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Transcriptional and Translational Regulation of Brain-Derived Neurotrophic Factor

Jürgen Tuvikene

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

Jürgen Tuvikene

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Aju päritolu neurotroofse teguri transkriptsiooni ja translatsiooni regulatsioon

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AP-1 Transcription Factors Mediate BDNF-Positive Feedback Loop in Cortical Neurons.
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III Koppel I, Jaanson K*, Klasche A*, **Tuvikene J***, Tiirik T, Pärn A, Timmusk T
Dopamine cross-reacts with adrenoreceptors in cortical astrocytes to induce BDNF expression, CREB signaling and morphological transformation.
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IV **Tuvikene J**, Esvald EE*, Rähni A*, Uustalu K*, Zhuravskaya A, Avarlaid A, Makeyev E, Timmusk T
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Author's Contribution to the Publications

Contribution to the papers in this thesis are as follows:

I The author performed part of the experiments and analysed their results. The author participated in editing the manuscript.

II The author planned all and performed the majority of the experiments. The author analysed all the results. The author wrote the manuscript draft and final manuscript.

III The author planned and performed part of the experiments and analysed their results. The author participated in editing the manuscript.

IV The author conceptualised the idea, planned all the experiments, performed part of the experiments, wrote the manuscript draft and final manuscript.

Introduction

Brain derived neurotrophic factor (BDNF), the second member of the neurotrophin family discovered in 1980s, has important roles in the proper functioning of the nervous system. BDNF is a potent survival factor for various neuron populations during the development of the nervous system, and is one of the key proteins in the regulation of synaptic plasticity in the adult organism. The expression of BDNF is strongly linked to neuronal activity, allowing activity-dependent refinement of neuronal morphology and synaptic connections – the molecular basis of memory. BDNF has an important role in the formation and active shaping of neuronal networks, leading to higher cognitive functions and complex behaviours. Dysregulation of BDNF expression and signalling have been implied in various disorders affecting the nervous system, including neuropsychiatric and neurodegenerative diseases. Deeper knowledge of the regulatory mechanisms of BDNF expression is necessary for understanding the basic principles of the functioning of the nervous system in both health and disease, allowing intelligent design of pharmaceuticals for treating various diseases affecting the nervous system.

In the current thesis, we have studied the transcriptional and posttranscriptional regulation of the BDNF gene expression. We provide novel mechanistic insight into the BDNF expression in both neurons and astrocytes and report novel translational mechanisms governing BDNF expression. These findings add insight into how BDNF is regulated to carry out its complex biological functions.

Abbreviations

AAV	adeno-associated virus
BAC	bacterial artificial chromosome
BDNF	brain-derived neurotrophic factor
ChIP	chromatin immunoprecipitation
CRISPRa	CRISPR activation
CRISPRi	CRISPR interference
DA	dopamine
EMSA	electrophoretic mobility shift assay
eRNA	enhancer RNA
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
NGF	nerve growth factor
NE	norepinephrine
NT3	neurotrophin 3
NT4/5	neurotrophin 4/5
PI-3K	phosphatidyl inositol-3-kinase
PLC	phospholipase C
p75NTR	p75 neurotrophin receptor
qPCR	quantitative polymerase chain reaction
RT-qPCR	reverse transcription qPCR
TrkB	tropomyosin receptor kinase B
UTR	untranslated region

1 Review of the Literature

The brain of an adult human contains approximately ~90 million neurons, together with about the same amount of non-neuronal supporting cells (Azevedo et al., 2009; Bartheld et al., 2016). During the development, many more neurons are generated and only a selection of neurons that have properly formed synaptic connections and receive sufficient trophic support will survive (Davies, 1988; Barde, 1989). As each neuron can form connections with tens to thousands of different cells, the number of synaptic connections in the brain is orders of magnitude higher than the number of neurons (Pakkenberg et al., 2003). All these connections need to be reliably regulated to form functional networks for the proper functioning of the nervous system. One of the key regulators of neuronal survival and synaptic connectivity are signalling proteins from the neurotrophin family (Chao, 2003). In mammals, the neurotrophin family consists of four members – nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3) and neurotrophin 4/5 (NT4/5). The second member of the neurotrophin family – BDNF – was discovered in late 1970s and early 1980s (Barde et al., 1978, 1980, 1982). Since then, the crucial role of BDNF in the development and maintenance of the nervous system has been studied in detail. In the following chapters, an overview of various aspects of BDNF biology, including transcriptional and translational regulation of BDNF, its functions, and others is given.

1.1 Functions of BDNF

BDNF has many important functions both during the development of an organism and in adulthood. In the current chapter, the main functions of BDNF are elaborated in more detail.

BDNF as a survival factor. BDNF is an important neurotrophin for survival of different neuronal populations in the developing nervous system (Barde, 1989). Application of exogenous BDNF protein promotes survival of various neurons both *in vitro* (Lindsay et al., 1985; Alderson et al., 1990; Hyman et al., 1991) and *in vivo* (Hofer and Barde, 1988). The importance of endogenous BDNF in cell survival *in vivo* is exemplified by the fact that majority of homozygous BDNF knock-out animals die within 2 days after birth, with some animals surviving up to 2-4 weeks (Jones et al., 1994). This premature death seems to originate from eating deficits due to the massive loss of sensory neurons, as BDNF is a survival factor for various spinal and cranial sensory neuron populations during the development of the nervous system (Ernfors et al., 1994; Jones et al., 1994). BDNF is also necessary for neuronal activity-dependent survival of neurons (Ghosh et al., 1994; Koh et al., 1995; Marini et al., 1998) and protects neurons under excitotoxic conditions (Cheng and Mattson, 1994). However, although BDNF can protect neurons from apoptosis, prolonged stress conditions can still lead to necrosis (Koh et al., 1995). In some cases, BDNF can also potentiate neuronal sensitivity to excitotoxicity (Fryer et al., 2000; Hu and Kalb, 2003).

BDNF as a differentiation factor. BDNF also acts as a differentiation factor in the nervous system. BDNF can induce differentiation of neural stem cells/neural progenitor cells into both neurons and astrocytes, depending on the receptor used for signalling (Ito et al., 2003; Cheng et al., 2007). BDNF has also been widely implicated in the maturation of

GABAergic neurons (Pappas and Parnavelas, 1997; Yamada et al., 2002; Sakata et al., 2009; Park and Poo, 2013). BDNF can also facilitate adult neurogenesis (Zigova et al., 1998; Choi et al., 2018), although there is also conflicting evidence reporting that BDNF does not to participate in adult neurogenesis (Galvão et al., 2008).

Modulating synapses and synaptic connectivity. Correct BDNF expression is crucial for the proper development of neurites, synapses and synaptic connections between neurons (reviewed in Park and Poo, 2013). BDNF modulates the formation of functional synapses between neurons (Vicario-Abejón et al., 1998, 2002). As the expression and secretion of BDNF are both induced by neuronal activity (West et al., 2014), BDNF can modulate synaptic signalling in an activity-dependent manner (Lu, 2003). Importantly, BDNF regulates proper maturation of inhibitory neurons and thus participates in regulating the excitatory-inhibitory balance in the nervous system (Huang et al., 1999). Furthermore, the neuronal activity-dependent induction of BDNF exon IV-containing transcripts has been shown to be required for this process (Hong et al., 2008). BDNF also modulates synaptic transmission between neurons, mainly through presynaptic mechanisms (Tyler et al., 2002). For example, BDNF regulates the probability of neurotransmitter release from the presynaptic terminus (Bradley and Sporns, 1999; Tyler et al., 2006). Furthermore, BDNF can also elicit fast changes in neurotransmitter release and membrane conductance (Numakawa et al., 1999, 2000, 2001; Jovanovic et al., 2000; Matsumoto et al., 2001; Blum et al., 2002) and facilitates glutamatergic transmission (Lemann et al., 1994).

BDNF has been shown to extensively regulate outgrowth and morphology of both axon (Cohen-Cory and Fraser, 1995; Song et al., 1997; Gallo and Letourneau, 1998; Jeanneteau et al., 2010) and dendrites (Wilson Horch et al., 1999; Danzer et al., 2002; Horch and Katz, 2002; Tanaka et al., 2008). However, the effect of BDNF on neurite morphology can depend on the cell type, as it has been shown that glutamatergic but not GABAergic cortical neurons need presynaptic BDNF for dendrite outgrowth (Kohara et al., 2003).

Synaptic plasticity and memory. In the adult organism/nervous system, one of the main roles of BDNF is regulating synaptic plasticity (reviewed in Park and Poo, 2013). It has been reported that homozygous BDNF knock-out mice show strongly impaired long-term potentiation (LTP) in the hippocampus (Korte et al., 1995) and applying exogenous BDNF rescues this deficit, indicating that the role of BDNF in LTP is acute, rather than arising from developmental changes in the brain (Patterson et al., 1996). The expression of BDNF is strongly induced upon neuronal activity (Zafra et al., 1990), providing the necessary signals for strengthening synaptic connections upon neuronal activity. BDNF itself can elicit LTP of hippocampal synapses (Kang and Schuman, 1995), but is also required for neuronal-activity dependent long-term strengthening of the synapses (Korte et al., 1995; Figurov et al., 1996; Patterson et al., 1996).

It has also been reported that BDNF is both necessary and sufficient for proper formation of long-term memories in mice (Bekinschtein et al., 2007, 2008; Cunha et al., 2010). For instance, mice with conditional BDNF knockout in the forebrain (Gorski et al., 2003) and hippocampus (Heldt et al., 2007) exhibit poorer spatial learning and memory, whereas such effect is not evident in BDNF heterozygous mice (Montkowski and Holsboer, 1997). BDNF is also necessary for proper aversive and appetitive learning in the amygdala (Heldt et al., 2014). The role of BDNF in memory functions has also been

reported in humans. For instance, a common polymorphism in the BDNF coding region that results in Val66Met amino acid substitution has been associated with poorer memory (Egan et al., 2003; Hariri et al., 2003; see also Chapter 1.3).

In addition to directly modulating synaptic connectivity, BDNF also participates in regulating neuronal activity-dependent myelination (Lundgaard et al., 2013; Geraghty et al., 2019). As neuronal activity-dependent myelination has been shown to be crucial for various memory functions (Gibson et al., 2014; McKenzie et al., 2014; Baraban et al., 2018; Pan et al., 2020), it is plausible these effects are, at least in part, conveyed by BDNF.

BDNF in neuropsychiatric, neurodevelopmental and neurodegenerative diseases. Dysregulation of BDNF expression and/or signalling has been implied in various neuropsychiatric and neurodevelopmental diseases (Autry and Monteggia, 2012), including major depression (reviewed in Jiang and Salton, 2013; Castrén and Kojima, 2017), autism spectrum disorders (Skogstrand et al., 2019), schizophrenia (Wong et al., 2010; Ray et al., 2014), bipolar disorder (Neves-Pereira et al., 2002; Okada et al., 2006), and obsessive-compulsive disorder (Hall et al., 2003a). BDNF is thought to be one of the main mediators of antidepressant effects in treating depression (Kozisek et al., 2008; Björkholm and Monteggia, 2016; Castrén and Kojima, 2017). BDNF levels are also changed in Rett syndrome (Chang et al., 2006; Wang et al., 2006; Rousseaud et al., 2015). Altered BDNF expression levels have also been shown in various neurodegenerative diseases, including Huntington's disease (Zuccato et al., 2001), Alzheimer's disease (Murray et al., 1994; Burbach et al., 2004), and amyotrophic lateral sclerosis (Nishio et al., 1998). Due to its role in various diseases and its potency to keep neurons alive, BDNF and its small-molecule mimetics have been proposed as potential drug candidates for various neuropsychiatric and neurodegenerative diseases (Nagahara and Tuszynski, 2011; Allen et al., 2013).

Energy metabolism. BDNF is an important mediator of organism energy balance. Heterozygous BDNF knock-out mice show severe late-onset obesity (Lyons et al., 1999), originating from both dysregulated food intake (hyperphagia) and impaired energy expenditure (Kernie et al., 2000; Liao et al., 2012; Fox et al., 2013; An et al., 2015). Nowadays, the role of BDNF in metabolism has been pinpointed to BDNF functions in the hypothalamic region (Unger et al., 2007; An et al., 2015), where BDNF acts downstream of the melanocortin-4 receptor signalling pathway (Xu et al., 2003). Hypothalamic BDNF is also associated with the regulation of thermogenesis (You et al., 2020). BDNF can also participate in metabolism in a central nervous system-independent manner by directly regulating insulin secretion from pancreatic β-cells (Fulgenzi et al., 2020).

The crucial role of BDNF in energy homeostasis also holds true in humans. It has been reported that a subpopulation of WAGR syndrome patients who have lost one allele of BDNF gene locus show obesity (Han et al., 2008). Furthermore, a SNP (rs12291063) found in the intronic region of the BDNF gene has been associated with lower BDNF expression in the hypothalamic region and a higher body mass index (Mou et al., 2015). Several other SNPs in BDNF gene have also been associated with various eating disorders, e.g. bulimia and anorexia (Ribasés et al., 2003, 2004). Severe early-onset obesity has been described in patients missing the BDNF gene locus (Harcourt et al., 2018), and in patients carrying mutations in the gene of TrkB – the receptor of BDNF (Yeo et al., 2004; Gray et al., 2007).

Pain. BDNF is an important modulator of pain sensitivity (reviewed in Pezet and McMahon, 2006). As such, BDNF signalling is associated with increased pain sensitivity (Sapio et al., 2019; Wang et al., 2019), chronic pain (Sikandar et al., 2018) and neuropathic pain (Coull et al., 2005; Chen et al., 2014). BDNF haploinsufficiency has been shown to increase pain sensitivity also in humans with WAGR syndrome (Sapio et al., 2019). To this end, inhibitors of BDNF-dependent signalling have been proposed as potential painkillers (Shih et al., 2017).

Drug addiction. As BDNF regulates the plasticity of brain reward circuits, it is not surprising that BDNF is associated with drug abuse and addiction (Ghitza et al., 2010). BDNF can also directly modulate the dopaminergic reward system by regulating the expression levels of dopaminergic receptors in the nucleus accumbens (Guillin et al., 2001). In humans, various polymorphisms in and near the BDNF gene are associated with vulnerability to substance abuse (Uhl et al., 2001; Cheng et al., 2005; Jia et al., 2011; Haerian, 2013).

BDNF is associated with positive reward feeling after cocaine use (Hall et al., 2003b) and heterozygous BDNF knockout mice show decreased rewarding effect of cocaine and decreased cocaine-seeking behaviour (St. Laurent et al., 2013). It has been shown that infusion of BDNF into nucleus accumbens increases behavioural sensitization to cocaine (Horger et al., 1999). BDNF is also required for synaptic sensitization after cocaine withdrawal (Pu et al., 2006; Lu et al., 2010). Increased BDNF expression and secretion in the neurons of nucleus accumbens is necessary for the persistence of cocaine addiction and increases the chances of relapsing (Graham et al., 2007). BDNF is also associated with opiate withdrawal symptoms (Akbarian et al., 2002) and behavioural changes after methamphetamine withdrawal (Ren et al., 2015). Furthermore, BDNF negatively regulates reward from morphine use (Koo et al., 2012).

Behaviour. BDNF regulates the proper functioning of circuits that give rise to various aspects of behaviour, including aggression (Lyons et al., 1999; Maynard et al., 2016), and sexual and maternal behaviour (Maynard et al., 2018). Alterations in BDNF expression and signalling have been associated with autism spectrum disorders (Garcia et al., 2012; Zheng et al., 2016; Skogstrand et al., 2019). Loss of BDNF causes hyperactivity in both mice (Jones et al., 1994; Rios et al., 2001) and humans (Gray et al., 2006).

1.2 Gene structure of the BDNF gene

In rodents, BDNF gene consists of eight 5' non-coding exons (exons I to VIII) and one 3' protein-coding exon (exon IX). In each transcript, one of the 5' exons is spliced together with the coding exon. In addition, transcription can start from the intron in front of exon IX, giving rise to an extended version of the coding exon (exon IXa) (Aid et al., 2007). In humans, two additional exons (Vh and VIIIh) have been described (Pruunsild et al., 2007). Additionally, in humans transcripts can be formed by splicing several of the 5' non-coding exons together, although this happens at a relatively low frequency (Pruunsild et al., 2007).

Each 5' exon has its own promoter, allowing precise spatiotemporal control of the BDNF gene expression at the transcriptional level throughout the lifespan of an organism and upon various stimuli. Alternative polyadenylation signals in exon IX can be used, yielding BDNF transcripts with either short or long 3' untranslated region (UTR) (Timmusk et al., 1993; Will et al., 2013). It has been shown that BDNF transcripts with the long

3’ UTR are less stable than those with short 3’ UTR and show increased localization to dendrites (Will et al., 2013). Furthermore, BDNF transcripts with long 3’ UTR can be stabilized upon external stimuli, e.g. neuronal activity (Fukuchi and Tsuda, 2010).

