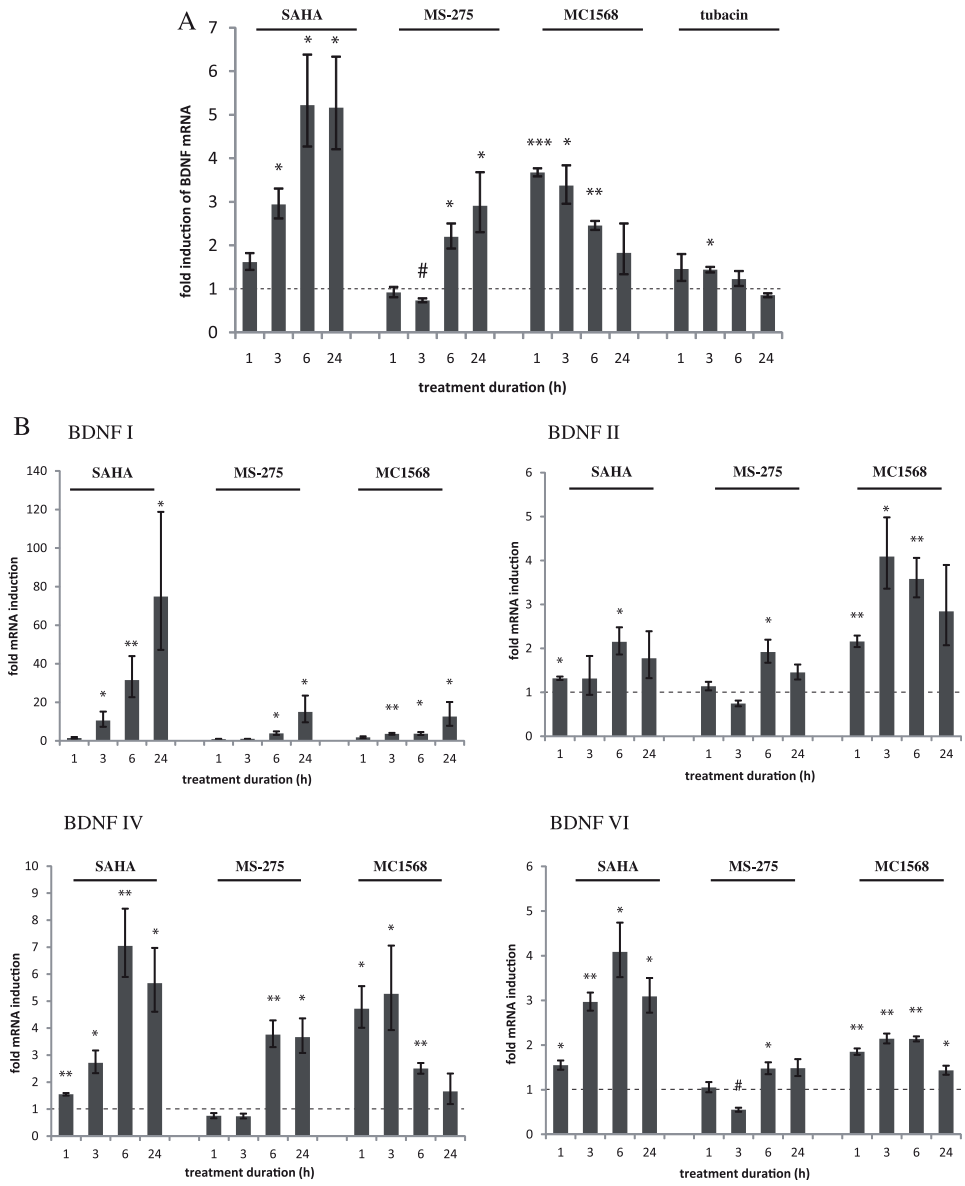

Fig. 3. *Bdnf* mRNA induction by class I and class II HDAC inhibitors shows different time courses. Primary rat cortical neurons were treated at 6 DIV with HDAC inhibitors (all 5 μM) or DMSO for the indicated times and total *Bdnf* mRNA levels **(A)** and levels of four major *Bdnf* transcripts **(B)** were analyzed by quantitative RT-PCR (three independent experiments). qRT-PCR data were normalized to cyclophilin B expression and are expressed here as fold induction relative to DMSO-treated controls at respective time points (indicated with dotted lines). Due to complex splicing of exon II, only one transcript variant (Genbank accession EF125678) was quantified. Statistically significant increases relative to DMSO-treated controls are denoted with asterisks – $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ – and significant decrease relative to controls with # $p < 0.05$ (*t*-test). Error bars – SEM.