In rodents and human, exon I contains an in-frame translation start codon ATG, and these transcripts possibly yield BDNF pre-pro-protein with an extended pre region (Aid et al., 2007; Pruunsild et al., 2007). In humans, BDNF exons VII and VIII also have an in-frame ATG. However, whether/how these alternative start codons influence BDNF expression and function is currently unknown. Jiang et al 2009 (Jiang et al., 2009) have reported that in human BDNF the alternative N-termini arising from the usage of exon I and VII could alter activity-dependent secretion of BDNF and negate the negative effect of Val66Met polymorphism on the activity-dependent secretion of BDNF protein.

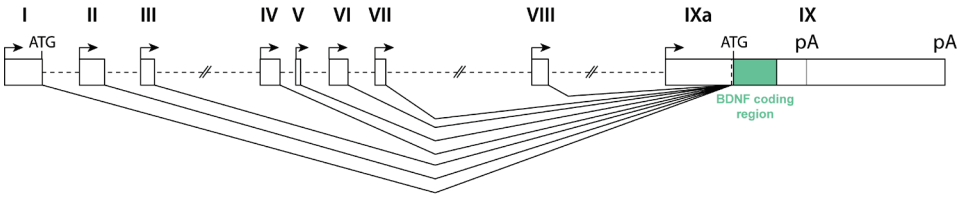

Figure 1: Murine BDNF gene structure and generated transcripts. Exons are shown as boxes, introns as dashed line, vertical dashed line in exon IX indicates splice acceptor site. Roman numerals above boxes show exon numbers. ATG indicates translation initiation codon. pA indicates polyadenylation sites. Transcription start sites are marked with arrows, possible splicing patterns are indicated with solid lines. BDNF coding region is marked green.

While the role of BDNF different 3’ UTRs has been established, the biological meaning of so many different 5’ exons remains obscure, considering that the protein encoded by different transcripts is (in almost all the cases) the same. As the initiation of transcription from each 5’ exon could happen independently and through specific regulatory mechanisms, such gene structure possibly provides spatiotemporal fine-tuning for BDNF expression, and to date, numerous specific functions for the BDNF 5’ exons has been found. For instance, it has been shown that neuronal activity-dependent induction of BDNF exon IV-containing transcripts is necessary for proper maturation of GABAergic neurons (Hong et al., 2008).

Using exon-specific BDNF knock-out mice, it has been reported that:

1) BDNF exon I-containing transcripts are required for proper sexual and maternal behaviour in female mice (Maynard et al., 2018);
2) BDNF exon I-containing transcripts in the hypothalamus participate in metabolism and thermoregulation (You et al., 2020);
3) male mice with knock-out of BDNF exon I or II-containing transcripts show extreme aggressive behaviour (Maynard et al., 2016);
4) exon IV-containing transcripts are required for proper GABAergic/inhibitory signalling and synaptic plasticity (Sakata et al., 2009);
5) BDNF exon IV-containing transcripts regulate GABAergic signalling and sleep (Martinowich et al., 2011)
6) BDNF exon IV-containing transcripts participate in sleep homeostasis and fear extinction (Hill et al., 2016);

7) BDNF exon IV-containing transcripts are required for proper formation of LTP and memory extinction (Sakata et al., 2013);
8) BDNF exon IV-containing transcripts regulate cholinergic transmission (Sakata and Overacre, 2017);
9) the lack of BDNF exon IV-containing transcripts promotes depressive-like behaviour (Sakata et al., 2010);
10) BDNF exon IV and VI-containing transcripts are required for proper GABAergic transmission (Maynard et al., 2016);
11) various BDNF transcripts differentially affect dendrite morphology in the hippocampus (Maynard et al., 2017).

Furthermore, it has also been reported that individual BDNF transcripts differentially contribute to the total pool of BDNF protein expression in different brain regions (Maynard et al., 2016). Therefore, it is plausible that different BDNF transcripts are expressed in different subpopulations of neurons in different brain regions, thereby regulating the plasticity of specific neural circuits and various aspects of behaviour. However, direct information of the spatial and cell type-specific expression pattern of different BDNF transcripts at a single-cell level is currently lacking.

1.3 Synthesis, processing and secretion of BDNF

BDNF is synthesised as a pre-pro-precursor protein at the rough endoplasmic reticulum (ER). The pre-region is a signal peptide for translocating the nascent BDNF polypeptide into the ER lumen, and is cleaved co-translationally upon translocation into the ER, yielding proBDNF (Lu et al., 2005). The synthesised BDNF forms strongly associated non-covalent homodimers in the ER (Radziejewski et al., 1992; Lessmann et al., 2003). It has also been shown that in overexpression conditions, BDNF can form heterodimers with other neurotrophins produced in the same cell (Radziejewski and Robinson, 1993; Heymach and Shooter, 1995). However, whether such heterodimers between different neurotrophins form also under physiological conditions *in vivo* is not known.

proBDNF can be cleaved to mature BDNF intracellularly by proprotein convertases (Seidah et al., 1996) and furin (Mowla et al., 2001), or in the extracellular space by plasmin and matrix metalloproteinases (Lee et al., 2001). It has been reported that processing of proBDNF to mature BDNF in the extracellular space is facilitated by neuronal activity (Nagappan et al., 2009). The activity of the tPa system is also under the control of neuronal activity, and processing of proBDNF to mature BDNF by plasmin is necessary for formation of LTP in the hippocampus (Pang et al., 2004). It has also been reported that BDNF, tPA and plasminogen could be colocalized into the same dense core vesicle (Lochner et al., 2008), opening the possibility that proBDNF is cleaved by plasmin also within the cell.

BDNF can be targeted into either constitutive or regulated secretory pathway (Lu et al., 2005). This targeting is dependent on both the interaction between BDNF pro-region and sortilin (Chen et al., 2005), and BDNF mature region and carboxypeptidase E (Lou et al., 2005). BDNF is secreted from neurons mainly through the regulated secretory pathway in a neuronal activity-dependent manner (Balkowiec and Katz, 2000). In addition to membrane depolarization and increase in cytoplasmic Ca^{2+} levels, BDNF can also be secreted in response to the activation of metabotropic GABA receptors in inhibitory synapses (Kuczewski et al., 2008, 2011) and cAMP/protein kinase A signalling (Cheng et al., 2011). Furthermore, it has been shown that different populations of secretory vesicles are used to transport and secrete BDNF in axons and dendrites (Dean et al.,

2012). In addition to neurons, BDNF is also produced and secreted by other cell types in the central nervous system, such as oligodendrocytes (Dougherty et al., 2000; Dai et al., 2003; Bagayogo and Dreyfus, 2009), microglia (Nakajima et al., 2001, 2002; Coull et al., 2005) and astrocytes (see Chapter 1.8).

A common polymorphism in the BDNF gene (rs6265) – causing Val66Met substitution in the BDNF pro domain, has been shown to disturb the interaction between proBDNF and sortilin, leading to decreased activity-dependent secretion of BDNF (Egan et al., 2003; Chen et al., 2004, 2005). BDNF Val66Met polymorphism knock-in mice exhibit increased anxiety (Chen et al., 2006), impaired aversive memory extinction learning (Yu et al., 2009; Soliman et al., 2010), increased drug reward extinction learning (Briand et al., 2012), and impaired synaptogenesis (Liu et al., 2012) and adult neurogenesis (Bath et al., 2008). The data obtained with Val66Met knock-in rodent animal models is reinforced by genetic association studies in humans, which have indicated that carriers of BDNF Val66Met allele show decreased memory performance (Egan et al., 2003; Hariri et al., 2003; reviewed in Dincheva et al., 2012), poorer short-term motor learning (McHughen et al., 2010), altered stress and reward responses (Peciña et al., 2014), and increased predisposition to eating disorders (Ribasés et al., 2003) and depression (Verhagen et al., 2010).

How much proBDNF is secreted from neurons under physiological conditions *in vivo* has been under debate for a long time and conflicting results exist. Matsumoto et al showed that neurons in the central nervous system secrete only mature BDNF, and no evidence was found for the secretion of proBDNF (Matsumoto et al., 2008). However, a year later two other groups reported secretion of proBDNF in addition to mature BDNF (Nagappan et al., 2009; Yang et al., 2009b). In the last decade, it has been shown that while very little proBDNF is stored in the secretory granules, the cleaved pro peptide of BDNF colocalizes with mature BDNF in the secretory vesicles and is also secreted (Dieni et al., 2012; Hempstead, 2015). It has been reported that the BDNF pro-peptide itself can signal similarly to proBDNF (see Chapter 1.4), causing retraction of axon growth cone (Anastasia et al., 2013), reducing the number of dendritic spines (Guo et al., 2016; Giza et al., 2018), inducing long-term depression in the hippocampus (Mizui et al., 2015) and altering fear-related neuronal circuitry *in vivo* (Giza et al., 2018). Interestingly, these effects of BDNF pro-peptide seem to be strongly influenced by the Val66Met polymorphism (see the previous references).

Whether BDNF is secreted from pre- or postsynaptic neurons has also been a question of active research, with conflicting evidence on where BDNF is localized in neurons (reviewed in Song et al., 2017). Using immunolabelling and electron microscopy, Dieni et al (Dieni et al., 2012) have reported that BDNF localizes mainly presynaptically in the hippocampus in vivo, with no detectable BDNF found in dendrites. In contrast, Harward et al have shown BDNF protein in both axonal and dendritic compartments (Harward et al., 2016). Experiments using BDNF-GFP knock-in mice have indicated that the majority (~70%) of BDNF localizes to dendrites, while axonal localization (~30%) is also present (Leschik et al., 2019). Transport of BDNF mRNA to dendrites for local translation has also been reported (An et al., 2008a; Will et al., 2013), further implying that BDNF could be secreted from the postsynaptic compartments. To date, it seems evident that BDNF is secreted from both pre- and postsynaptic neurons (Song et al., 2017) and that BDNF secretion from presynaptic termini needs higher frequency electrical stimulation than from postsynaptic termini (Matsuda et al., 2009).

1.4 BDNF-dependent signalling pathways

BDNF can bind to two types of receptors, namely Tropomyosin receptor kinase B (TrkB), a member of the receptor tyrosine kinase superfamily, and p75 neurotrophin receptor (p75NTR), a member of the tumour necrosis factor superfamily (Reichardt, 2006).

BDNF and TrkB. Mature BDNF preferentially binds to TrkB (Reichardt, 2006). According to the classical model of BDNF-TrkB signalling, the binding of BDNF causes receptor dimerization and auto-phosphorylation of the intracellular kinase domain (Reichardt, 2006). By recruiting different adapter proteins to the phosphorylated tyrosine residues, several intracellular signalling cascades are started. The three main pathways activated downstream of TrkB receptor are: 1) mitogen-activated protein kinase (MAPK) pathway; 2) phosphatidyl inositol-3-kinase (PI3K)-Akt pathway; 3) phospholipase C-γ1 (PLCγ1)-diacylglycerol (DAG)-inositol tris-phosphate (IP3) pathway (Reichardt, 2006). Activation of MAPK pathway is generally associated with changes in transcription, PI3K-Akt pathway with increased cell survival (Reichardt, 2006), and PLCγ1-DAG-IP3 pathway with the regulation of synaptic plasticity (Minichiello et al., 2002; Gruart et al., 2007; Minichiello, 2009). TrkB signalling can also regulate the activity of Rho GTPases to regulate cytoskeleton organization (Reichardt, 2006). TrkB signalling regulates neuronal morphology, axonal and dendritic growth through regulating the dynamics of both microtubules and actin, providing a basis for structural plasticity after TrkB signalling (Gonzalez et al., 2016).

In synapses, TrkB localizes to both pre- and postsynaptic membrane, allowing secreted BDNF to signal bidirectionally to both pre- and postsynaptic neuron (Gomes et al., 2006). Binding of BDNF to TrkB induces endocytosis of the ligand-receptor complex, and retrograde transport to convey the signal to the cell body and nucleus by the means of signalling endosome (Bronfman et al., 2007, 2014; Harrington and Ginty, 2013; Gonzalez et al., 2016). Decrease of cell-surface TrkB expression and thereafter proteasomal TrkB degradation helps to attenuate TrkB signalling even upon continuous presence of BDNF protein in the environment (Frank et al., 1996; Sommerfeld et al., 2000). It has been reported that acute treatment of neurons with BDNF causes transient activation of TrkB signalling that is quickly attenuated by a decrease of cell-surface expression of TrkB. In contrast, gradually increasing BDNF concentration causes retention of TrkB on the cell membrane and sustained TrkB signalling (Ji et al., 2010; Guo et al., 2018). Furthermore, neuronal activity prior to BDNF-Trk signalling can also elicit sustained TrkB signalling (Guo et al., 2014). Acute and sustained TrkB signalling differentially affect synaptic transmission and dendrite morphology (Ji et al., 2010; Guo et al., 2018).

In addition to the classical model of ligand-mediated dimerization of TrkB, it has been reported that TrkB could exist as preformed dimers at the plasma membrane even without a ligand (Shen and Maruyama, 2012). Recently, it has been reported that TrkB can also signal as a monomer at the plasma membrane and that dimers are formed only after internalization of the receptor (Zahavi et al., 2018). Furthermore, it has been shown that activated monomeric TrkB signals through the MAPK pathway, while endosomal TrkB dimers signal through PI3K-Akt pathway (Zahavi et al., 2018).

A truncated form of TrkB, missing the intracellular kinase domain, named TrkB.T1 has been reported (Klein et al., 1990). Originally, this receptor isoform was considered to be dominant-negative for BDNF-dependent signalling (Eide et al., 1996). Since then, however, a multitude of evidence has accumulated showing that TrkB truncated receptor

variants are able to convey signals into the cell (reviewed in Fenner, 2012). For example, TrkB.T1 has been shown to induce Ca^{2+}-signalling in astrocytes (Rose et al., 2003).

TrkB is also the cognate receptor for NT4/5 (Berkemeier et al., 1991; Ip et al., 1992; Klein et al., 1992), and the downstream signalling pathways of TrkB activation could differ depending on the ligand (Minichiello et al., 1998; Fan et al., 2000). For instance, while TrkB signalling through the Shc adaptor is required for NT4/5-dependent cell survival, removing the site for this adaptor only modestly affects BDNF-dependent cell survival (Minichiello et al., 1998). In addition to BDNF and NT4/5, NT3 can bind and activate TrkB, although with lower efficiency (Klein et al., 1991; Soppet et al., 1991; Squinto et al., 1991; Strohmaier et al., 1996).

The importance of TrkB *in vivo* is exemplified by the severe phenotype of TrkB knock-out animals. Notably, most of homozygous TrkB-TK knock-out mice, harbouring a deletion of the tyrosine kinase domain of the TrkB, die within first postnatal week, possibly due to feeding deficits (Klein et al., 1993) much alike BDNF knock-out animals (Ernfors et al., 1994; Jones et al., 1994). TrkB-TK knock-out animals fail to respond to different external stimuli and show severe reduction in numbers of neurons in various parts of the peripheral nervous system, including trigeminal, nodose and petrosal ganglia, facial motor nucleus and dorsal root ganglia (Klein et al., 1993; Barbacid, 1994). Furthermore, in humans a *de novo* mutation in the TrkB gene, impairing the activation of MAPK signalling pathway, has been shown to cause obesity, problems with memory and nociception (Yeo et al., 2004). TrkB also has functions outside the central nervous system. For example, TrkB knock-out mice exhibit severe defects in the formation of heart microvasculature (Anastasia et al., 2014). Overactive TrkB signalling is associated with various forms of cancers (DeWitt et al., 2014; Cocco et al., 2018; Gatalica et al., 2019; Joshi et al., 2019).

BDNF and p75NTR. Pro-neurotrophins, including proBDNF preferentially bind to the low-affinity neurotrophin receptor p75NTR (Reichardt, 2006). Activation of p75NTR generally leads to the activation of three main pathways: 1) nuclear factor-κB (NFκB) pathway, associated with increased cell survival; 2) Jun N-terminal kinase pathway, often associated with apoptosis; 3) Rho-GTPases, associated with cytoskeleton remodelling (Reichardt, 2006). Remarkably, signalling through p75NTR by proBDNF often elicits results that are diametrically different to those of TrkB signalling (Lu et al., 2005). P75NTR has been shown to interact with TrkB receptor, increases its affinity for BDNF but not NT4/5 and NT3, and modulates the signalling through TrkB receptor (Bibel et al., 1999; Vesa et al., 2000).

proBDNF signalling through p75NTR causes neuronal apoptosis (Bamji et al., 1998; Teng et al., 2005; Taylor et al., 2012), long-term depression of synaptic connectivity (Woo et al., 2005), and reduction of neurite outgrowth (Zagrebelsky et al., 2005; Yang et al., 2009a; Sun et al., 2012). However, the effects of p75NTR signalling strongly depend on the exact cellular context and co-receptors of p75NTR. For example, pro-survival signalling through p75NTR has also been reported (DeFreitas et al., 2001). proBDNF signalling through p75NTR decreases excitability of cortical pyramidal neurons (Gibon et al., 2015, 2016), whereas p75NTR signalling has also been described to maintain depolarizing GABA currents during the development and thus increases general excitability of the neuronal network (Riffault et al., 2018).