2011). These include three calcium-response elements – *CaRE1*, *CaRE2* and *CaRE3* which bind calcium-response factor (CaRF) (Tao et al., 2002), upstream stimulatory factors (USFs) 1 and 2 (Chen et al., 2003) and the cAMP/Ca^{2+} response element binding protein (CREB) (Shieh et al., 1998; Tao et al., 1998), respectively. Recently, it has been shown that *CaRE1* element in promoter IV also binds myocyte enhancer factor 2 (MEF2), which participates in activation of promoter IV by neuronal activity (Lyons et al., 2012). Class II HDACs are known to interact with and MEF2 and CREB and modulate their activity (Li et al., 2012; Lu et al., 2000a; Miska et al., 1999). Therefore, we hypothesized that *CaRE1* and *CaRE3* (also known as *CRE*) regulatory elements could mediate promoter IV activation by HDAC inhibitors. 24 h treatment with SAHA robustly increased luciferase activity in neurons transfected with wild-type promoter IV construct (Fig. 7B). Mutation of the *CaRE1* element did not alter the amplitude of promoter IV induction, while *CRE*

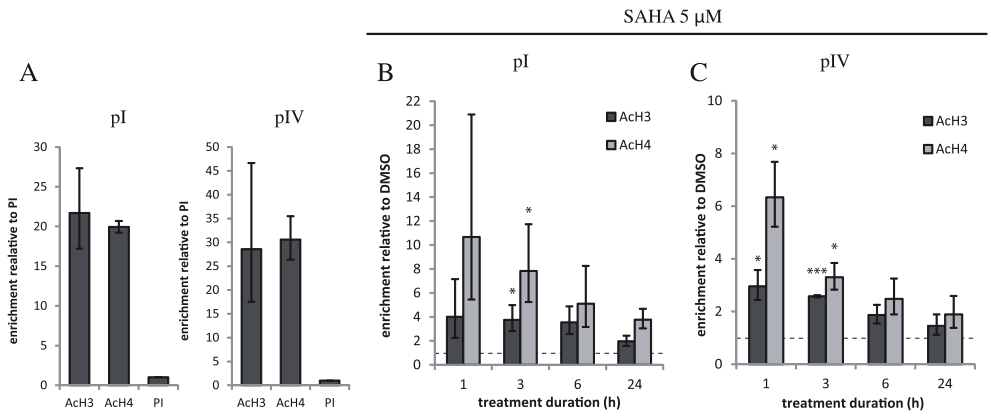

Fig. 4. SAHA rapidly induces histone acetylation at *Bdnf* promoters. **(A)** Validation of *Bdnf* promoter I (pI) and IV (pIV) chromatin immunoprecipitation analysis. Lysates of primary cortical neurons were immunoprecipitated with AcH3 or AcH4 antibodies or rabbit preimmune serum (PI) and analyzed with BDNF promoter-specific primers by qRT-PCR. Results were normalized with respective promoter levels in input DNA and values shown are enrichments relative to immunoprecipitation with preimmune serum ($n = 2$). **(B,C)** ChIP analysis of AcH3 and AcH4 levels at *Bdnf* promoters I and IV in neurons (6 DIV) treated with 5 μM SAHA or DMSO for indicated times ($n = 3$). Results were normalized with input levels and are expressed as enrichment relative to DMSO-treated controls at indicated time points (dotted lines). Statistically significant effects relative to DMSO-treated controls are denoted with asterisks $^{*}p < 0.05$, $^{***}p < 0.001$ (*t*-test) Error bars − SEM.

mutation nearly − but not completely − abolished the induction. In addition, luciferase activity was strongly stimulated by SAHA treatment in neurons transfected with 3xCRE-luc carrying three tandem copies of a consensus *CRE* sequence (Fig. 7C). In contrast, SAHA was unable to induce activity of 3xMEF2-luc reporter with three tandem MEF2-response elements, in fact it effectively repressed this activity.

3.7. CRE is critical for promoter IV induction by both class I- and class II-selective HDAC inhibitors

It has been reported that *cis*-elements mediating *Bdnf* promoter IV activation by class I inhibitor valproic acid in rat cortical neurons are located within −710 and −74 bp and all three calcium response elements including the *CRE* element are dispensable for this effect (Yasuda et al., 2009). However, we observed that *CRE* has a critical role in upregulation of promoter IV by SAHA (Fig. 7B). To study the relevance of the *CRE* element for promoter IV induction by other HDAC inhibitors, we transfected neurons with wild-type or *CRE*-mutated promoter IV-luciferase constructs (0.5 kb promoter sequence) and treated them with SAHA, MS-275, MC1568 (all 5 μM) and sodium valproate. Valproate was used in two concentrations: 0.5 mM as used in Yasuda et al. (2009), and 5 mM, concentration saturating *Bdnf* mRNA induction in our experiments (Fig. 7D). To analyze the contribution of additional regulatory elements located further upstream from promoter IV transcription start site (TSS), we also transfected constructs carrying 4.2 kb genomic sequence 5′ of the TSS. Following transfection, neurons were treated for 24 h with SAHA, MS-275 and sodium valproate and for 6 h with MC1568 (*Bdnf* exon IV mRNA induction by MC1568 was greatly diminished by 24 h). Our analysis showed no statistically significant differences between 0.5 kb (pIV wt) and 4.5 kb promoter IV constructs in induction of luciferase activity by any of the used treatments (Fig. 7E). In contrast, *CRE* mutation clearly reduced promoter IV induction by all HDAC inhibitors tested. This reduction was statistically significant for all treatments except for 0.5 mM valproate treatment, while a tendency towards this effect could be noticed even with 0.5 mM valproate (Fig. 7E). Although *CRE* mutation almost abolished *Bdnf* promoter IV induction by HDAC inhibitors, a small (1.5–2.5×) *CRE*-independent induction compared to DMSO-treated controls remained, which was statistically significant ($p < 0.05$) for MS-275 and MC1568 treatments. In experiments reported by Yasuda et al. (2009), neurons were treated with 0.5 mM valproate for 2–3 days. However, in our hands the *Bdnf* promoter IV induction by valproate appeared *CRE*-dependent even when treatment duration was extended to 48 h (Fig. 7F). Again, no difference in induction amplitude was noted between reporters carrying 0.5 kb and 4.5 kb of promoter IV sequence. Taken together, our results show that induction of *Bdnf* promoter IV by HDAC inhibitors is *CRE*-dependent regardless of their class selectivity.