P75NTR knock-out animals show decreased sympathetic innervation in the peripheral nervous system (Lee et al., 1992, 1994; Stucky and Koltzenburg, 1997). In the central

nervous system, knockout of p75NTR causes increased apoptosis of neural progenitor cells, and loss of neurons in various parts of the brain, including cortex and striatum (Peterson et al., 1997; Meier et al., 2019). It should be noted that the effects seen in these knock-out animals could be attributed to p75NTR-dependent signalling by other neurotrophins, i.e. NGF, NT3, NT4/5 in addition to BDNF.

1.5 Autocrine actions of BDNF

BDNF, having a relative high pI (~10) (Barde et al., 1982), is positively charged under physiological conditions, restricting long-distance diffusion of BDNF protein in the extracellular space. Therefore, secreted BDNF is expected to mainly act on the same or neighbouring cells in an autocrine or paracrine manner, respectively, in a site-specific way. In most brain regions, at least some subpopulations of neurons express both BDNF and its receptor TrkB (Kokaia et al., 1993; Miranda et al., 1993). Co-expression of BDNF and TrkB mRNA, mainly in glutamatergic neurons, has also been reported using single-cell RNA-sequencing methods (Tasic et al., 2016, 2018), suggesting autocrine BDNF-TrkB signalling could be relevant for the proper functioning of excitatory neurons.

To date, various effects of autocrine BDNF signalling have been reported. For example, autocrine BDNF-TrkB signalling increases neuronal survival in both peripheral and central nervous system (Acheson et al., 1995; Davies and Wright, 1995; Hansen et al., 2001; Jiang et al., 2005). Autocrine actions of BDNF also regulate neuronal morphology, being necessary for proper axon formation (Cheng et al., 2011) and dendritic outgrowth (Wirth et al., 2003). Autocrine BDNF-TrkB action after glutamate signalling at the level of a single dendritic spine has been shown to be important for both functional and structural synaptic plasticity (Harward et al., 2016).

BDNF-TrkB signalling has also been reported to induce transcription of BDNF mRNA itself in cultured neurons (Yasuda et al., 2007; Zheng and Wang, 2009; Nakajima et al., 2015; Esvald et al., 2020) and also *in vivo* in the dentate gyrus after BDNF-induced LTP (Wibrand et al., 2006; Esvald et al., 2020). BDNF-TrkB transcriptional feedback loop has been implicated in memory consolidation in inhibitory avoidance paradigm (Bambah-Mukku et al., 2014). Using TrkB knockout mice, it has been shown that TrkB signalling participates in kainate-induced BDNF mRNA induction (Saarelainen et al., 2001), indicating that BDNF-TrkB signalling could contribute to the upregulation of BDNF expression *in vivo* after neuronal activity. Silencing of TrkB expression also decreases BDNF mRNA levels in zebrafish (Sahu et al., 2019), suggesting BDNF transcriptional autoregulation also exists in other vertebrates in addition to mammals. Impairing retrograde transport of BDNF-TrkB complexes to the cell soma decreases BDNF mRNA expression, suggesting a role of retrograde transport in BDNF transcriptional autoregulation (Negrete-Hurtado et al., 2020). In addition to transcriptional positive feedback loop, it has been suggested that BDNF-TrkB signalling could increase local translation of BDNF mRNA (Leal et al., 2017). It has also been reported that neurotrophin-dependent signalling could induce BDNF secretion (Krüttgen et al., 1998), further reinforcing BDNF autocrine signalling.

1.6 Transcriptional regulation of the BDNF gene expression

Since the discovery of BDNF almost 40 years ago, numerous stimuli and transcription factors have been identified in the regulation of BDNF gene expression (reviewed in West et al., 2014). In neurons, the expression of BDNF is strongly induced in response to neuronal activity through the activation of kainate, AMPA and NMDA-type glutamate receptors (Zafra et al., 1990, 1991; Metsis et al., 1993); increase in intracellular Ca^{2+} levels through voltage-gated calcium channels (Zafra et al., 1990; Ghosh et al., 1994); activation of GABA receptors (Berninger et al., 1995); increase in intracellular cAMP levels (Benito et al., 2011; Nakajima et al., 2015); activation of TrkB signalling (Yasuda et al., 2007; Zheng and Wang, 2009; Nakajima et al., 2015). *In vivo*, the expression of BDNF is also upregulated in response to various stimuli, including fear memory consolidation in the amygdala (Rattiner et al., 2004a, 2004b), hippocampal contextual learning (Hall et al., 2000), formation of social recognition memory (Broad et al., 2002), environmental enrichment (Falkenberg et al., 1992; Young et al., 1999), brain injury (Ballarín et al., 1991; Hughes et al., 1993; Yang et al., 1996; Hicks et al., 1997), physical activity (Neeper et al., 1995, 1996), exposure to behavioural tests (Falkenberg et al., 1992), kindling with electricity (Ernfors et al., 1991), visual stimulus (Castrén et al., 1992), and others (reviewed in West et al., 2014).

In the following sections, a short overview of the major currently known transcription factors regulating the BDNF gene expression is given.

BDNF promoter I. BDNF exon I-containing transcripts show mainly neural expression patterns (Aid et al., 2007; Pruunsild et al., 2007). While the basal expression levels of BDNF exon I-containing transcripts in neurons is much lower than of BDNF exon IV-containing transcripts, the expression of exon I-containing transcripts is greatly increased in response to neuronal activity (Metsis et al., 1993; Timmusk et al., 1993; Pruunsild et al., 2011). It has been reported that CREB, binding to a conserved CRE element in BDNF promoter I regulates the induction of BDNF exon I-containing transcripts after membrane depolarization (Tabuchi et al., 2002; Pruunsild et al., 2011). There is also a USF binding site overlapping the CRE element (Tabuchi et al., 2002). The USF binding site is not conserved between rodents and humans and it has been reported that USF-s participate in depolarization-induced activation of promoter I in rat (Tabuchi et al., 2002). However, in human BDNF it has been shown that the site binds USF, but only regulates the basal activity and not depolarization-dependent activity of BDNF promoter I (Pruunsild et al., 2011). Neuronal activity-dependent regulation of BDNF promoter I activation is also dependent on NPAS4/Arnt2 dimer binding to the PASRE cis-element (Lin et al., 2008; Pruunsild et al., 2011). Approximately 6.5 kb upstream of BDNF exon I, a MEF2D binding site has been described. This region has been shown to regulate BDNF expression in hippocampal (Flavell et al., 2008) but not in cortical neurons (Lyons et al., 2012), indicating differential regulation of BDNF exon I-containing transcripts in different brain regions by MEF2 family transcription factors.

BDNF promoter II. The expression of BDNF promoter II is mainly restricted to neural tissues, with virtually no expression in non-neuronal tissues (Aid et al., 2007; Pruunsild et al., 2007). One of the most well-described transcription factors regulating the activity of BDNF promoter II is NRSF/REST, binding to the NRSE *cis*-element within BDNF exon II (Palm et al., 1998; Timmusk et al., 1999). While REST is generally thought to repress the

expression of neural genes in non-neural tissues and cells (Schoenherr and Anderson, 1995), it has also been reported to repress the expression of BDNF in neural tissues (Timmusk et al., 1999; Hu et al., 2011; Paonessa et al., 2016). Overactive repression of BDNF promoter II activity by REST has been implicated in the pathophysiology of Huntington's disease (Zuccato et al., 2001, 2003, 2007) and inhibition of REST function has been shown to increase BDNF expression in Huntington's disease models (Soldati et al., 2011; Conforti et al., 2013, 2013).

Conflicting results exist whether REST is the reason for mainly neural expression pattern of BDNF exon II-containing transcripts. Although Timmusk et al reported that mutating the NRSE element does not cause ectopic activation of the BDNF promoter II region in non-neural tissues (Timmusk et al., 1999), it has been shown since then that silencing REST induces the expression of BDNF in the liver in vivo (Sedaghat et al., 2013) and optogenetic inhibition of REST activity increases BDNF expression in N2A cells (Paonessa et al., 2016).

Another transcription factor, SCIRR69, belonging to the CREB/ATF family, regulates the expression of BDNF exon II-containing transcripts upon mechanical injury in neurons, thus possibly participating in repairing and regeneration of damaged neural tissue (Liu et al., 2013).

BDNF promoter IV. BDNF promoter IV is by far the most studied BDNF promoter due to its strong/high expression and inducibility upon various stimuli, including neuronal activity (West et al., 2014). Numerous neuronal-activity regulated cis-elements have been described in BDNF promoter IV and various transcription factors binding to BDNF promoter IV have been identified (West et al., 2014).

Originally, three Ca^{2+}-responsive cis-elements (CaRE1-3) were identified in the BDNF promoter IV (Shieh et al., 1998; Tao et al., 1998, 2002; Chen et al., 2003b). CREB binding to the CRE cis-element (CaRE3) in BDNF promoter IV is the main mediator of depolarization-dependent induction of BDNF exon IV-containing transcripts in neurons (Shieh et al., 1998; Tao et al., 1998; Hong et al., 2008). Furthermore, activity-dependent induction of promoter IV through the CRE site in promoter IV has been shown to be necessary for the proper development of inhibitory neurons (Hong et al., 2008). It has also been suggested that CREB acts as a nucleating factor for other transcription factors binding BDNF promoter IV (Hong et al., 2008). CREB also mediates TrkB signalling-dependent induction of BDNF exon IV-containing transcripts (Esvald et al., 2020).

It has been reported that transcription factor XBP1 could also regulate the expression of BDNF exon IV-containing transcripts after neuronal activity (Martínez et al., 2016; Saito et al., 2018). Interestingly, the cis-element that XBP1 binds to overlaps the CRE element in promoter IV (Martínez et al., 2016; Saito et al., 2018), opening up a possibility of competitive inhibition between CREB and XBP1 in the regulation of BDNF promoter IV activity.

In addition to CREB, neuronal activity-dependent upregulation of BDNF exon IV-containing transcripts has been shown to be directly regulated by calcium-activated transcription factor CaRF through the 3' part of the CaRE1 cis-element (Tao et al., 2002). In CaRF knock-out mice, the levels of BDNF exon IV-containing transcripts are decreased in the cortex, but not in the hippocampus, suggesting brain region-specific regulation of BDNF promoter IV activity by CaRF (McDowell et al., 2010). Another family of neuronal activity-regulated transcription factors – MEF2 family, also regulates the neuronal

activity dependent induction of promoter IV by binding the 5' part of the CaRE1 cis-element (Hong et al., 2008; Lyons et al., 2012). The CaRE2 cis-element binds members of the USF family (Chen et al., 2003b; Pruunsild et al., 2011) to regulate membrane depolarization-dependent induction of the promoter region (Pruunsild et al., 2011).

Numerous other transcription factors have been reported to regulate the activity of BDNF promoter IV. The neuronal activity-dependent expression of BDNF exon IV-containing transcripts is also regulated by NPAS4/Arnt2 dimer binding to the PASRE cis-element in the promoter IV region (Lin et al., 2008; Pruunsild et al., 2011). NFkB binding to BDNF promoter IV has been shown to mediate neuroprotection upon signalling through NMDA receptors (Lipsky et al., 2001). The expression of BDNF exon IV-containing transcripts is repressed by BHLHB2 transcription factor (Jiang et al., 2008). After neuronal activity, the binding of BHLHB2 to BDNF promoter IV decreases and the repression of BDNF promoter IV is relieved (Jiang et al., 2008). Members of the NFAT transcription factor family also regulate the activation of BDNF promoter IV after both neuronal activity (Vashishta et al., 2009) and TrkB signalling (Groth and Mermelstein, 2003; Ding et al., 2018). TrkB signalling-dependent binding of C/EBPβ to BDNF promoter IV has been shown to be required for memory consolidation *in vivo*, although the exact cis-element has not been described (Bambah-Mukku et al., 2014).

The activity of BDNF promoter IV is also regulated through epigenetic mechanisms. Inhibiting endogenous demethylases completely abolishes depolarization-dependent induction of BDNF exon IV-containing transcripts, and targeted demethylation of BDNF promoter IV has been shown to increase the expression of BDNF exon IV-containing transcripts (Liu et al., 2016), indicating the importance of DNA methylation in the regulation of BDNF promoter IV. It has been shown that MeCP2 binds to methylated CpG dinucleotides in BDNF promoter IV to repress the activity of the promoter (Chen et al., 2003a; Martinowich et al., 2003). This repression is relieved upon neuronal activity, mainly through phosphorylation of MeCP2 which helps dissociate MeCP2 from DNA (Chen et al., 2003a), and demethylation of the CpG dinucleotides by chromatin remodelling complexes (Martinowich et al., 2003). It has also been reported that in animal models of Rett syndrome, caused by inactivating mutations in the MeCP2 gene, a decrease in BDNF levels results in an earlier onset of the disease (Chang et al., 2006). Furthermore, overexpressing BDNF in MeCP2-deficient animals delays the onset of the disease and increases the lifespan of the animals, indicating a potential functional role of BDNF in the disease/Rett syndrome (Chang et al., 2006). In addition to cytosine methylation, N6-methylation of adenine at BDNF promoter IV has also been implied in the activity-dependent upregulation of BDNF exon IV-containing transcripts, although the exact mechanism is not known (Li et al., 2019).

BDNF promoter VI. Not much is known about the transcription factors regulating BDNF promoter VI. However, in contrast to BDNF exon I and II-containing transcripts, which are mainly expressed in the nervous system, BDNF exon VI-containing transcripts are expressed in both the nervous system and in many non-neural tissues, e.g. heart and lung (Timmusk et al., 1993; Aid et al., 2007; Pruunsild et al., 2007). This suggests that the regulation of BDNF promoter VI could be remarkably different from that of other BDNF promoters. Transcription factors C/EBPβ and SP1 have been indicated to regulate the activity of BDNF promoter VI (Takeuchi et al., 2002).

BDNF promoter IXa. Little is known about the transcriptional regulation of BDNF promoter IXa. It has been reported that human BDNF promoter IXa contains partially overlapping CRE and PASRE cis-elements, conferring responsiveness to neuronal activity through CREB and NPAS4, respectively (Pruunsild et al., 2011). As these sites partially overlap, possible interference can be expected in the regulation of BDNF promoter IXa by these transcription factors. Of note, the CRE cis-element in the BDNF promoter IXa has acquired responsiveness to BDNF-TrkB signalling during the evolution of primates through two single nucleotide changes (Esvald et al., 2020).

1.7 BDNF and translation

BDNF-dependent regulation of translation. It is well-established that BDNF signalling induces local translation of synaptic mRNAs (reviewed in Santos et al., 2010). It has been reported that BDNF signalling induces local translation in the hippocampus (Aakalu et al., 2001) and that this upregulation of local translation is required for BDNF-dependent induction of LTP (Kang and Schuman, 1996). Interestingly, BDNF signalling-dependent potentiation of translation occurs in both axon (Zhang and Poo, 2002) and dendrites (Takei et al., 2004). The BDNF-dependent increase of local translation requires mTOR signalling pathway (Schratt et al., 2004; Takei et al., 2004), potentiating both translation initiation (Takei et al., 2001, 2004; Kanhema et al., 2006) and elongation (Inamura et al., 2005; Kanhema et al., 2006; Takei et al., 2009).

Translational regulation of BDNF. Less is known about local translation of the mRNA of BDNF itself. For local translation to happen, the respective mRNAs first need to be transported to synapses (Holt et al., 2019a). It has been reported that while BDNF transcripts with short 3' UTR are mainly restricted to the soma of the neuron, BDNF transcripts with long 3' UTR are additionally targeted to dendrites for local translation (An et al., 2008). Furthermore, BDNF transcripts are targeted to dendrites in a neuronal activity-dependent manner (Tongiorgi et al., 1997). Using a mouse model where BDNF long 3' UTR region was removed, An et al. showed impaired dendritic targeting of BDNF, reduced size of dendritic spines and impaired LTP in the dendrites but not in the soma region. It has also been shown that dendritically targeted BDNF long 3' UTR transcripts are essential for the energy homeostasis function of BDNF (Liao et al., 2012).

In addition to 3' UTR region, different 5' exons can affect the localization of BDNF transcripts, with exons I and IV mainly targeting the transcript to soma, and exons II and VI to dendrites in neuronal-activity paradigm (Pattabiraman et al., 2005; Chiaruttini et al., 2008). Furthermore, this spatial segregation of BDNF transcripts based on 5' exon also shows distinct regulation of the morphology of either proximal or distal dendrites for BDNF transcripts containing exons I and IV, and exons IIc and VI, respectively (Baj et al., 2011).

While the translation of BDNF transcripts with short 3' UTR seems to be constitutively active, BDNF long 3' UTR has been associated with translational repression under basal conditions (Timmusk et al., 1994; Lau et al., 2010), and the BDNF transcripts with long 3' UTR are a subject to significant increase in translation upon neuronal activity (Lau et al., 2010). Local translation of BDNF has been shown to regulate dendritic spine morphology (Verpelli et al., 2010; Orefice et al., 2013). BDNF signalling can also induce the release of various mRNAs, including mRNA of TrkB and BDNF itself, from P-bodies in the dendrites, increasing their availability for translation (Zeitelhofer et al., 2008; Leal et al., 2017).

1.8 BDNF in astrocytes

Astrocytes are the main supporting cells in the central nervous system, regulating brain homeostasis and modulating synaptic connections between neurons (Verkhratsky et al., 2019). In addition to neurons, astrocytes also express BDNF, although at much lower levels than neurons (Hisaoka-Nakashima et al., 2016). While there is no clear consensus about which BDNF transcript is expressed the highest, the major BDNF transcripts expressed in astrocytes seem to be BDNF exon IV- and VI-containing transcripts (Kruse et al., 2007; Takasaki et al., 2008; Zhang et al., 2014; Rousseaud et al., 2015; Hisaoka-Nakashima et al., 2016). Of note, big variations have been described in BDNF expression between astrocytes of different mouse strains (Wei et al., 2010).

The expression of BDNF in astrocytes can be induced upon various stimuli, including membrane depolarization (Wu et al., 2004), monoamine signalling (e.g. norepinephrine, dopamine) (Zafra et al., 1992; Inoue et al., 1997; Miklič et al., 2004; Mojca Jurič et al., 2006; Jurič et al., 2008; Day et al., 2014), ATP (Takasaki et al., 2008), glutamate acting through metabotropic glutamate receptors (Wu et al., 2004), forskolin/cAMP signalling (Zafra et al., 1992; Miklič et al., 2004), neuropeptides (e.g. VIP, PACAP) (Pellegri et al., 1998), α-melanocyte-stimulating hormone (Caruso et al., 2012), antidepressants such as fluoxetin (Mercier et al., 2004; Kinboshi et al., 2017; Kinoshita et al., 2018) and amitryptiline (Kajitani et al., 2012; Hisaoka-Nakashima et al., 2016), prostaglandins (Toyomoto et al., 2004), and TNFα-NFκB signalling (Saha et al., 2006). In addition to inducing BDNF expression, glutamate also induces BDNF secretion from astrocytes (Jean et al., 2008), allowing astrocytes to release BDNF in response to neuronal glutamatergic signalling.

Little is known about the biological relevance of astrocyte-synthesized BDNF. Using co-cultures of primary neurons and BDNF-deficient astrocytes, Pins et al (Pins et al., 2019) have shown that astrocyte-synthesised BDNF regulates neuronal dendritic growth and spine density *in vitro*. Using astrocyte-conditioned media, it has been shown that astrocytes could modulate inhibitory synapses by regulating BDNF-TrkB signalling between neurons (Elmariah et al., 2005). Astrocytes can also activate plasminogen, enhancing the catalytic activity of plasmin, thereby participating in the processing of secreted proBDNF to mature form (Briens et al., 2017).

Astrocytic BDNF is also necessary for oligodendrocyte maturation under cellular stress conditions (Miyamoto et al., 2015). Miyamoto et al have shown that BDNF in astrocyte-conditioned media promotes oligodendrocyte precursor cell maturation *in vitro*. They also showed, using GFAP-CRE dependent heterozygous conditional BDNF knockout animals, that astrocytes secrete BDNF that promotes oligodendrocyte maturation after white matter damage *in vivo*. However, it should be noted that GFAP promoter does not drive expression only in astrocytes, but also in neural progenitor cells, leading to knock-out in both astrocytes and some neuronal populations (Casper and McCarthy, 2006), which could confound the results obtained by Miyamoto et al. Furthermore, it has been reported that human GFAP promoter drives transgene expression in also oligodendrocyte precursor cells, whereas mouse GFAP promoter does not (Casper and McCarthy, 2006). As Miamoto et al do not report, which GFAP-CRE mouse line they used, their conclusions should be taken with caution.

Using tamoxifen-inducible CRE under the control of human GFAP promoter to obtain astrocyte-specific BDNF knockout, Fulmer et al showed that signalling through metabotropic glutamate receptors induces BDNF expression in astrocytes and this astrocyte-synthesized BDNF supports remyelination after demyelination induced by

chopper chelator cuprizone. Thus, modulating astrocyte-derived BDNF could be useful for treating neurological diseases involving myelination deficits (Fulmer et al., 2014). Therefore, the evidence suggests that astrocyte-synthesized BDNF participates in myelination.

Astrocytic BDNF has been implied in various diseases. Dysregulation of BDNF expression and/or secretion in astrocytes has been suggested to contribute to lower BDNF levels in Huntington's disease (reviewed in Gray, 2019). It has been shown that overexpression of N-terminal fragment of mutant huntingtin inhibits BDNF expression (Wang et al., 2012b), processing and secretion in astrocytes (Wang et al., 2012a). In contrast, Hong et al (Hong et al., 2016) found no significant changes in BDNF mRNA and protein levels, but did note reduced BDNF secretion in Huntington's disease model astrocytes. Astrocytic BDNF could also have a role in Rett syndrome, as MeCP2 knock-out animals show increased BDNF levels in astrocytes (Maezawa et al., 2009). Astrocytic BDNF signalling has also been associated with neuropathic pain (Kitayama et al., 2016) after spinal cord injury (Matyas et al., 2017).

In addition to expressing and secreting BDNF, astrocytes also express the receptors of BDNF. Astrocytes have been shown to express high levels of TrkB (Holt et al., 2019b). However, almost all of the expressed TrkB in astrocytes is the truncated TrkB.T1 isoform (Frisén et al., 1993; Zhang et al., 2014; Holt et al., 2019b) that lacks the intracellular kinase domain. The expression of TrkB.T1 in astrocytes peaks in a month old animals (Holt et al., 2019b). Using TrkB.T1 and p75NTR, astrocytes can sequester BDNF secreted from neurons and release it upon various stimuli (Rubio, 1997; Alderson et al., 2000; Bergami et al., 2008; Vignoli et al., 2016; Vignoli and Canossa, 2017). It has been suggested that astrocytes could be the main recipient of neuron-secreted BDNF (Stahlberg et al., 2018). In addition, Stahlberg et al found that instead of recycling, BDNF is mostly directed to degradation in astrocytes.

In addition to sequestering and recycling BDNF, BDNF can also convey signals in astrocytes. It has been shown that activation of TrkB.T1 induces Ca^{2+}-signalling through the IP_3 pathway (Rose et al., 2003), affecting astrocyte morphology (Ohira et al., 2005, 2007; Holt et al., 2019b). In addition to morphological changes, signalling through TrkB.T1 regulates astrocyte proliferation and migration speed (Matyas et al., 2017). Astrocyte migration can also be regulated by p75NTR, although brain region-specific differences seem to exist, as p75NTR regulates astrocyte migration in striatal, but not in cortical and hippocampal astrocytes (Cragnolini et al., 2018). BDNF-dependent signalling has also been reported to elicit antiapoptotic effect in astrocytes, leading to general neuroprotection (Saba et al., 2018). BDNF signalling also modulates cholesterol homeostasis in both neurons and astrocytes (Spagnuolo et al., 2018).

2 Aims of the Study

The main aim of the thesis was to investigate the molecular mechanisms of transcriptional and translational regulation of BDNF gene expression. The more specific aims of the study were as follows:

- Study the role of alternative translation start codons of BDNF in translatability, processing and secretion of BDNF;
- Study the mechanism of BDNF transcriptional autoregulation in neurons;
- Study the mechanism of BDNF expression in astrocytes;
- Study the role of distal enhancer regions in BDNF gene expression.

3 Materials and Methods

The following methods, described in more detail in the respective publications, were used in this study:

- Growing of cell lines (HEK293, HEK293FT, PC12) – Publications I, II, III, Manuscript
- Growing of primary cells (rat primary cortical neurons, rat primary cortical astrocytes) – Publications I, II, III, Manuscript
- Growing and differentiation of mouse embryonic stem cells – Manuscript
- Molecular cloning – Publications I, II, III, Manuscript
- CRISPR/Cas9-mediated deletion of enhancer region in mouse embryonic stem cells – Manuscript
- RNA extraction, cDNA synthesis, qPCR – Publications I, II, III, Manuscript
- Metabolic labelling – Publication I
- Western blot analysis – Publications I, II, III
- The purification and use of adeno-associated virus vectors – Publications I, II
- The purification and use of lentivirus vectors – Publications III, Manuscript
- CRISPR activation and interference systems – Manuscript
- Transfection, luciferase reporter assay – Publications I, II, III, Manuscript
- Electrophoretic mobility shift assay – Publication II
- *In vitro* DNA pulldown coupled with mass-spectrometry - Manuscript
- Chromatin immunoprecipitation – Publication II, Manuscript
- Immunocytochemistry – Publication II, III

4 Results and Discussion

4.1 The role of alternative translation initiation codons of BDNF in translatability, posttranslational processing and secretion of BDNF

It has been reported for various ER-targeted proteins that although the ER-targeting signal peptide is removed co-translationally, it could affect the fate of the protein, including proper folding, glycosylation patterns, localization, etc. (Hegde and Bernstein, 2006). A conserved in-frame translation start codon exists in BDNF exon I (Aid et al., 2007; Pruunsild et al., 2007), that could possibly give rise to BDNF protein with an extended pre-region (signal peptide targeting the protein to ER). As the BDNF exon I-containing transcripts are greatly inducible in response to various stimuli, we set out to determine whether the in-frame start codon affects BDNF translatability, processing and secretion (Publication I). For this, we generated V5-tagged BDNF overexpression constructs with different 5' UTRs together with mutation in either exon I or exon IX ATG.

Both the BDNF exon I and exon IX translation initiation codons (GCCACA**AUG**UU and AGAGUG**AUG**AC, respectively) seem to be in a suitable context for translation initiation, with the exon I AUG context slightly (~10%) better based on previously reported mass-analysis of translation initiation efficiency (Noderer et al., 2014). Here, we report that the translation start codon in exon I is used for translation initiation in both cultured rat primary neurons and in PC12 cells. Furthermore, our results indicate that the usage of the start codon in BDNF exon I results in much higher (3-6-fold) steady-state BDNF protein levels than using the canonical start codon in BDNF exon IX. We also noted that BDNF exon I and exon IV 5' UTRs inhibit translation efficiency, as evident from luciferase reporter assay and western blot analysis.

Using metabolic labelling with ^{35}S in PC12 cells, we analysed the fate of the synthesised BDNF proteins employing different translation start codons. Our metabolic labelling experiments indicated higher translation efficiency when using BDNF exon I AUG. We found slight differences in the co-translational or post-translational of the BDNF protein depending on the used signal peptide. However, we found no remarkable differences in the stability or constitutive secretion of BDNF with different pre-sequences. Thus, the difference in steady-state BDNF levels when using different translation initiation codons arises from increased efficiency of translation initiation.

To determine whether the usage of different signal peptides affects the regulated secretion of BDNF, we used both transient overexpression of different BDNF-V5 proteins in PC12 cells and AAV-mediated overexpression of different BDNF-V5 variants in cultured cortical neurons. We depolarized the cells with KCl to induce regulated secretion and measured the secreted BDNF protein from the cell culture media using Western blot analysis. Our results did not indicate any significant difference between the regulated secretion of BDNF using different N-terminal signal peptides.

Collectively, we report that exon I-containing BDNF transcripts could contribute significantly more to the total pool of BDNF protein than previously thought through an increased efficiency of translation initiation. We found no evidence of different stability or secretion of the BDNF protein translated using the exon I AUG.

In humans, in-frame translation start codons also exist in BDNF exons VII and VIII (Pruunsild et al., 2007) that are missing in rodents (Aid et al., 2007). The role human BDNF exon I and exon VIII alternative start codons in BDNF secretion has been investigated previously (Jiang et al., 2009). However, Jiang et al report that the usage of both exon I

and exon VIII start codons increases regulated secretion of BDNF in AtT-20 cells, whereas our study did not confirm this finding for BDNF exon I-containing transcripts. This discrepancy could be attributable to the usage of different cell lines, as the regulated secretion machinery could function differently in a cell-type specific manner.

Although the basal expression levels of BDNF exon I-containing transcripts in neurons are relatively low, the quantity of BDNF exon I-containing transcripts could reach comparable levels with BDNF exon IV-containing BDNF transcripts after neuronal activity (Pruunsild et al., 2011). Thus, considering the higher translation efficiency, it is plausible that BDNF exon I-containing transcripts contribute the majority of BDNF protein after neuronal activity and other stimuli. Furthermore, a study using BDNF exon I knockout animals suggests that BDNF exon I-containing transcripts could contribute up to 50% of total BDNF protein pool in the cortex and hypothalamus *in vivo* in 4-week old mice (Maynard et al., 2016). In contrast, no reduction of BDNF protein levels were seen in the hippocampus in these animals, indicating that different BDNF transcripts contribute to total BDNF protein levels in different brain regions. The significant contribution of BDNF exon I-containing transcripts to BDNF protein could (partially) help to explain the severe phenotype of BDNF exon I knock-out animals, who show extremely agressive behaviour in males (Maynard et al., 2016) and defects in maternal care and sexual receptiveness in females (Maynard et al., 2018).

4.2 The mechanism of BDNF transcriptional autoregulation in neurons

It has been reported that BDNF-TrkB signalling can induce the expression of BDNF mRNA levels, thus forming a positive feedback loop in cortical neurons (see Chapter 1.5). However, the mechanism of this phenomenon is largely unknown. Here, we set out to thoroughly characterize the molecular mechanisms of BDNF positive feedback loop (Publication II). First, we found that the expression of all major BDNF transcripts (I, II, III, IV, VI, IXa) was increased after BDNF-TrkB signalling in rat cultured cortical neurons. Using inhibitors of the main signalling pathways downstream of TrkB, we determined that this positive feedback loop mainly functions through the MAPK cascade pathway, with ERK1/2 and ERK5 being the most important mediators. These results are mostly in accord with those published by Nakajima et al. (2015), although they also reported a role of PI3K-Akt-signalling pathway in addition to the MAPK pathway in BDNF autoregulation.

AP-1 transcription factors, consisting of members of the Jun and Fos families, are important mediators of stimulus-dependent transcription in neurons (Morgan and Curran, 1991; Herdegen and Leah, 1998) and it has been reported that BDNF-TrkB signalling induces AP-1 family-dependent transcription (Gaiddon et al., 1996; Okamoto et al., 2003). Therefore, we chose to investigate whether AP-1 proteins could mediate the BDNF-TrkB positive feedback loop. For this, we used AAV-mediated overexpression of A-Fos, a dominant negative for AP-1 proteins (Ahn et al., 1998), and found that this greatly impaired the BDNF-TrkB signalling-dependent expression of BDNF exon I-containing transcripts, and slightly decreased the induction of BDNF exon III- and VI-containing transcripts in cultured cortical neurons.

Using luciferase reporter assay, we found that overexpression of AP-1 proteins increased the activity of human BDNF promoter I, with different dimers of AP-1 proteins eliciting different results. Using bioinformatic analysis, we determined the presence of two putative AP-1 *cis*-elements in human BDNF promoter I. Using mutagenesis and luciferase reporter assay, we found that these two *cis*-elements were responsible for the BDNF-TrkB signalling and AP-1 protein-dependent activation of human BDNF promoter I.

We also verified that AP-1 proteins regulated the activity of rat BDNF promoter I using luciferase reporter assay.

Next, we showed that the AP-1 *cis*-elements in human BDNF promoter I region bound AP-1 proteins *in vitro* using EMSA. In HEK293 cell line, overexpression of constitutively active c-Fos and c-Jun increased the expression of BDNF exon I-containing transcripts, indicating that AP-1 proteins can regulate the expression of these transcripts also in the endogenous context. The binding of AP-1 proteins to human BDNF promoter I region was also detected in HEK293 cells overexpressing AP-1 proteins using ChIP assay. Furthermore, we found TrkB signalling-dependent binding of endogenous AP-1 proteins to the BDNF promoter I region in rat cultured cortical neurons.

The effect of AP-1 proteins on the activation of BDNF promoters III and VI was most likely to be indirect, as no functional AP-1 cis-elements we discovered in these promoter regions, Furthermore, no significant AP-1 binding could be detected to those promoter regions using ChIP assay.

Collectively, our results show that AP-1 proteins are direct mediators of BDNF-TrkB signalling-dependent induction of BDNF exon I-containing transcripts, and indirect regulators of BDNF exon III and VI-containing transcripts.