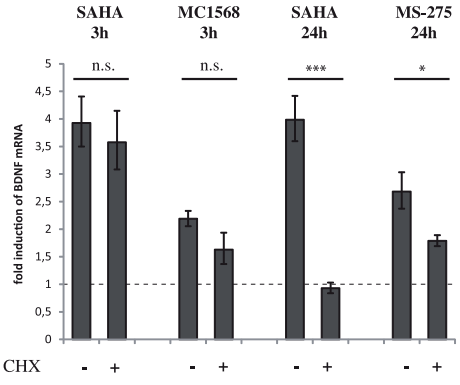

Fig. 5. Inhibition of protein synthesis decreases late, but not early *Bdnf* induction by HDAC inhibitors. Cortical neurons (6 DIV) were treated for 3 h or 24 h with SAHA, MS-275 or MC1568 (all 5 μM) together with or without 10 μM cycloheximide (CHX). *Bdnf* mRNA levels were quantified by qRT-PCR and are expressed as fold induction relative to DMSO-treated controls (dotted line); ($n = 3$) $^{*}p < 0.05$ $^{***}p < 0.01$; n.s. − not significant (*t*-test) Error bars − SEM.

3.8. Class II HDAC inhibition rapidly increases c-fos and Arc mRNA levels

Finally, we tested the effect of HDAC inhibitors on expression of c-*fos* and *Arc*, immediate early genes that are associated with synaptic plasticity and memory formation (Bramham et al., 2010;

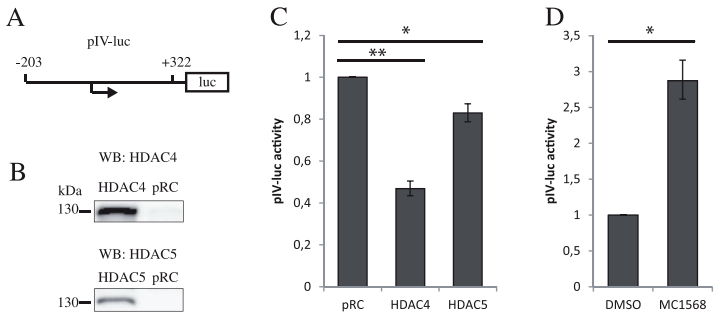

Fig. 6. HDAC4 and HDAC5 repress and MC1568 induces *Bdnf* promoter IV activity. **(A)** Schematic of the firefly luciferase construct carrying 0.5 kb of *Bdnf* promoter IV sequence. Arrow indicates the transcription start site (TSS), numbers designate bases upstream and downstream of the TSS. **(B)** Verification of HDAC4 and HDAC5 expression in HEK293 cells transfected with respective expression constructs (Western blot). pRC − control cells transfected with empty pRC vector. **(C)** Cortical neurons were co-transfected at 6 DIV with *Bdnf* pIV-luc reporter and empty pRC vector or expression vectors coding for HDAC4 or HDAC5; luciferase activity was measured 24 h later ($n = 3$). **(D)** Cortical neurons were transfected with *Bdnf* pIV-luc reporter and treated on the following day with MC1568 (5 μM) or 0.1% DMSO for 6 h before measuring luciferase activity ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$ (*t*-test). Error bars − SEM.

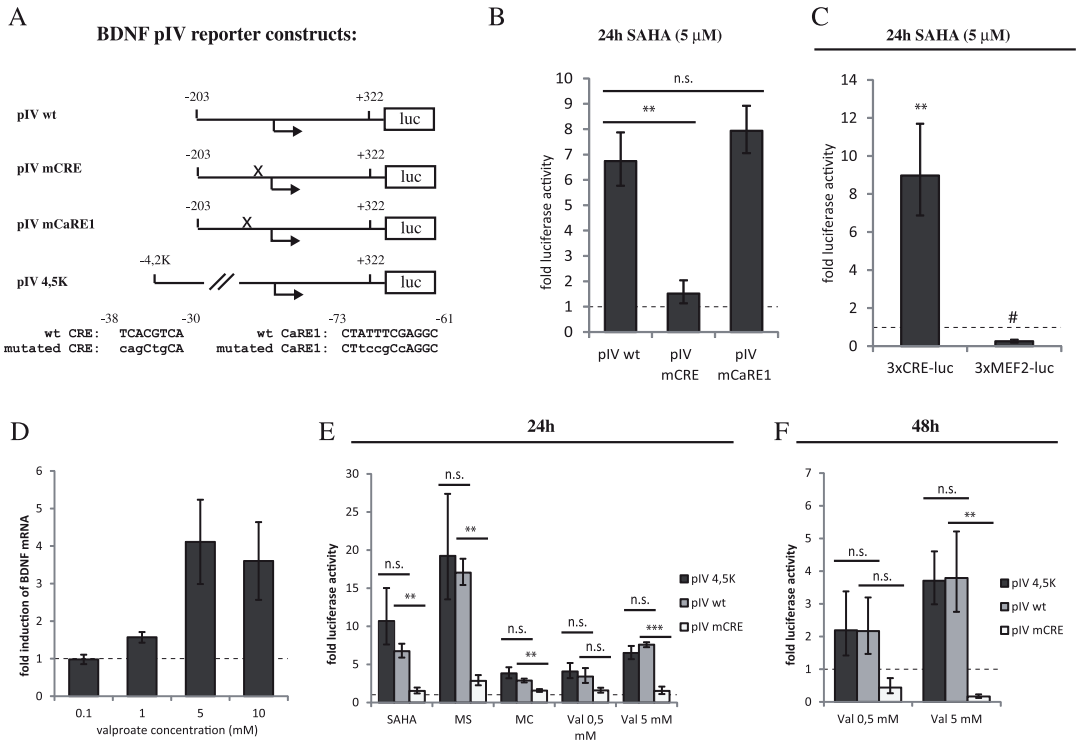

Fig. 7. HDAC inhibitors induce *Bdnf* promoter IV activity in a *CRE*-dependent manner. **(A)** Schematic of firefly luciferase constructs carrying 0.5–4.5 kb of *Bdnf* promoter IV sequence. Arrow indicates the transcription start site (TSS), numbers designate bases upstream and downstream of the TSS. mCRE and mCaRE1 indicate mutations (see below), generated after (Hong et al., 2008) and (Lyons et al., 2012), respectively. **(B, C)** Cortical neurons were transfected with indicated constructs at 6DIV and treated on the same day for 24 h with 5 μM SAHA or DMSO before measuring luciferase activity. 3xCRE-luc (Promega) and 3xMEF2-luc (Lu et al., 2000b) are luciferase reporters carrying 3 tandem copies of binding sites for CREB or MEF2, respectively. Shown are results from at least three independent experiments. $^{**}p < 0.01$ − significant increase relative to DMSO-treated controls (dotted lines), $\#p < 0.05$ − significant decrease relative to DMSO-treated controls, n.s. − not significant (*t*-test). **(D)** Cortical neurons were treated with indicated concentrations of sodium valproate or DMSO (dotted line) for 24 h and *Bdnf* mRNA levels were quantified with qRT-PCR ($n = 2$) **(E)** Cortical neurons (6DIV) were transfected with indicated luciferase reporters and treated on the same day for 6 h with 5 μM MC1568 (MC) or 24 h with 5 μM SAHA, 5 μM MS-275 (MS) and 0.5/5 mM valproate (Val). The values shown are means of four independent experiments and are expressed as fold induction of luciferase activity relative to DMSO-treated controls (dotted line). $^{**}p < 0.01$, $^{***}p < 0.001$, n.s. − not significant (ANOVA with Tukey's post hoc tests). **(F)** Cortical neurons were transfected with indicated reporters and treated with 0.5/5 mM valproate or DMSO (dotted line) for 48 h before measuring luciferase activity ($n = 3$). $^{**}p < 0.01$, n.s. − not significant (ANOVA with Tukey's post hoc tests). Error bars indicate SEM.

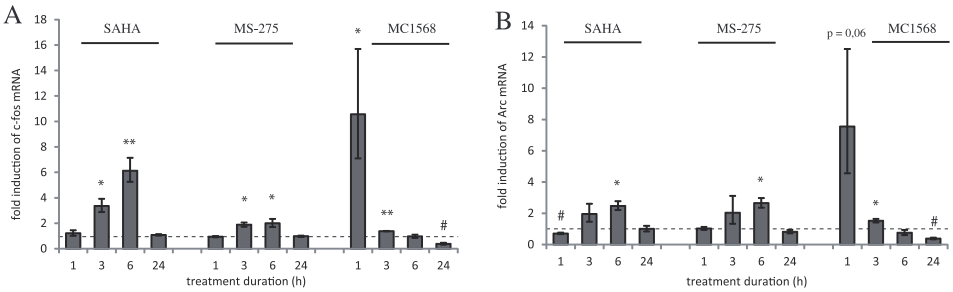

Fig. 8. Induction of c-*fos* and *Arc* mRNA by HDAC inhibitors of different selectivity. Cortical neurons were treated at 6 DIV with HDAC inhibitors (all 5 μM) or DMSO for times indicated and c-*fos* **(A)** and *Arc* **(B)** mRNA levels were analyzed by qRT-PCR. mRNA levels were normalized to cyclophilin B expression and are expressed here as fold induction relative to DMSO-treated controls at indicated times (dotted lines). Shown are data from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ significant increase relative to DMSO-treated controls, $\#p < 0.05$ − significant decrease relative to controls (*t*-test). Error bars − SEM.

Cohen and Greenberg, 2008). *CRE*-dependent transcription (Kawashima et al., 2009; Sassone-Corsi et al., 1988) and regulation by class II HDACs (Schlumm et al., 2013; Sugo et al., 2010) has been reported for both genes. Our analysis showed that c-*fos* and *Arc* were regulated by all three HDAC inhibitors (Fig. 8). mRNA levels of both genes were robustly increased after 1 h treatment with class II-selective inhibitor MC1568, returning to baseline levels between 3 and 6 h treatment and dropping to about 40% of levels in DMSO-treated cultures by 24 h. In contrast, treatment with SAHA and MS-275 resulted in a gradual increase of c-*fos* and *Arc* mRNA between 1 and 6 h and return to baseline levels by 24 h.