The expression of AP-1 proteins is strongly induced upon various stimuli in the central nervous system, including neuronal activity, and c-Fos is one of the archetypal neuronal activity-regulated gene in the nervous system (Kovács, 2008; Lyons and West, 2011; Benito and Barco, 2015). c-Fos has been implicated in the regulation of BDNF expression, as c-Fos knockout animals exhibit impaired BDNF induction in response to kainic acid (Zhang et al., 2002). It has also been suggested that AP-1 transcription factors participate in the formation of long-term memories (Alberini, 2009). Although it has not been directly shown whether AP-1 proteins engage in the neural activity-dependent induction of BDNF expression, it is plausible that AP-1 proteins participate in integrating signals of neuronal activity to regulate synaptic plasticity through the expression of BDNF.

The *in vivo* relevance of BDNF-TrkB signalling-dependent induction of BDNF mRNA is still largely unknown, as there are currently no good means for detaching the BDNF transcriptional autoregulatory loop from neuronal-activity induced BDNF expression without seriously compromising TrkB signalling itself. Identifying the *cis*-elements responsible for the TrkB signalling-dependent induction of BDNF, that at the same time do not participate in neuronal activity-dependent BDNF expression would allow deciphering the physiological relevance of the transcriptional feedback loop of BDNF. Thus, it would be of interest to investigate whether the AP-1 *cis*-elements we have described in BDNF promoter I also participate in neuronal activity-dependent activation of this promoter.

BDNF transcriptional autoregulation can occur in an autocrine manner in neurons that express both BDNF and TrkB. However, in addition to the autocrine action of BDNF, paracrine BDNF-TrkB signalling to neighbouring cells could also increase BDNF expression in these cells. Thus, TrkB signalling-dependent induction of BDNF expression could potentially participate in the formation of larger neuronal networks, instead of only affecting the cells in its immediate vicinity.

It is plausible that the reinforcing BDNF expression through the TrkB-BDNF signalling provides sufficient amounts of BDNF for its various biological functions (see Chapter 1.1). It has been reported that BDNF positive feedback loop is necessary for memory consolidation *in vivo* (Bambah-Mukku et al., 2014) and it has been suggested that BDNF autoregulatory loop might explain the delayed effect of antidepressants (Castrén and Antila, 2017).

4.3 The regulation of BDNF expression in astrocytes.

It has been reported that various stimuli increase BDNF expression in astrocytes (see Chapter 1.8). However, the molecular mechanisms of BDNF expression in astrocytes have been poorly studied. Here, we focused on the induction of BDNF expression in astrocytes by catecholamines dopamine (DA) and norepinephrine (NE) (Publication III), as these two monoamines have been extensively shown to induce BDNF expression in astrocytes (Zafra et al., 1992; Inoue et al., 1997; Miklič et al., 2004; Mojca Jurič et al., 2006; Jurič et al., 2008; Day et al., 2014). We first confirmed the induction of BDNF in cultured cortical astrocytes in response to these treatments using RT-qPCR. Furthermore, we showed that DA and NE treatment of cultured astrocytes also increased BDNF protein levels in astrocytes. To the best of our knowledge, this is the first time BDNF protein has been detected in astrocytes using Western blot analysis.

We next set out to determine the receptors responsible for the induction of BDNF expression in response to DA and NE. First, we used specific agonists of dopaminergic and adrenergic receptors and measured BDNF mRNA expression levels. We found that while dopaminergic receptors failed to significantly increase BDNF expression, agonists of α1 and β adrenergic receptors induced the expression of BDNF mRNA. By using selective inhibitors of different catecholamine receptors combined with DA treatment, we noted that inhibitors of adrenergic receptors strongly decreased the DA-dependent induction of BDNF expression, whereas inhibition of dopamine receptors had a limited effect on BDNF induction. In agreement with these results, RT-qPCR analysis showed that our cultured rat cortical astrocytes expressed high levels of noradrenergic receptors, but low levels of dopamine receptors. Thus, our results indicate that DA-dependent expression of BDNF in astrocytes is mainly mediated by adrenoreceptors, rather than dopamine receptors.

Next, we determined which BDNF transcripts are expressed in our cultured cortical astrocytes using RT-qPCR and found that our cultured astrocytes mainly expressed BDNF exon VI-containing transcripts, and to a lesser extent exon IV-containing transcripts. Using lentiviral-mediated overexpression of a dominant-negative form of CREB family, termed A-CREB (Ahn et al., 1998), we showed that the induction of BDNF in response to DA was dependent on CREB family transcription factors. Using luciferase reporter assay in cultured cortical astrocytes, we found that the activity of BDNF promoters IV and VI was not induced in response to DA and NE treatment. In contrast, the activity of human and rat BDNF bacterial artificial chromosome (BAC) constructs, encompassing the whole BDNF gene locus, was induced by catecholamines, indicating that an unknown distal enhancer element within or near the BDNF gene locus regulates the catecholamine-dependent induction of BDNF in astrocytes. As the induction was dependent on CREB family transcription factors, we also determine whether the well-described CRE element (see Chapter 1.6) in BDNF promoter IV participated in the induction of BDNF in response to DA and NE. However, we found that mutating the BDNF promoter IV CRE element in the BAC context did not change the inducibility of the reporter, indicating that the promoter IV CRE is not required for DA-dependent BDNF expression in astrocytes.

We also noted remarkable morphological transformation of the astrocytes in response to DA treatment, an effect that was completely blocked by the application of β adrenoreceptor inhibitor. However, it has been reported by Galloway et al. that DA induces morphological changes in astrocytes through D1 and D2 dopaminergic receptors (Galloway et al., 2018). Unfortunately, Galloway et al. did not study the DA-dependent

induction of BDNF expression in their model system. Nevertheless, considering the very low expression levels of dopaminergic receptors in our cultured astrocytes, our results strongly support the idea that both the BDNF expression and morphological changes are elicited by the activation of adrenoreceptors by DA.

Collectively, we found that DA induces BDNF expression in astrocytes mainly through β-adrenergic receptors in a CREB-dependent manner, and that this induction depends on an unknown enhancer region within the BDNF gene locus. Our results also suggest that astrocytes surrounding dopaminergic synapses *in vivo* could potentially „listen" to the dopaminergic signalling between neurons and modulate the synaptic efficacy through expression and secretion of BDNF. Furthermore, it has been reported that astrocyte-secreted BDNF increases survival of dopaminergic neurons (Datta et al., 2018). Therefore, DA-dependent expression of BDNF in astrocytes could be important in the pathophysiology of Parkinson's disease, as astrocyte dysfunction has been described in this disease (Booth et al., 2017). Astrocytes have also been implied in psychiatric diseases affecting the dopaminergic system, including schizophrenia (Takahashi and Sakurai, 2013) and major depression (Marathe et al., 2018). As a general dysregulation of BDNF has been indicated these diseases (see Chapter 1.1 for references), further work is needed to determine whether astrocytic BDNF somehow participates in their pathophysiology.

4.4 The role of distal enhancer regions in BDNF gene expression.

Enhancers are distal regulatory elements often underlying cell type and developmental stage-specific expression of genes (Nord and West, 2020). While the role of proximal promoter regions in regulating BDNF gene expression have been well characterized (see Chapter 1.6), while very little information is available about distal regulatory regions of the BDNF gene. It has been suggested that enhancer regions participate in TrkB signalling-dependent induction of BDNF expression (Esvald et al., 2020). Furthermore, our results obtained in astrocytes suggest that catecholamine-dependent induction of BDNF expression in astrocytes is regulated by an enhancer element located within or near the BDNF gene locus (Publication III). Therefore, we set out to determine the distal regulatory elements governing the expression of BDNF (Manuscript).

First, we used ChIP-sequencing data from the ENCODE project and CAGE-sequencing from FANTOM5 project to predict potential enhancer regions in the BDNF gene locus. We found that the region downstream of BDNF exon III (termed +3 kb region, as distance from BDNF exon I transcription start site) showed numerous enhancer-associated characteristics, including DNaseI hypersensitivity, H3K4me1 and H3K27ac histone modifications and binding of RNA polymerase II in murine brain tissue, and bidirectional transcription, another hallmark of enhancers (Nord and West, 2020). Data from a recently published human brain cell type-specific ATAC-sequencing and ChIP-sequencing experiments (Nott et al., 2019) indicated neuron-specific open chromatin and H3K27ac markings at the +3 kb region. We further used data from FANTOM5 project to determine the expression of +3 kb enhancer eRNA in different human tissues and cell types. We found that the enhancer is mainly active in neural tissues – brain, spinal cord, olfactory region, and the strongest eRNA expression could be seen in hair follicle cells, neurons, trabecular meshwork cells and neuronal stem cells. As the +3 kb region shows enhancer-associated characteristics that are evolutionarily conserved, we chose to further study this region in the regulation of BDNF expression. We decided to test the functionality of the enhancer in two main cell types in the brain – neurons and astrocytes.

As enhancers act as bidirectional promoters (Kim et al., 2010; Nord and West, 2020), we cloned the respective +3 kb region, and +11 kb intronic region as a negative control in front of luciferase coding sequence in either orientation, and used the generated constructs in luciferase reporter assay. We found that the +3 kb region showed great bidirectional transcriptional activity in neurons, and its activity was slightly potentiated upon stimulating the neurons with BDNF or KCl. In astrocytes, the region showed much weaker but still detectable bidirectional transcription, but no change in transcription from the enhancer region could be detected upon stimulating the cells with DA.

Next, we generated luciferase reporter vectors containing BDNF promoter in front of the luciferase coding sequence and the enhancer region downstream of the luciferase expression cassette. Using these constructs in luciferase reporter assay, we found that adding the +3 kb enhancer region to the plasmid in either orientation activated the transcription from BDNF promoters I and IV, the main active BDNF promoters in neurons, ~2-3-fold. In contrast, in astrocytes the +3 kb enhancer region failed to activate transcription from BDNF promoters IV and VI, the main active BDNF promoters in astrocytes.

We next determined whether the +3 kb enhancer region affects BDNF expression in the endogenous context. For that, we used CRISPR/dCas9 system where nuclease-deficient dCas9 was fused to either repressor domain KRAB (CRISPR interference, CRISPRi) or two VP64 activator domains (CRISPR activation, CRISPRa). We targeted these activator and repressor forms to the +3 kb enhancer region using enhancer-specific gRNAs and measured BDNF expression using RT-qPCR. We found that in cultured cortical neurons, activating the +3 kb enhancer region increased the expression of BDNF exon I, II and III-containing transcripts, whereas the levels of other transcripts remained unchanged. Likewise, repressing the +3 kb enhancer region with CRISPRi decreased the expression BDNF exon I, II and III-containing transcripts. In cultured cortical astrocytes, activating the enhancer region greatly induced the expression of all BDNF transcripts, whereas repressing the enhancer decreased only the DA-induced levels of BDNF exon II-containing transcripts. These results, together with the data obtained from luciferase reporter assays, indicate that the +3 kb enhancer region regulates the expression of the first cluster of BDNF promoters (promoter I, II and III) in neurons, but is in a mostly inactive state in astrocytes.

We also measured the expression of enhancer RNAs (eRNAs) from the +3 kb enhancer region using strand-specific reverse transcription and qPCR, and found that the eRNA expression was slightly upregulated in response to different stimuli in neurons, suggesting that the region is a stimulus-dependent enhancer. Furthermore, we noted ~6-fold lower eRNA transcription from +3 kb enhancer region in cultured astrocytes than in cultured neurons.

To further confirm that the +3 kb enhancer region is a functional enhancer, we used CRISPR/Cas9 to delete the respective region in mouse embryonic stem cells. We then differentiated the obtained cells into excitatory neurons using overexpression of neurogenin 2 and measured BDNF expression levels. Consistent with our other results, deleting the enhancer region strongly decreased the expression of BDNF exon I, II and III-containing transcripts, but had minor or no effect on other BDNF transcripts.

To investigate the molecular mechanisms regulating the activity of the +3 kb enhancer region, we first performed *in vitro* DNA pulldown coupled with mass-spectrometric analysis to uncover the transcription factors that bind the +3 kb enhancer region. In total, we determined 21 transcription factors binding the +3 kb region, including USFs, EGR1,

TCF4, NeuroD2, NeuroD6 and JunD. We next used previously published ChIP-sequencing data and confirmed the binding of EGR1, TCF4 and NeuroD2 to the enhancer region, and further found activity-dependent binding of AP-1 proteins, NPAS4 and CBP. Using ChIP-qPCR we also determined binding of CREB and TCF4 to the +3 kb enhancer region in our cultured neurons.

We next used overexpression of a panel of dominant negative forms of different transcription factors to determine functionally important transcription factors regulating the activity of +3 kb enhancer region in luciferase reporter assay. Our results indicate that CREB, AP-1 proteins and ATF2 participate in regulating the activity of the +3 kb enhancer. We also investigated the role of E-box binding proteins in the regulation of +3 kb enhancer using luciferase reporter assay and found that silencing the expression of TCF4 decreased the activity of the enhancer region. Based on the ChIP-sequencing data we further uncovered an E-box element (CAGATG) in the enhancer and found that mutating this *cis*-element decreased the activity of the enhancer region ~2-fold. We further determined that this E-box participates in the enhancer's ability to potentiate the activity of BDNF promoter I in reporter assay. These results suggest an important role of various pro-neural basic helix-loop-helix transcription factors (e.g. TCF4 and its heterodimerization partners from the NeuroD family) regulating the activity of +3 kb enhancer region, potentially also conferring the neuron-specific activity of the enhancer.

Collectively, our results indicate that the +3 kb enhancer region is a neuron specific enhancer that governs the neuronal expression of the first cluster of BDNF promoters (BDNF exon I, II and III-containing transcripts). This enhancer provides an explanation for the mostly neural expression pattern of these BDNF transcripts (Aid et al., 2007; Pruunsild et al., 2007). It has previously been suggested that BDNF promoters I and II are regulated as a functional unit (Timmusk et al., 1999; Maynard et al., 2016). Here, we describe the +3 kb enhancer region regulating the whole first cluster of BDNF exons. However, what the exact nature of the interplay between these BDNF promoters and the +3 kb enhancer region is, and whether coordinated regulation of the expression of BDNF exon I, II and III-containing transcripts has any biological meaning is still unknown. Considering the important role of BDNF exon I and II-containing transcripts in regulating various aspects of behaviour (see Chapter 1.2), it is plausible that the +3 kb enhancer region is an important regulator of BDNF expression to carry out these functions. Future work is required to determine the *in vivo* role of this novel enhancer region.

5 Conclusion

The main findings of this thesis are as follows:

- The alternative translation start codon in BDNF exon I increases translation efficiency compared to the canonical start codon in BDNF exon IX, but the extended signal peptide in does not significantly change stability and secretion of the BDNF protein.
- BDNF-TrkB signalling induces the expression of all major BDNF transcripts in cortical neurons, mainly through MAPK pathway.
- AP-1 transcription factors directly mediate the BDNF-TrkB signalling-dependent induction of BDNF exon I-containing transcripts through two novel AP-1 *cis*-elements in BDNF promoter I.
- Dopamine induces BDNF expression in astrocytes mainly through β-adrenoreceptors in a CREB signalling-dependent manner. This induction depends on a yet unidentified enhancer region within or near the BDNF gene locus.
- The +3 kb enhancer region in the BDNF gene governs neuron-specific transcription of the first cluster of BDNF exons – exon I, II and III-containing BDNF transcripts. The enhancer contains a functional E-box element, linking the expression of BDNF and various pro-neural basic helix-loop-helix transcription factors.

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Abstract

Transcriptional and Translational Regulation of Brain-Derived Neurotrophic Factor

Brain-derived neurotrophic factor (BDNF) is a secreted protein belonging to the neurotrophin family. Since its discovery around 40 years ago, various important biological roles of BDNF have been described. BDNF promotes the survival of various neuron populations in the developing nervous system and regulates the plasticity of synaptic connections in the adulthood. BDNF is also an important regulator of organism energy balance. Dysregulation of BDNF expression and signalling has been implied in various neuropsychiatric and neurodegenerative disorders, making BDNF and its signalling pathways an attractive therapeutic target.

In rodents, BDNF transcripts are formed by splicing together one of the eight 5' non-coding exons (exons I-VIII) with the 3' protein-coding exon (exon IX). Each 5' exon is controlled by its own promoter, allowing precise spatiotemporal control of the BDNF gene expression at the transcriptional level throughout the lifespan of an organism. To date, various roles of different BDNF transcripts have been described, including the regulation of social behaviour for BDNF exon I-containing transcripts and the regulation of GABAergic signalling and memory functions for BDNF exon IV-containing transcripts.

BDNF is synthesised as a pre-pro-BDNF precursor protein. The pre-region, a signal peptide targeting the nascent BDNF protein into endoplasmic reticulum, is cleaved co-translationally, yielding proBDNF. Further intra- or extracellular processing by different proteases converts proBDNF into mature BDNF. Synthesised BDNF is transported to neurites and is secreted mainly through the regulated secretory pathway in a neuronal activity-dependent manner.

BDNF can activate two types of receptors, namely TrkB and p75NTR, with mature BDNF preferentially activating TrkB and proBDNF p75NTR. Remarkably, signalling through TrkB and p75NTR BDNF often elicit diametrically different outcomes, with TrkB signalling promoting cell survival, long-term potentiation and neurite outgrowth, and p75NTR signalling inducing neuronal apoptosis, long-term depression and reduction of neurite outgrowth.

The expression of BDNF is induced by various stimuli, and one of the most well-studied activators of BDNF expression in neurons is neuronal activity. To date, various transcription factors and their regulatory DNA elements have been described in the activity-dependent expression of BDNF and most of the research in this field has focused on the regulation of BDNF promoters I and IV. Many neurons in the central nervous system express both BDNF and its receptor TrkB, and various autocrine actions of BDNF have been identified. For instance, autocrine BDNF-TrkB signalling increases neuronal survival and is important for synaptic plasticity. Furthermore, BDNF-TrkB signalling also induces transcription of BDNF itself, forming a positive feedback loop. However, the exact mechanisms of this feedback loop are poorly understood.

In addition to neurons, BDNF is also expressed in astrocytes, the main cell type supporting neuronal functions in the central nervous system. Like in neurons, the expression of BDNF in astrocytes is also regulated by external stimuli, including various neurotransmitters and antidepressants. BDNF synthesised in astrocytes has been shown to be important for cell survival and proper myelination. Dysregulation of astrocytic BDNF expression and secretion has been implied in various diseases, e.g. Huntington's

disease. However, the molecular mechanism of the regulation of BDNF expression in astrocytes are much less known than in neurons.

In the current thesis, we set out to investigate the molecular mechanism of the transcriptional and translational regulation of BDNF expression. First, we focused on the posttranscriptional regulation of BDNF expression. An evolutionarily conserved in-frame translation initiation codon in BDNF exon I has been described, potentially giving rise to BDNF protein with 8 amino-acids longer pre-region than the canonical BDNF protein initiated from exon IX. Here, we analysed the usage of the translation initiation codon in exon I in BDNF expression and found that the use of this translation start codon provides much greater translation efficiency than the start codon in exon IX, whereas the extended signal peptide sequence synthesised from BDNF exon I-containing transcripts has no profound effect on the stability and secretion of the BDNF protein.

Second, we investigated the molecular mechanism governing TrkB signalling-dependent induction of BDNF transcription in neurons. We describe that BDNF-TrkB signalling induces the expression of major BDNF transcripts, mainly through the MAPK pathway. We also report two novel AP-1 *cis*-elements in BDNF promoter I that mediate the induction of BDNF exon I-containing transcripts by AP-1 family transcription factors after BDNF-TrkB signalling.

Third, we studied the mechanisms regulating the transcription of BDNF in astrocytes. Deciphering the mechanism of dopamine-dependent induction of BDNF expression, we found that this induction is mainly mediated by β-adrenoreceptors in a CREB signalling-dependent manner. Furthermore, catecholamine-dependent regulation of BDNF expression is mediated by a yet unidentified enhancer region within or near the BDNF gene locus.

Finally, we set out to find novel distal regulatory regions participating in BDNF gene expression in neural cells. While the regulation of BDNF expression by its proximal promoter regions has been well characterized, little is known about the distal regulatory regions of the BDNF gene. Here, we found a conserved intronic region downstream of BDNF exon III that governs the neuron-specific transcription of the first cluster of BDNF exons – exon I, II and III-containing BDNF transcripts.

Collectively, our results increase the understanding of the already very complex regulation of BDNF gene expression at both transcriptional and posttranscriptional level and helps to understand how BDNF carries out its diverse biological functions. As dysregulation of BDNF expression and signalling is linked to various neurological disorders, precise knowledge of the regulation of BDNF gene expression is a prerequisite for developing novel therapeutics involving BDNF.

Lühikokkuvõte
Aju päritolu neurotroofse teguri transkriptsiooni ja translatsiooni regulatsioon

Aju-päritolu neurotroofne tegur (*brain-derived neurotrophic factor*, BDNF) on sekreteeritav valk, mis kuulub neurotrofiinide perekonda. Tänaseks on BDNF-il kirjeldatud mitmeid olulisi rolle, näiteks suurendab BDNF neuronite elulemust arenevas organismis, reguleerib sünaptiliste ühenduste plastilisust täiskasvanueas ning osaleb organismi metabolismi regulatsioonis. Häireid BDNF geeni avaldumises ning signaliseerimises on seostatud mitmete neuroloogiliste haigustega, mistõttu on BDNF ja temast sõltuvad signaalirajad paljulubavad ravimmärklauad.

Näriliste BDNF geen sisaldab üheksat eksonit ning iga transkripti moodustamiseks splaissitakse kokku üks kaheksast 5' eksonist (eksonid I-VIII) valku kodeeriva 3' eksoniga (ekson IX). Iga 5' eksoni ekspressiooni reguleerib oma promootorala, mis võimaldab reguleerida BDNF transkriptide avaldumist ajaliselt ja ruumiliselt kogu organismi eluea jooksul. Ehkki erinevad BDNF transkriptid kodeerivad sama BDNF valku, on neil kirjeldatud erinevaid füsioloogilisi rolle. Näiteks osalevad transkriptid, mis sisaldavad BDNF eksonit I, sotsiaalse käitumise regulatsioonis, samas kui transkriptid, mis sisaldavad BDNF eksonit IV, mõjutavad ajus närviaktiivsust pärssivat signaliseerimist ning mälu.

BDNF valk sünteesitakse esmalt pre-pro-eellasvalguna endoplasmaatilise retiikulumi (ER) membraanil. Pre-regioon on signaalpeptiid, mis suunab sünteesitava valgu ER-i, ning see eemaldatakse proteolüütiliselt translatsiooni ajal. Selle tulemusel saadakse proBDNF valk, mille edasine proteolüütiline lõikamine nn küpseks BDNF valguks võib toimuda nii raku sees kui ka väljaspool. Sünteesitud proBDNF ja küps BDNF transporditakse neuronites jätketesse, kus seda väljutatakse rakust reguleeritud sekretsiooniraja kaudu, eelkõige närviaktiivsuse tulemusena.

BDNF aktiveerib raku pinnal kahte tüüpi retseptoreid – TrkB ja p75NTR, kusjuures küps BDNF aktiveerib valdavalt TrkB retseptorit ning proBDNF p75NTR retseptorit. Nende kahe retseptori kaudu antakse rakule sageli vastupidiseid signaale – enamasti toetab TrkB signaliseerimine rakkude ellujäämist, sünaptiliste ühenduste tugevnemist ning neuriitide väljakasvu, samas kui p75NTR retseptorist lähtuvad signaalirajad põhjustavad neuronites apoptoosi, sünaptiliste ühenduste nõrgenemist ning neuriitide tagasitõmbumist.

BDNF-i avaldumist kutsuvad esile mitmed stiimulid. Kõige enam on BDNF geeni avaldumist uuritud närviaktiivsuse järel ning tänaseks on kirjeldatud mitmeid BDNF geeni avaldumises osalevaid transkriptsioonitegureid ja nende seondumissaite BDNF geeni promootorites. Seejuures on enamik uuringutest keskendunud BDNF promootoritele I ja IV. Paljudes neuronites avalduvad mõlemad, nii BDNF kui ka tema retseptor TrkB, mistõttu on BDNF valgul ka autokriinseid rolle. Näiteks suurendab autokriinne BDNF-TrkB signaliseerimine rakkude elulemust ning on oluline sünaptilises plastilisuses. Lisaks on näidatud, et BDNF-TrkB signaliseerimine suurendab BDNF geeni enda avaldumist ning moodustab seeläbi positiivse tagasisidemehhanismi, mille molekulaarsed alused ei ole hästi kirjeldatud.

Lisaks neuronitele on BDNF geen avaldunud ka astrotsüütides, mis on peamine tugirakutüüp kesknärvisüsteemis ning toetab neuronite funktsioone. Nagu ka neuronites on astrotsüütides BDNF geeni avaldumine reguleeritud väliste stiimulite, muuhulgas erinevate neurotransmitterite ning antidepressantide poolt. Astrotsüütides sünteesitud BDNF-il on oluline roll närvisüsteemi rakkude elus püsimisel ning neuronite jätkete

müeliniseerimises. BDNF-i avaldumise ja sekretsiooni häireid astrotsüütides on samuti seostatud mitmete haigustega, näiteks Huntingtoni tõvega. Samas aga on BDNF-i avaldumise mehhanismidest astrotsüütides palju vähem teada kui neuronites.

Käesolevas väitekirjas uurisime BDNF-i avaldumise molekulaarseid aluseid nii transkriptsioonilisel kui posttranskriptsioonilisel tasandil. Esmalt keskendusime BDNF-i avaldumise posttranskriptsioonilisele regulatsioonile. BDNF geeni ekson I sisaldab evolutsioonis konserveerunud alternatiivset translatsiooni initsiatsioonikoodonit, mis võib potentsiaalselt anda 8 aminohappe võrra pikema pre-regiooniga BDNF valgu kui teised BDNF transkriptid, milles BDNF-i kodeeriv järjestus algab eksonis IX. Käesolevas töös uurisime BDNF eksonis I olena alternatiivse translatsiooni initsiatsioonikoodoni rolli BDNF valgu sünteesis ning leidsime, et seda kasutatakse palju suurema efektiivsusega BDNF valgu sünteesiks kui eksonis IX paiknevat kanoonilist initsiatsioonikoodonit. Samas ei mõjutanud eksonit I sisaldavatelt transkriptidelt kodeeritud pikem pre-regioon BDNF valgu stabiilsust ega sekretsiooni.

Järgmiseks uurisime TrkB signaliseerimisest sõltuva BDNF geeni avaldumise molekulaarseid mehhanisme neuronites. Kirjeldasime, et TrkB signaliseerimine suurendab peamiste BDNF transkriptide avaldumist ning seda põhiliselt MAPK signaaliraja kaudu. Samuti kirjeldasime BDNF promootoris I kaks uut AP-1 DNA-elementi, mille kaudu reguleerivad AP-1 perekonda kuuluvad transkriptsioonitegurid BDNF eksonit I sisaldavate transkriptide avaldumist TrkB signaliseerimise järel.

Kolmandaks uurisime BDNF geeni avaldumise molekulaarseid mehhanisme astrotsüütides. Avastasime, et dopamiin kutsub esile BDNF geeni avaldumise peamiselt läbi β-adrenoretseptorite ning selles protsessis osaleb transkriptsioonitegur CREB. Lisaks näitavad meie tulemused, et BDNF geeni avaldumine katehhoolamiinide toimel sõltub BDNF geeni lookuses asuvast distaalsest regulatoorsest alast ehk enhanseralast.

Viimaseks uurisime BDNF geeni ekspressiooni reguleerivaid enhanseralasid. Ehkki BDNF geeni promootoralade rolli BDNF geeni avaldumises on palju uuritud, on vähe teada BDNF geeni distaalsetest regulatoorsetest aladest. Oma töös kirjeldasime, et BDNF eksonist III allavoolu paikneb intronis varasemalt kirjeldamata enhanserala, mis reguleerib neuronites, aga mitte astrotsüütides BDNF geeni esimese klastri eksonitelt (eksonid I-III) algavate transkriptide avaldumist.

Käesoleva väitekirja tulemused laiendavad meie teadmisi keerukast BDNF geeni avaldumise regulatsioonist nii transkriptsioonilisel kui posttranskriptsioonilisel tasandil ning aitavad mõista, kuidas BDNF mõjutab erinevaid bioloogilisi protsesse. Kuna BDNF-i avaldumise ja sekretsiooni häireid on seostatud mitmete neuroloogiliste haigustega, on BDNF geeni avaldumise mehhanismide täpne tundmine oluline eeldus uute BDNF-iga seotud ravimite loomisel.

Appendix

Publication I

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ORIGINAL ARTICLE

Efficient use of a translation start codon in BDNF exon I

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Abstract

The brain-derived neurotrophic factor (BDNF) gene contains a number of 5′ exons alternatively spliced with a common 3′ exon. BDNF protein is synthesized from alternative transcripts as a prepro-precursor encoded by the common 3′ exon IX, which has a translation start site 21 bp downstream of the splicing site. BDNF mRNAs containing exon I are an exception to this arrangement as the last three nucleotides of this exon constitute an in-frame AUG. Here, we show that this AUG is efficiently used for translation initiation in PC12 cells and cultured cortical neurons. Use of exon I-specific AUG produces higher levels of BDNF protein than use of the common translation start site, resulting from a higher translation rate. No differences in protein degradation, constitutive or regulated secretion were detected between BDNF isoforms with alternative 5′ termini. As the BDNF promoter preceding exon I is known to be highly regulated by neuronal activity, our results suggest that the function of this translation start site may be efficient stimulus-dependent synthesis of BDNF protein.

Keywords: BDNF, secretion, signal peptide, translation.

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Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, has important roles in the developing and adult nervous system (Bibel and Barde 2000; Binder and Scharfman 2004). BDNF is a secreted protein synthesized as prepro-precursor protein into the endoplasmic reticulum (ER). Endoplasmic reticulum-targeting signal peptide is cleaved cotranslationally, the resulting proBDNF undergoes N-terminal glycosylation in the pro-domain (Mowla *et al.* 2001) and is processed into mature protein either intracellularly by proprotein convertases (Seidah *et al.* 1996; Wetsel *et al.* 2013), or extracellularly by plasmin and metalloproteinases (Lee *et al.* 2001; Pang *et al.* 2004). BDNF is secreted from neurons by either a constitutive or a regulated pathway (Lessmann *et al.* 2003) and sorting of BDNF to the regulated secretion pathway involves interaction of a sorting receptor sortilin with the BDNF pro-domain (Chen *et al.* 2005). This interaction is impaired by a common human polymorphism that results in Val66Met substitution in proBDNF and defective activity-dependent BDNF secretion in cultured neurons (Egan *et al.* 2003).

A number of alternative transcripts are generated from the BDNF gene by the use of alternative promoters and alternative splicing (Aid *et al.* 2007; Pruunsild *et al.* 2007). In general, BDNF transcripts are spliced together from one of several alternative 5′ exons and a 3′ exon containing the commonly used translation initiation site. 5′ exons are mainly untranslated, with the potential exception of exon I (and exons VII and VIII in human, but not in rodents), comprising start codons in-frame with the start codon in exon IX (Aid *et al.* 2007; Pruunsild *et al.* 2007). Use of these alternative ATGs would generate preproBDNF isoforms with longer signal peptides (8 amino acids for exon I, 15 aa for exon VII and 82 aa for exon VIII) compared to the isoform produced using the start codon in exon IX (termed ‘the common isoform’ below). After signal peptide removal, all isoforms generate proBDNF species with identical primary sequences (Fig. 1a). It is known, however, that use of alternative signal peptides can result in physiologically relevant differences in the properties of the

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Abbreviations used: AAV, adeno-associated virus; BDNF, brain-derived neurotrophic factor; DIV, days *in vitro*; ER, endoplasmic reticulum; MOI, multiplicity of infection; ORF, open reading frame; UTR, untranslated region.