4. Discussion

HDAC inhibitors are effective in increasing *Bdnf* expression both in cell culture and *in vivo*, making histone deacetylases attractive targets for pharmacological intervention aimed at modulating *Bdnf* expression (Chen et al., 2006; Chiu et al., 2011; Zeng et al., 2011; Yasuda et al., 2009). However, understanding of the molecular mechanisms mediating these effects is limited. Effects of HDAC inhibitors on gene expression are commonly interpreted as direct consequences of alterations in histone acetylation levels at gene promoters. Although these undoubtedly have an important role, other mechanisms are likely to contribute. First, HDAC inhibitors alter expression levels of a large set of genes (e.g. Fass et al., 2013; Fukuchi et al., 2009) and therefore induction of a gene may be secondary to activation/repression of its regulators. Second, the effects of HDAC inhibitors may be mediated by other acetylated proteins rather than histones (Choudhary et al., 2009) or even non-HDAC targets (e.g. Bantscheff et al., 2011; Chen et al., 1999). Finally, class II HDACs that very inefficiently deacetylate histones (Lahm et al., 2007) may have role.

In this study, we compared the effects of four HDAC inhibitors of different selectivity on *Bdnf* mRNA expression in cultured neurons. Selectivity of MS-275 for class I HDACs and that of tubacin for HDAC6 is widely accepted. SAHA had previously been seen as a typical non-selective HDAC inhibitor until this view was challenged by a recent study using improved HDAC assays (Bradner et al., 2010). MC1568 is believed to be a class II selective HDAC inhibitor (Mai et al., 2005; Nebbioso et al., 2009). However, it has been reported that it increased histone acetylation at 20 μM in breast cancer cells, indicating that in higher concentrations it can inhibit class I HDACs as well (Duong et al., 2008). Here, we tested its effect on class I HDAC and HDAC6 specific substrates (acetylated histone 3 and acetylated tubulin, respectively) in primary neurons and HEK293 cells. Our analysis showed that at 5 μM MC1568 did not affect acetyl-H3 and acetyl-tubulin levels in neurons. In HEK293 cells, MC1568 increased tubulin, but not H3 acetylation, confirming class II HDAC selectivity of MC1568 at this concentration. As natural substrates for class IIa HDACs are unknown, it is not possible to assess the activity of MC1568 on these enzymes by similar immunoblot experiments. Therefore, the inhibitory activity of MC1568 for class IIa HDACs has to be unambiguously determined in further studies, e.g. using the assay described by Bradner et al. (2010). Nevertheless, on the grounds of our selectivity analysis, class I HDACs and HDAC6 could be excluded as mediators of its effects on gene expression reported here.

Comparison of *Bdnf* mRNA induction time courses revealed that MC1568 treatment produced a much faster induction of *Bdnf* mRNA than MS-275, suggesting that class II HDACs may participate more directly in *Bdnf* transcriptional regulation than class I proteins. *Bdnf* induction by MC1568 treatment was transient, gradually decreasing between 1 and 24 h. Mechanisms underlying such dynamics remain to be determined, but this effect could be advantageous in therapeutic use as excessive stimulation of neurotrophin expression can lead to deleterious side effects (Price et al., 2007). Class I/IIb inhibitor SAHA significantly induced *Bdnf* mRNA earlier than MS-275 (at 3 h), suggesting contribution of HDAC6 or other non-class I HDAC targets. Indeed, a weak but statistically significant induction of *Bdnf* mRNA was observed in neurons after 3 h of treatment with the selective HDAC6 inhibitor tubacin. Time course analysis of major *Bdnf* transcripts showed that all of them were induced more rapidly by MC1568 and SAHA than with MS-275, supporting the results of total *Bdnf* mRNA analysis. Accumulation of *Bdnf* exon I mRNAs was markedly slower and reached higher amplitudes than other transcripts following treatment with all three HDAC inhibitors. This phenomenon appears to be a central feature of exon I mRNA transcription as it is more highly induced than exon IV mRNA and peaks later regardless of the stimulus or type of neurons used (Marmigère et al., 2001; Metsis et al., 1993; Pruunsild et al., 2011; Tian et al., 2009). Exon I and IV *Bdnf* mRNAs are more highly induced by neuronal activity than exon II and exon VI mRNAs (Metsis et al., 1993; Pruunsild et al., 2011; Timmusk et al., 1993). Here, we show that similarly to neuronal activity, HDAC inhibitors induced exon I and exon IV mRNAs more potently than exon II and VI mRNAs.

The observed delay in *Bdnf* mRNA induction by class I HDAC inhibition suggested that mechanisms other than increased acetylation at *Bdnf* promoters may be responsible for these effects. Our analysis showed that SAHA treatment rapidly increased histone acetylation at *Bdnf* promoters I and IV, contrasting with relatively slow induction *Bdnf* mRNA. It could be argued that time consumed in *Bdnf* mRNA synthesis may have caused this delay. However, the effects of MC1568 on *Bdnf* in the same experiment showed that

Bdnf mRNA can be induced faster, suggesting that the late phase of *Bdnf* mRNA induction by HDAC inhibitors may depend on altered expression of other proteins that participate in *Bdnf* transcriptional regulation. This hypothesis was supported by our findings that protein synthesis inhibitor cycloheximide significantly reduced the late (24 h) *Bdnf* induction by SAHA and MS-275 treatment, but not the early phase (3 h) of induction by SAHA and MC1568 treatment.