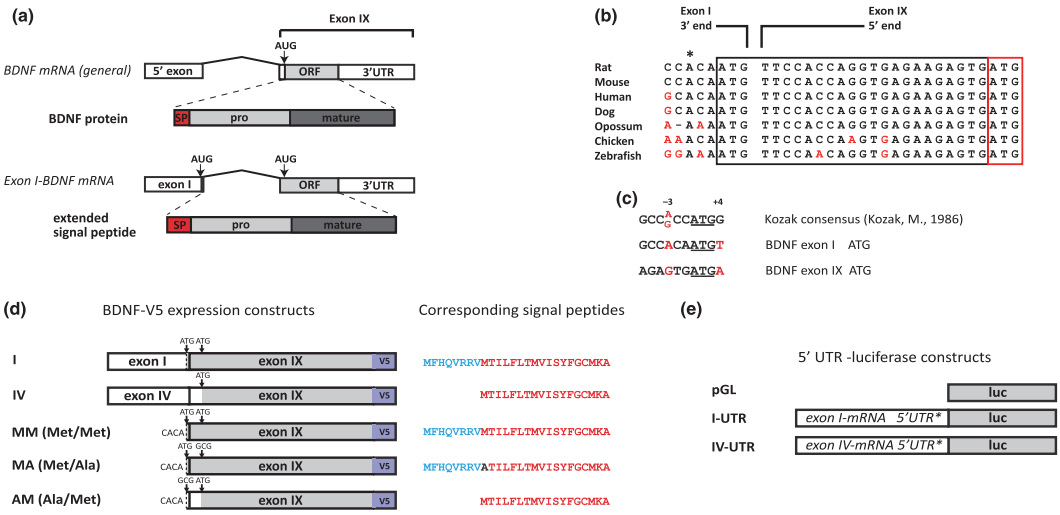

Fig. 1 (a) Synthesis of brain-derived neurotrophic factor (BDNF) protein from alternatively spliced mRNAs. Alternative 5′ exons generally contribute untranslated regions (UTR) to different BDNF transcripts. However, exon I contains a conserved in-frame ATG that can be used as a translation start codon, extending the signal peptide (SP) by 8 amino acids. (b) Conservation of exon I-specific ATG, its context nucleotides and the first 24 nucleotides from exon IX in vertebrates [data from a UCSC Genome Browser (http://genome-euro.ucsc.edu)]. Black box indicates sequence encoding the transcript I-specific N-terminal extension to the BDNF signal peptide, red box indicates the ATG in exon IX, and asterisk indicates the highly conserved -3 position from exon I ATG. (c) Context nucleotides of translation start codons in BDNF exons I and IX. (d) Schemes of V5-tagged BDNF expression constructs used in this study and sequences of corresponding signal peptides generated from these constructs. Constructs I and IV contain rat BDNF 5′ exons I and IV, exon IX sequence to the end of the open reading frame (ORF) and a C-terminal V5-His tag (V5). Construct MM contains BDNF sequence from exon I-specific ATG to the end of the ORF without UTR; 4 exon I nucleotides upstream of the ATG have been included to ensure the natural translation initiation context (b). MA – the commonly used BDNF start codon in exon IX is mutated to GCG (Met→Ala) in construct MM; AM – exon I-specific start codon is mutated to GCG in construct MM. White – untranslated regions; gray – BDNF protein-coding sequence; violet – V5 tag; solid vertical lines – splicing sites; hatched vertical lines – exon I-specific ATG. (e) Schemes of luciferase constructs. pGL: pGL4.15 vector. I-UTR and IV-UTR: constructs with exon I and IV-specific 5′-UTRs inserted 5′ of luciferase start codon. * exon I mRNA-specific 5′-UTR contains sequence from exon I transcription start site to the start codon contained in exon I; exon IV mRNA-specific 5′-UTR contains sequence from exon IV transcription start site to the start codon in exon IX.

synthesized protein via affecting protein folding and post-translational modifications (Hegde and Bernstein 2006; Braakman and Hebert 2013). Kinetic and qualitative aspects of protein folding and modification (e.g., glycosylation) can in turn regulate several aspects of protein fate such as subcellular sorting and degradation (Moremen *et al.* 2012; Braakman and Hebert 2013).

Exon I-containing BDNF transcripts are widely expressed in the brain, but absent or expressed at very low levels in non-neuronal tissues (Timmusk *et al.* 1993; Aid *et al.* 2007; Pruunsild *et al.* 2007). Levels of exon I-containing BDNF mRNAs can be robustly increased by neuronal activity (Metsis *et al.* 1993; Timmusk *et al.* 1993) and a number of transcription factors and corresponding *cis* regulatory elements have been identified that keep BDNF promoter I activity under tight control (West *et al.* 2014). In fact, promoter I is the most dynamically regulated of BDNF promoters (Metsis *et al.* 1993; Tabuchi *et al.* 2002; Tian *et al.* 2009; Pruunsild *et al.* 2011; Koppel and Timmusk 2013). Finally, relatively high expression of exon I mRNA has been observed in human parietal cortical tissue (Garzon and Fahnestock 2007). Therefore, exon I mRNAs are expected to contribute a significant amount to the pool of BDNF protein generated from alternative BDNF transcripts, and this amount may increase further upon transcriptional up-regulation of BDNF by neuronal activity.

Here, we investigated the use of BDNF exon I-specific translation start codon in PC12 cells and cultured cortical neurons. Our results show that exon I-specific start site is used for translation initiation and leads to higher steady-state protein levels than the common BDNF isoform translated from a start codon in exon IX. This difference resulted from more efficient synthesis of the exon I-specific isoform, whereas no differences were observed in degradation and secretion between these isoforms.

Methods

Cell culture

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 treated with 0.25% trypsin (Life Technologies, Grand Island, NY, USA) and 0.5 mg/mL DNase I (Roche Molecular Biochemicals, Indianapolis, IN, USA) digestion, followed by mechanical dissociation. Cells were plated on poly-L-lysine (0.1 mg/mL) coated dishes at a density of 1–2 × 10^5 cells/cm^2 in Neurobasal A medium (Life Technologies), supplemented with B27 (Life Technologies), 1 mM L-glutamine (GE Healthcare Life Sciences, Piscataway, NJ, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (PAA Laboratories). At day 2 *in vitro* (DIV), half of the medium was replaced with fresh medium and 5-fluoro-2-deoxyuridine (Sigma, St Louis, MO, USA) (10 μM) was added to inhibit growth of mitotic cells. PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum, 5% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (all from PAA Laboratories).

DNA constructs, transfections, and luciferase assays

Rat BDNF coding sequence with indicated 5′ untranslated region (UTR) sequences were cloned into the pcDNA3.1/V5-His-TOPO expression vector (Life Technologies). Site-directed mutagenesis was performed as previously described (Pruunsild *et al.* 2011) using Phusion DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) and primers flanking the mutation site by 13–15 nucleotides. All constructs were verified by sequencing. For constructing adeno-associated viral vectors (AAV), BDNF-V5 open reading frames (ORFs) from MM and AM constructs were subcloned into rAAV vector under the human synapsin I promoter as described earlier (Klugmann *et al.* 2005; Zhang *et al.* 2007). rAAV and helper plasmids were kindly provided by H. Bading. For western blot analysis, neurons grown in 6-well plates were transfected at 6 DIV using 2 μg of DNA at 1 : 2 w/v ratio with Lipofectamine 2000 and collected for analysis 2 days later. For luciferase assays, neurons grown in 96-well plates were transfected at 6 DIV with 0.25 μg of DNA (equal amounts of Firefly and Renilla luciferase plasmids combined) at 1 : 2 w/v ratio with Lipofectamine 2000. Cells were incubated in the transfection medium (unsupplemented Neurobasal A) for 3–4 h with gentle agitation to improve transfection efficiency, after which the medium was replaced with conditioned growth medium. 2 days after transfection, cells were lysed in Passive Lysis Buffer (Promega, Madison, WI, USA) and luciferase activity was measured using Dual-Glo Luciferase Assay System (Promega). Relative luciferase activity was calculated by dividing Firefly luciferase signals with Renilla luciferase signals and expressed as relative luciferase units. PC12 cells were transfected with Lipofectamine 2000 or LipoD293 reagent (Signagen) according to manufacturer's instructions and used for western blot or metabolic labeling experiments 24–48 h later.

Western blotting

For preparation of cell lysates, cortical neurons or PC12 cells were lysed directly in Laemmli buffer and boiled for 5 min. For analysis of secreted protein, 5X Laemmli buffer was added to concentrated media samples and boiled for 5 min. Proteins (10–20 μL of lysates or 40 μL of concentrated media) were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Membranes were blocked with 5% skimmed milk in TBST (Tris-buffered saline and 0.05% Tween 20) and incubated with anti-V5 antibody (R960-25; Life Technologies) at 1 : 5000 dilution or anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody (clone 6C5; Millipore Corporation, Bedford, MA, USA) at 1 : 5000 dilution in TBST with 2% skimmed milk. Horseradish peroxidase-conjugated secondary antibodies (Thermo Scientific) were used at 1 : 5000 dilution in TBST with 2% skimmed milk. Bands were visualized with SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific) and ImageQuant LAS 4000 imager (GE Healthcare Life Sciences, Piscataway, NJ, USA) and quantified using ImageQuant TL software (GE Healthcare).

RNA extraction and quantitative RT-PCR

RNA was isolated from transfected neurons with RNeasy Mini kit (Qiagen, Valencia, CA, USA) and treated with TURBO DNase (Life Technologies) according to the manufacturer's instructions. 2 U of DNase was added to 20 μL reactions in two aliquots to ensure complete degradation of plasmid DNA (1 U at the beginning and 1 U at after 20 min incubation, total incubation time 40 min). cDNA was synthesized from 1 μg of DNase-treated RNA with RevertAid reverse transcriptase (Thermo Scientific) using oligo(dT) primers. Quantitative PCR was performed on a LightCycler 480 instrument using LightCycler 480 SYBR Green I Master (Roche Molecular Biochemicals). All qPCR reactions were performed in triplicate and target expression was normalized to cyclophilin B mRNA levels. The following primers were used: analysis of BDNF 5′-UTR-luciferase mRNA: FFluc F: 5′-CCAGCGCCATTCTACCCACT-3′; FFluc R: 5′-CATGGCTTTGTGCAGCTGCTC-3′; analysis of BDNF-V5 mRNA: V5His F, 5′-AGCCTATCCCTAACCCTCTCC-3′; V5His R, 5′-AGCGGGTTTAAACTCAATGG-3′; Cyclophilin B: cycB F, 5′-AGATCGAAGTGGAGAAACCCTTTG-3′; cycB R, 5′-TAAAAATCAGGCCTGTGGAATGTG-3′.

Metabolic labeling

For pulse labeling, PC12 cells grown in 6-well plates were transfected with indicated constructs and 2 days later were washed once with 1 mL pre-warmed Hank's Balanced Salt Solution (HBSS) medium, incubated for 30 min in 1 mL pre-incubation medium (DMEM without cysteine/methionine + 10% horse serum and 5% fetal bovine serum), followed by labeling for 0.5–1 h in 600 μL in pre-incubation medium containing 0.2 mCi/mL ^{35}S cycteine/methionine (EasyTag Express35S; PerkinElmer, Waltham, MA, USA). Cells were washed once in ice-cold phosphate-buffered saline (PBS) and collected by centrifugation (200 *g*) in 1 mL ice-cold PBS. Cells were lysed in 300 μL radioimmunoprecipitation assay (RIPA) buffer containing 0.1% sodium dodecyl sulfate (SDS), 1x Complete Protease Inhibitor Cocktail (Roche Molecular Biochemicals), and 1 mM phenylmethylsulfonyl fluoride (PMSF); sonicated to reduce viscosity and centrifuged at 16 000 *g* to remove cell debris. 700 μL 1X PBS was added to lysates and BDNF-V5 protein was immunoprecipitated overnight with 30 μL Anti-V5 Agarose Affinity Gel

(A7345; Sigma), according to manufacturer's instructions. Immunoprecipitated protein was eluted from beads by boiling for 5 min with 50 μL Laemmli buffer and separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Gels were fixed for 5 min in 10% acetic acid + 10% methanol, dried for 1 h at 70°C, and exposed to Kodak BioMax MR film (Eastman Kodak, Rochester, NY, USA) for 3–5 days. For chase analysis, after labeling cells were washed with 1 mL pre-warmed HBSS buffer and incubated for indicated times in 1 mL DMEM + 10% horse serum and 5% fetal bovine serum with two times excess of cysteine and methionine. Secreted protein was immunoprecipitated from chase media, using 30 μL Anti-V5 Agarose Affinity Gel per sample and immunoprecipitates were processed as described above for cell lysates.

AAV generation and infection of cultured neurons

Chimaeric AAV1/AAV2 virions were produced as described previously (Zhang *et al.* 2007), with minor modifications. HEK293 cells were grown in 10 cm dishes (10 dishes per batch) in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin (PAA Laboratories). Cells were transfected in DMEM + 10% fetal bovine serum without antibiotics, using 20 μg PEI and 10 μg plasmids per 10 cm dish (5 μg of mini-adenovirus helper plasmid, 1.25 μg of AAV1 helper plasmid, 1.25 μg of AAV2 helper plasmid, and 2.5 μg of rAAV plasmid containing MM or AM BDNF-V5 ORFs under human synapsin I promoter). Three days after transfection, cells were collected by centrifugation at 800 *g* in PBS pre-warmed to 37°C, resuspended in 150 mM NaCl/20 mM Tris-HCl pH 8.0, frozen and thawed once followed by 1 h incubation at 37°C with 0.5% Na-deoxycholate and 50 U/mL benzonase (Sigma). Cell debris was collected by 15 min centrifugation at 3000 *g* and supernatant was subjected to another freeze–thaw cycle and centrifugation at 3000 *g* to remove debris. Virions were purified from the supernatant using HiTrap Heparin HP affinity columns (GE Healthcare) and desalted using Amicon Ultra-4 units with 100K cutoff filters (Millipore Corporation). Infection efficiency was tested by anti-V5 immunocytochemistry in cortical neurons 5 days after infection. For secretion experiments, cortical neurons were infected at 2 DIV with AAVs at 1–2 multiplicity of infection, simultaneously with medium change. Half of the medium was changed again at 5 DIV and neurons were used for secretion experiments at 7 DIV.

Analysis of regulated secretion

PC12 cells were grown in 10 cm dishes and transfected with indicated constructs using Lipofectamine 2000. Cortical neurons were grown in 10 cm dishes and infected with AAVs at 2 DIV. Secretion experiments were performed 2 days after transfection of PC12 cells and at 5 days after infection of neurons. PC12 cells were incubated for 30 min in 4 mL of fresh DMEM (serum and antibiotic-free) or DMEM containing 50 mM KCl; neurons were incubated for 30 min in 4 mL of fresh unsupplemented Neurobasal A medium with or Neurobasal A containing 25 mM KCl. Media were collected, supplemented with Complete Protease Inhibitor Cocktail (Roche Molecular Biochemicals) + 1 mM phenylmethylsulfonyl fluoride and concentrated with Amicon Ultra-4 units with 10K cutoff filters (Millipore Corporation) at 4°C. Concentrates were desalted with 2 × 3 mL PBS in the same columns and concentrated to approximately 100 μL. 5X Laemmli buffer was added to samples and boiled for 5 min. Cell lysates were performed by direct lysis in 1 mL Laemmli buffer. Levels of BDNF-V5 protein in secretion media and cell lysates were determined by western blotting with anti-V5 antibody (R960-25; Life Technologies).

Results

BDNF exon I–specific ATG is used for translation initiation

For all rodent BDNF transcripts, with the exception of those containing exon I, the translation initiation AUG is in exon IX (Fig. 1a). In this study, we analyzed the use of putative start codon in exon I for BDNF translation initiation. We did not study exon VII and VIII-specific translation initiation because in-frame start codons in these exons, although present in the human gene, are lacking in the rodent BDNF gene. In contrast, start codon in exon I and the 21 nucleotides preceding the common (exon IX-specific) start codon are highly conserved in vertebrates (Fig. 1b).

Context nucleotides of the start codon are known to be critical for translation initiation. A favorable context is defined by a purine in position −3 together with a G in position +4, and adequate context by fulfilling one of these requirements (Kozak 1986). Accordingly, exon I-specific ATG appears to lie in adequate initiation context (Fig. 1c). To experimentally determine whether exon I-specific ATG can be used for translation initiation, we transfected rat primary cortical neurons with C-terminally tagged BDNF expression constructs indicated in Fig. 1(d). These constructs contain either full 5′ untranslated regions of BDNF transcripts I and IV (constructs I and IV) or truncated exon I UTR limited to nucleotides −4…−1 relative to the first ATG (constructs MM, MA and AM), followed by full BDNF ORF and a V5-His tag. Exon IV was selected as a representative BDNF transcript with a translation start codon contributed by exon IX. Western blot analysis showed that BDNF was expressed and processed to mature protein from both exon I and exon IV UTR constructs, with significantly higher expression from the exon I UTR construct (Fig. 2a and c). As the UTRs may contribute to the different translatability of exon I and exon IV constructs, we tested the efficiency of BDNF synthesis from exon I and exon IX-specific ATGs using UTR-less constructs as indicated in Fig. 1(d). In this experiment, expression from exon I-specific UTR-less construct MM (designating methionins encoded by exon I and exon IX specific ATGs) was compared with constructs where either exon I or exon IX-specific ATG were mutated to GCG (AM and MA, designating respective Met→Ala mutations) (Fig. 2b and d). This analysis confirmed that exon I-specific ATG is used for translation initiation and indicates that use of this start codon results in higher BDNF protein levels than use of start codon in exon IX, independently of the effects of 5′-UTRs. Furthermore, luciferase assay with UTRs from either exon I or exon IV inserted in