Among class II HDACs, HDAC4 and HDAC5 are highly expressed in neurons (Broide et al., 2007) and regulate gene expression through interaction with transcription factors such as MEF2, SRF and CREB (Davis et al., 2003; Li et al., 2012; Lu et al., 2000a; Miska et al., 1999). HDAC4 has been implicated in regulation of *Bdnf* transcription through suppression of MEF2- and CREB-dependent transcriptional activity in cerebellar neurons (Li et al., 2012). This agrees well with our results showing that *Bdnf* promoter IV activity is downregulated by HDAC4 and HDAC5 overexpression and induced by class II HDAC inhibitor MC1568. In the present study, we found that mutation of *CRE*, but not MEF2 binding site *CaRE1* in *Bdnf* promoter IV significantly decreased its induction by HDAC inhibition. The *CRE* element is indispensable for promoter IV activation by neuronal activity (Hong et al., 2008; Shieh et al., 1998; Tao et al., 1998), but its role in promoter IV induction by HDAC inhibitors is not as well characterized. Non-selective HDAC inhibitor trichostatin A (TSA) has been shown to enhance *CRE*-dependent transcription (Canettieri et al., 2003; Fass et al., 2003) and crebinostat, another *CRE*-activating HDAC inhibitor, induced expression of *Bdnf* transcript IV in primary mouse forebrain neurons (Fass et al., 2013). However, a study by Yasuda et al. concluded that exclusion of all three *CaRE* elements (including *CRE*) from *Bdnf* promoter IV does not affect its induction by valproic acid (Yasuda et al., 2009). This is in contradiction with the results of the current study, as here we found that promoter IV induction by all tested HDAC inhibitors – including sodium valproate – was drastically decreased by the *CRE* mutation. This discrepancy may possibly arise from different transfection and drug treatment protocols: in our experiments transfections and HDAC inhibitor treatments were applied simultaneously at 6 DIV, whereas in Yasuda et al. (2009) valproate treatment was started at 7 DIV, one day before transfections. In addition, it is possible that the effect of *CaRE1-3* deletion on promoter IV induction by valproate was missed in Yasuda et al. (2009) as they did not include the wild type promoter IV control in experiments examining the effect of *CaRE1-3* deletion on promoter IV induction by valproate. Taken together, our results indicate that the *CRE* element is critical for the induction of *Bdnf* promoter IV by HDAC inhibitors.

Finally, we were interested if the differential effects of class I and class II HDAC inhibitors on *Bdnf* expression can be generalized to other genes that share common regulation mechanisms with *Bdnf*. Like *Bdnf*, c-*fos* and *Arc* are immediate early genes (IEG) rapidly induced by neuronal activity and have a *CRE*-dependent component in their transcriptional regulation (Lyons and West, 2011). C-fos and Arc have important roles in synaptic plasticity, Arc being required for consolidation of long-term memory (Bramham et al., 2010; Cohen and Greenberg, 2008). Similarly to *Bdnf* mRNA, robust induction of both c-*fos* and *Arc* mRNAs was detected after 1 h treatment of neurons with MC1568, while induction by SAHA and MS-275 was comparatively delayed and more moderate. Together, these data indicate that inhibition of both class I and II HDACs induce transcription of IEGs in neurons and imply a more direct transcriptional role for class II HDACs. Overexpression of mutant HDAC9 that is retained in the nucleus has been reported to downregulate c-*fos* expression in cortical neurons (Sugo et al., 2010). In addition, HDAC4 has recently been shown to regulate *Arc* expression in hippocampal neurons (Schlumm et al., 2013). The exact molecular mechanisms mediating regulation of these genes by class II HDACs remain to be determined as transcription of c-*fos* and *Arc* is known to be regulated by CREB, MEF2 and SRF proteins (Lyons and West, 2011), all of which have been shown to interact with class II HDACs (Davis et al., 2003; Li et al., 2012; Lu et al., 2000a; Miska et al., 1999). Whatever the mechanisms involved, activation of *Bdnf*, *Arc* and c-*fos* transcription by MC1568 suggests that class II selective HDAC inhibitors may have potential as therapeutic agents for psychiatric and neurodegenerative diseases associated with synaptic plasticity impairment.

5. Conclusions

In this study, we provide evidence for regulation of *Bdnf* expression in neurons by class II HDACs and suggest that *Bdnf* induction by class I HDAC inhibitors may be an indirect effect of altering the expression of its transcriptional regulators. Our results indicate that the *CRE* element in *Bdnf* promoter IV is critical for its induction by both class I and II HDAC inhibitors. In addition to *Bdnf*, class II HDAC inhibition rapidly induced the expression of two immediate early genes associated with synaptic plasticity and memory function – c-*fos* and *Arc*.

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ABSTRACT

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family of neurotrophic factors, supports survival and differentiation of several neuron populations in the developing peripheral and central nervous system. In addition, BDNF has important roles in regulation of neurotransmission in the adult nervous system. Impairments in BDNF function have been associated with a number of human nervous system disorders and therapeutic strategies oriented at modulating BDNF expression or function are currently being actively sought. To this end, it is important to understand how BDNF expression is regulated. In this thesis, I have used two approaches to study BDNF regulation. First, BAC (bacterial artificial chromosome) transgenic mice carrying large fragments of rat or human BDNF loci were generated and characterized. Second, I have investigated regulation of BDNF expression by chromatin modifying drugs.

For generating human BDNF-BAC transgenic mice, we used a BAC construct carrying 169 kb of the human BDNF locus (including 67 kb of the BDNF gene), modified to encode BDNF protein C-terminally tagged with EGFP. Rat BDNF-BAC transgenic mice were generated using a BAC construct carrying 207 kb of the rat BDNF locus (including 50 kb of the BDNF gene), where the BDNF coding sequence was replaced with a beta-galactosidase (LacZ) coding sequence. BDNF-BAC transgenic mice carrying either rat or human BDNF loci recapitulated endogenous BDNF expression in several brain regions and non-neuronal tissues. In addition, expression of transgenic BDNF mRNA in the cortex and hippocampus was induced by neuronal activity. Together, our results show that the BAC constructs used in these studies contained most regulatory sequences necessary for recapitulating activity-dependent and tissue-specific BDNF expression. Importantly, human BDNF locus inserted into the mouse genome enables to study regulation of the human gene *in vivo,* allowing to perform experiments that are otherwise not feasible. For example, these mice can be used for studying molecular mechanisms of human BDNF regulation and *in vivo* testing of compounds that modulate human BDNF expression. Finally, shortcomings in the ability of transgenic constructs to recapitulate the expression of a particular gene can be used as clues for locating tissue-specific regulatory elements. Comparison of BAC-driven BDNF mRNA expression in transgenic mice with expression patterns of endogenous BDNF and previously reported BDNF transgenes suggests that a regulatory element necessary for BDNF expression in the heart may be located downstream of the BDNF gene. Further studies are warranted to confirm the existence of such element.

Chromatin modifying drugs - compounds targeting enzymes that add or remove covalent modifications to DNA or histones - are under active research as perspective therapies for nervous system disorders, especially neurodegenerative disorders. As altered expression of BDNF has been

implicated in several nervous system disorders, we decided to study the effects of different classes of chromatin drugs on BDNF expression in cultured rat cortical neurons. Among the tested compounds, histone deacetylase inhibitors showed the most consistent ability to increase BDNF mRNA expression. DNA methyltransferase inhibitors, in contrast, were ineffective in modulating BDNF expression. Thus, we proceeded to analyze regulation of BDNF expression by histone deacetylase inhibitors in more detail. HDAC inhibitors with different isoform selectivity were effective in increasing BDNF mRNA in neurons. Further analysis suggested that that distinct mechanisms may operate in BDNF induction by class I selective and class II selective HDAC inhibitors. Specifically, these experiments indicate that class II HDACs probably act as direct transcriptional regulators of BDNF expression, whereas induction of BDNF expression by class I HDAC inhibitors is mediated by indirect mechanisms. Similar effects of class-selective HDAC inhibitors on BDNF expression were observed in neurons and cardiac endothelial cells, suggesting that the mechanisms involved are not neuron-specific, but of a more general nature. Finally, potent HDAC-independent and promoter IV-selective induction of BDNF expression was observed with one tested compound. The molecular target mediating this induction remains to be determined.

KOKKUVÕTE

Neurotroofsed tegurid on sekreteeritavad valgud, mis on olulised mitmete neuronipopulatsioonide elushoidmiseks kesk- ja piirdenärvisüsteemi arengus ning lisaks omavad mitmesuguseid funktsioone neuronite diferentseerumisel ning diferentseerunud neuronite talitluses. Aju-päritoluline neurotroofne tegur (*Brain-Derived Neurotrophic Factor*, BDNF) on neurotrofiinide perekonda kuuluv valk, mida iseloomustab kõrgel tasemel avaldumine kesknärvisüsteemi neuronites. BDNF valk eraldati aastal 1982 ning seda kodeeriv geen kloneeriti aastal 1990. Möödunud mõne aastakümne jooksul on BDNF funktsiooni ja avaldumise regulatsiooni intensiivselt uuritud, kuna juba varakult ilmnes, et lisaks "klassikalisele" neurotroofsele funktsioonile ehk neuroneid elushoidvatele omadustele osaleb BDNF juba väljakujunenud ja närvivõrgustikesse lülitunud neuronite talitluse reguleerimises. Näiteks arvatakse, et BDNF-il võib olla määrav roll pikaajalises sünaptilises ülekande võimenduses (*long term potentiation*), mis praeguste arusaamade kohaselt on mälu rakulis-molekulaarseks aluseks.

Häireid BDNF avaldumises või talitluses on seostatud mitmete närvisüsteemi haigustega, sealhulgas psühhiaatriliste ja neurodegeneratiivsete häiretega, ning vastavalt on BDNF sihtmärgiks mitmesugustele ravistrateegiatele. Osa ravistrateegiaid keskendub endogeense BDNF tasemete tõstmisele või normaliseerimisele, mõjutades BDNFi avaldumist vahendavaid molekulaarseid mehhanisme. Mõistagi on selliste lähenemiste väljatöötamise eelduseks BDNFi avaldumise mehhanismide hea tundmine. Käesolevas töös uurisin ma BDNF geeni regulatsioonimehhanisme kahel viisil. Esiteks valmistasime ja iseloomustasime BAC transgeensed hiired, uurimaks lähi- ja kaugmõjus toimivate regulaatoralade funktsiooni BDNF lookuses. Teiseks analüüsisin ma kromatiini seisundit mõjutavate ainete (nn kromatiiniravimite) mõju BDNF avaldumisele.

Selles töös valmistati ja iseloomustati hiireliinid, millesse on viidud 169 kb inimese BDNF lookust (sisaldab BDNF geeni pikkusega 67 kb) ja 207 kb roti BDNF lookust (sisaldab BDNF geeni pikkusega 50 kb). Kasutades transgeneesiks suuri genoomseid fragmente, mis sisaldavad lisaks tervele geenile sellest kümneid kuni sadu kilobaase üles- ja allavoolu jäävaid järjestusi, saab tagada transgeeni suhteliselt autonoomse, naabruses asuvate regulatooralade mõju eest kaitstud avaldumise. Nii roti kui inimese BDNF geeniga BAC hiirtes järgis transgeeni ekspressioonimuster suuresti endogeense BDNF ekspressiooni kesknärvisüsteemis ja mitteneuraalsetes kudedes. Sarnaselt endogeensele BDNF geenile avaldusid transgeenidelt alternatiivselt splaisitud BDNF mRNAd, mille tase oli tõstetav neuraalse aktiivsuse indutseerimisel kainaathappe süstimisega kõhuõõnde. Loodud BAC-BDNF hiireliinid on edasipidi kasutatavad tööriistadena BDNFi geeni regulatsioonimehhanismide uurimiseks *in vivo*. Näiteks võimaldavad inimese

BDNF geeni sisaldavad hiired läbi viia eksperimente, mis inimese geeni uurimiseks selle loomulikus kontekstis (ehk inimese genoomis) oleks mõeldamatud.

Kromatiiniravimid on madalamolekulaarsed ühendid, mille sihtmärgiks on DNA või histoonide kovalentseid modifikatsioone lisavad või eemaldavad ensüümid. Selliseid ühendeid uuritakse käesoleval ajal intensiivselt kui potentsiaalseid ravimeid närvisüsteemi haiguste tarvis. Kromatiiniravimite toime avaldub läbi muutuste geenide avaldumises ning BDNF on üks nendest geenidest, mille avaldumise tõstmine on eesmärgiks närvisüsteemi haiguste ja eeskätt neurodegeneratiivsete haiguste ravimisel. Käesolevas töös analüüsisin erinevatesse aineklassidesse kuuluvate kromatiiniravimite mõju BDNF mRNA ekspressioonile roti ajukoore neuronites. Uuritud ainete seas indutseerisid BDNF mRNA avaldumist kõige efektiivsemalt histoondeatsetülaaside (HDAC) inhibiitorid, mille toimet uurisin täpsemalt. Imetajates on leitud 11 HDAC valku, mis struktuuri ja funktsiooni alusel jagatakse kahte klassi. Selgus, et nii klass I kui klass II HDAC valkudele selektiivsed inhibiitorid tõstavad neuronites BDNF mRNA taset. Saadud tulemused viitavad klass II HDAC valkude vahetule BDNF transkriptsioonilisele mõjule, mis on seni kirjeldamata BDNF regulatsiooni mooduseks. Klass I HDAC valkudele selektiivsete inhibiitorite BDNF avaldumist stimuleeriv toime on varasemast teada, kuid tüüpiliselt interpreteeritakse seda otsese transkriptsiooni soodustava mõjuna läbi histoonide atsetüleerimise BDNF promootoritel. Käesoleva töö andmed viitavad pigem võimalusele, et klass I HDAC inhibiitorite toimes BDNFile on määravad kaudsed mõjud, tõenäoliselt muutused BDNF transkriptsiooni regulaatoriteks olevate valkude avaldumises.

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high and low exploratory activity. Brain Research, 1194, 110 - 117.

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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

2. Cis- and trans-regulators of BDNF gene and their uses; Owner: Tallinn University of Technology; Authors: Priit Pruunsild, Mari Sepp, Ester Orav, Indrek Koppel, Tõnis Timmusk; Priority
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Helen Verev, bakalaureusetöö, 2006, juhendajad Indrek Koppel ja Tõnis Timmusk, Neurotroofse faktori GDNF-i alternatiivne splaising ja ekspressioon inimese kudedes, Tallinna Tehnikaülikool

Publikatsioonid:

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Roos, J.L.; Fisher, S.E.; Wade-Martins, R.; Rouleau, G.A.; Stein, J.F.; Karayiorgou, M.; Geschwind, D.H.; Ragoussis, J.; Kendler, K.S.; Airaksinen, M.S.; Oshimura, M.; Delisi, L.E.; Monaco, A.P.
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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,
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number: US61/456,930; Priority date: 15.11.2010

DISSERTATIONS DEFENDED AT TALLINN UNIVERSITY OF TECHNOLOGY ON *NATURAL AND EXACT SCIENCES*

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