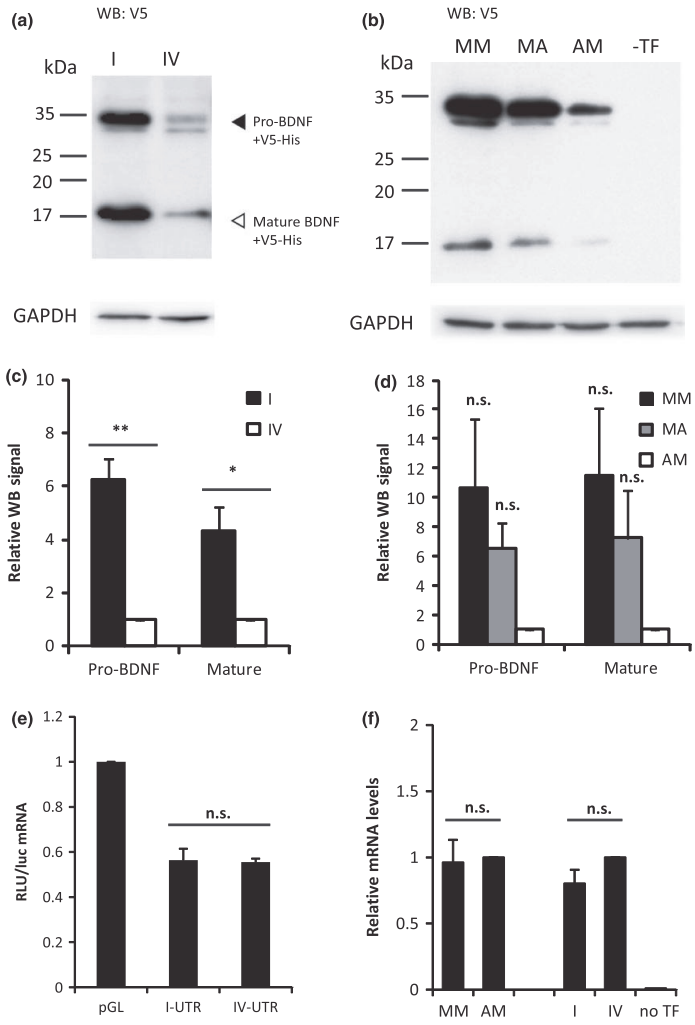

Fig. 2 In-frame ATG in exon I is used as a translation start site in rat primary cortical neurons. (a and b) Western blot analysis (anti-V5) of cell lysates from cultured cortical neurons transfected with rat brain-derived neurotrophic factor (BDNF)-V5 expression constructs containing BDNF untranslated regions (UTRs) I and IV (a) or UTR-less constructs (b). -TF indicates untransfected cells and GAPDH is shown as loading control. Note that image in (a) was obtained with a longer exposure time than image in (b). (c) Quantification of western blot signals in (a) from three independent experiments. *$p < 0.05$, **$p < 0.01$ (*t*-test). (d) Quantification of western blot signals in (b) from three independent experiments. n.s., not significant relative to AM (*t*-test). For proBDNF-V5, signals of both bands were summed. (e) Luciferase assay in primary cortical neurons transfected with constructs carrying BDNF exon I and exon IV-specific 5′-UTRs in front of the firefly luciferase open reading frame (ORF). This data are expressed as relative luciferase units (RLU) normalized with luciferase mRNA levels. n.s., not significant (*t*-test); $n = 3$. (f) RT-qPCR analysis of BDNF-V5 mRNA levels in cultured neurons transfected with BDNF-V5 constructs; no TF – untransfected cells; n.s., not significant (*t*-test); $n = 4$; error bars – SEM.

front of the firefly luciferase ORF (Fig. 1e) indicated that both UTRs inhibited translation to a similar extent (Fig. 2e). Finally, RT-qPCR analysis of mRNA transcribed from transfected plasmids showed that the observed differences in protein levels were not caused by different mRNA expression levels (Fig. 2f).

Of note, relatively higher mature BDNF/proBDNF ratios were observed when neurons were transfected with 5′-UTR I and IV-containing constructs (Fig. 2a) compared to neurons transfected with MM/MA/AM constructs (Fig. 2b). It is known that BDNF over-expression can overwhelm cellular proprotein processing machinery and therefore mature BDNF/proBDNF ratio depends on proBDNF expression levels (Matsumoto *et al.* 2008). In our experiments, over-expressed BDNF levels in cells transfected with 5′-UTR-containing constructs were markedly lower than in cells transfected with MM/MA/AM constructs (data not shown), explaining the observed differences in proBDNF processing.

Enhanced translation rate and not higher stability or secretion underlies higher levels of BDNF with exon I-specific N-terminus

Steady-state protein levels are determined by an equilibrium between protein synthesis and degradation and, in case of secreted proteins, efficiency of secretion. Characteristically to secreted proteins, BDNF is processed from proprotein to

the mature form, both of which can be secreted (Mowla *et al.* 1999, 2001). To investigate if higher levels of BDNF protein generated from exon I ATG-containing expression constructs may have been caused by slower intracellular degradation or less effective secretion, we performed ^{35}S metabolic labeling experiments in PC12 cells. PC12 cells were used because of the poor transfection efficiency of cortical neurons and hence low sensitivity for this assay. PC12 cells are a widely used model system for neurosecretion and are relatively easy to transfect. Analysis of steady-state protein levels in PC12 cells transfected with BDNF MM, MA, and AM expression constructs reflected the expression differences observed in cortical neurons (Fig. 3a and b). Differently from neurons where two proBDNF species were seen, four proBDNF-V5 bands were detected in PC12 cell lysates, probably reflecting differences in glycosylation. In addition, proBDNF-V5 band with the lowest apparent molecular weight may correspond to unglycosylated 28 kDa BDNF species (here migrating higher owing to the V5 tag) described previously (Mowla *et al.* 2001). The only N-linked glycosylation consensus site in the BDNF protein is asparagine 123 in the pro-domain (Lessmann and Brigadski 2009). Inactivation of this site by N123Q mutation shifted the migration of proBDNF bands by several kDa and only two prominent bands were observed (Fig. 3c). This confirms that N123 is indeed used for glycosylation and agrees with a previous report (Mowla *et al.* 2001). Mature BDNF signal was very low or undetectable in N123Q mutants, suggesting that glycosylation is necessary for efficient proBDNF processing. In agreement with previous findings (Mowla *et al.* 2001), total levels of N-glycosylation deficient BDNF protein were lower than glycosylated BDNF levels (data not shown), indicating that glycosylation positively affects BDNF stability.

Pulse labeling with ^{35}S Met/Cys showed that significantly higher levels of over-expressed proBDNF-V5 protein with the exon I-specific signal peptide (construct MM) was synthesized in PC12 cells within 1 h compared to the common BDNF isoform (AM) (Fig. 4a and c). Mature BDNF-V5 was not quantified as corresponding signal could not be reliably detected by 1 h labeling. Interestingly, two proBDNF-V5 bands unique to either MM (1 in Fig. 4b) or AM (2 in Fig. 4b) were detected, indicating differences in co-/posttranslational processing of BDNF isoforms. Chase of the ^{35}S signal revealed that pro- and mature BDNF-V5 species of both isoforms were efficiently secreted from PC12 cells (Fig. 4d and e). In accordance with previous findings (Mowla *et al.* 2001), mainly glycosylated proBDNF-V5 was secreted. Our analysis showed that the MM/AM proBDNF intensity ratio seen after 1 h pulse labeling (Fig. 4c) remained constant for both proBDNF and mature BDNF within the 1–6 h chase time frame both in cell lysates (Fig. 4d and f) and in culture media (Fig. 4e and g), indicating lack of differences in protein degradation or constitutive secretion. Therefore, the observed differences in steady-state protein levels can be attributed to better efficiency of translation from exon I-specific ATG.

BDNF isoforms with alternative N-termini display no differences in regulated secretion

Enhanced regulated secretion of exon I-specific BDNF isoform relative to the common isoform with exon IX-derived

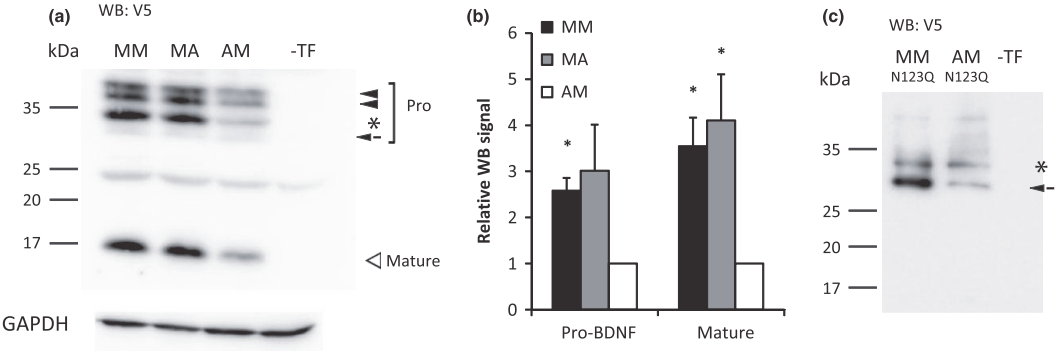

Fig. 3 Expression of brain-derived neurotrophic factor (BDNF) proteins with alternative N-termini in PC12 cells. (a) Western blot analysis (anti-V5) of cell lysates from PC12 cells transfected with BDNF-V5 expression constructs MM, AM and AM. Filled triangles indicate glycosylated full-length proBDNF-V5, asterisk indicates unglycosylated full-length proBDNF-V5, and arrow indicates a proBDNF-V5 species that may correspond to the '28 kDa' proBDNF described by Mowla *et al.* 2001. -TF indicates untransfected cells. (b) Quantification of western blot signals in (a) from three independent experiments. *$p < 0.05$ relative to AM (*t*-test). (c) Western blot analysis (anti-V5) of cell lysates from PC12 cells transfected with BDNF-V5 expression constructs with N123Q mutation eliminating the single N-glycosylation consensus site in the proBDNF region. Asterisk indicates unglycosylated full-length proBDNF-V5 and arrow indicates '28 kDa' proBDNF-V5 species.

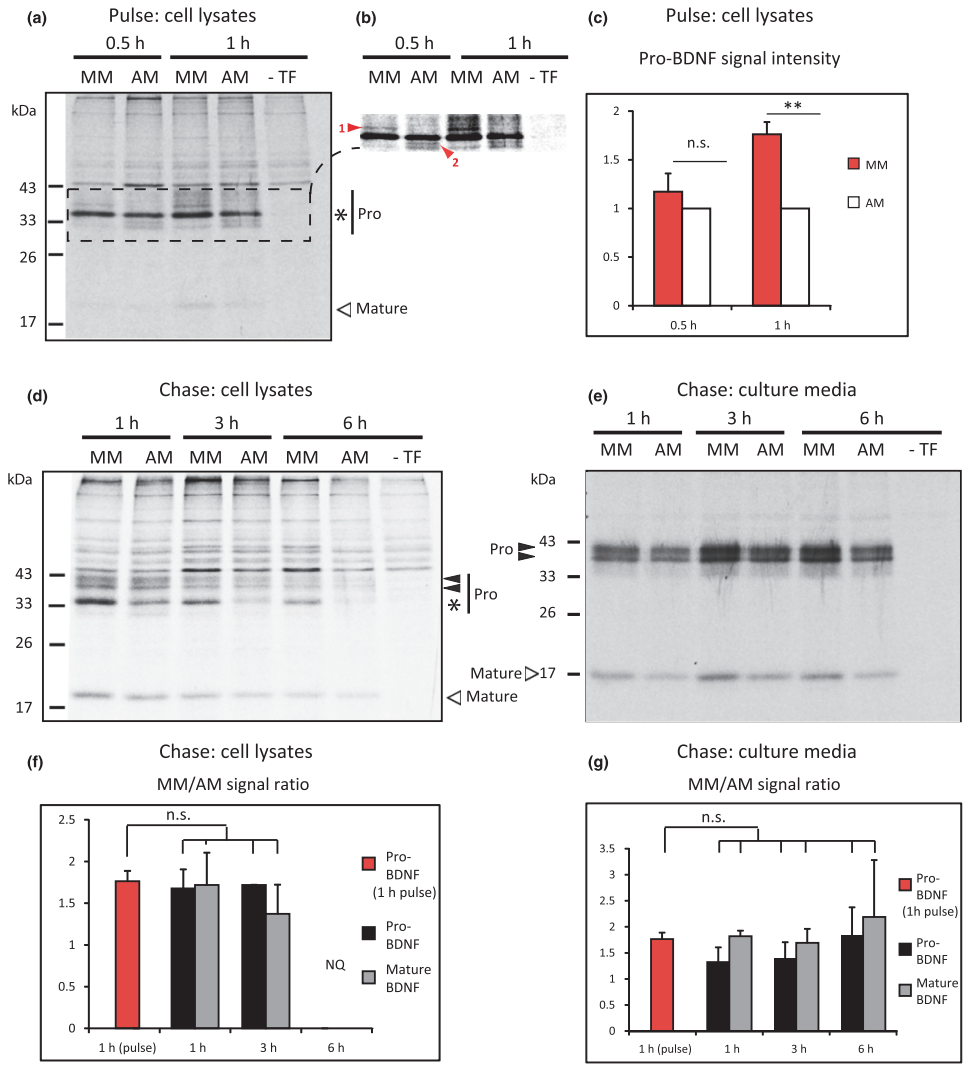

Fig. 4 Pulse-chase analysis of alternative brain-derived neurotrophic factor (BDNF) isoforms in PC12 cells. (a) PC12 cells transfected with MM and AM constructs were labeled with 0.5 or 1 h pulses of [35S]Cys-Met, lysed, immunoprecipitated with anti-V5 agarose, separated with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and analyzed with autoradiography. Asterisk indicates unglycosylated proBDNF-V5 and open triangle indicates mature BDNF-V5. -TF indicates untransfected cells. (b) Highly contrasted inset from (a) showing bands unique to either MM (arrow 1) or AM (arrow 2). (c) Quantification of proBDNF-V5 bands. Total intensity of proBDNF-V5 bands were quantified in three independent experiments and are expressed relative to band intensities in AM lanes. n.s., not significant; $^{**}p < 0.01$ (*t*-test). (d–g) Cells were pulse labeled for 1 h and incubated in chase medium for 1, 3, or 6 h. Cell lysates (d) and media (e) were immunoprecipitated with anti-V5 agarose and analyzed as in (a). Filled triangles indicate glycosylated proBDNF-V5 and asterisk indicates unglycosylated proBDNF-V5. (f) Quantification of proBDNF-V5 and mature BDNF-V5 bands in chase cell lysates. Signal from different proBDNF-V5 bands was summed. Shown are ratios of MM and AM band intensities from three independent experiments, as compared with MM/AM ratio after 1 h pulse labeling (red column). n.s., not significant between 1 h pulse and any other group (*t*-test); NQ, not quantified as signal above background was not detected in all experiments. (g) Quantification of proBDNF-V5 and mature BDNF-V5 bands in chase media. Signals from different proBDNF-V5 bands were summed. n.s., not significant between 1 h pulse and any other group (*t*-test); $n = 3$. Error bars – SEM.

start codon has been reported in AtT-20 cells (Jiang *et al.* 2009). We sought to test whether a similar difference could be seen in PC12 cells. Here, a robust depolarization-induced secretion was observed for both isoforms and both proBDNF and mature BDNF species in PC12 cells (Fig. 5a and c). However, the exon I-BDNF isoform (MM) did not display more efficient regulated secretion, that is, higher induction of secretion by membrane depolarization compared to the common isoform (AM). Instead, we observed a tendency (albeit not significant) for enhanced regulated secretion of the common isoform (Fig. 5c). BDNF-V5 levels in cell lysates did not increase upon depolarization, indicating that increased levels of the protein in the culture medium samples could be attributed to regulated secretion (Fig. 5b and d). In fact, small decreases in proBDNF-V5 levels were detected, reflecting loss from the cellular pool by secretion. Considering the contradiction with results reported by Jiang *et al.* 2009 and the fact that AtT-20 or PC12 cell lines may not ideally recapitulate neurosecretion, we analyzed regulated secretion of MM and AM proteins also in cultured neurons. Cortical neurons were infected with AAV encoding V5-tagged MM and AM proteins and secretion experiments were performed as with PC12 cells. Again, we did not observe significant differences between depolarization-induced secretion of MM and AM isoforms (Fig. 6). Note the higher ratios of mature BDNF/proBDNF in AAV-infected neurons (Fig. 6b) as compared to chemically transfected neurons (Fig. 2b). Viral transduction leads to lower expression in higher number of cells than Lipofectamine transfection (data not shown), probably allowing more effective processing of proBDNF.

Discussion

Owing to its multiple roles in the nervous system, regulation of BDNF expression has been thoroughly investigated. It is well established that BDNF expression is governed by alternative differentially regulated promoters (West *et al.* 2014). The role of alternative 5′ exons in BDNF translation regulation, however, is less well understood. A well-studied function for these 5′-UTRs is their acting as a dendritic targeting code, together with sequence elements in the BDNF coding region and 3′-UTR (An *et al.* 2008; Chiaruttini *et al.* 2009; Baj *et al.* 2011). In addition, there is evidence that alternative 5′-UTRs may also regulate BDNF translation efficiency (Vaghi *et al.* 2014). Finally, use of alternative start codons can serve as an additional mechanism for regulating BDNF translation. In a recent study using human BDNF over-expression in mouse pituitary tumor AtT-20 cells, Jiang and colleagues showed that both exon I and exon VII-specific isoforms displayed better regulated secretion than the common BDNF isoform (Jiang *et al.* 2009). In addition, impairment of regulated BDNF secretion by the Val66Met substitution (Egan *et al.* 2003) was overridden by the use of variant signal sequences (Jiang *et al.* 2009). Here, we have

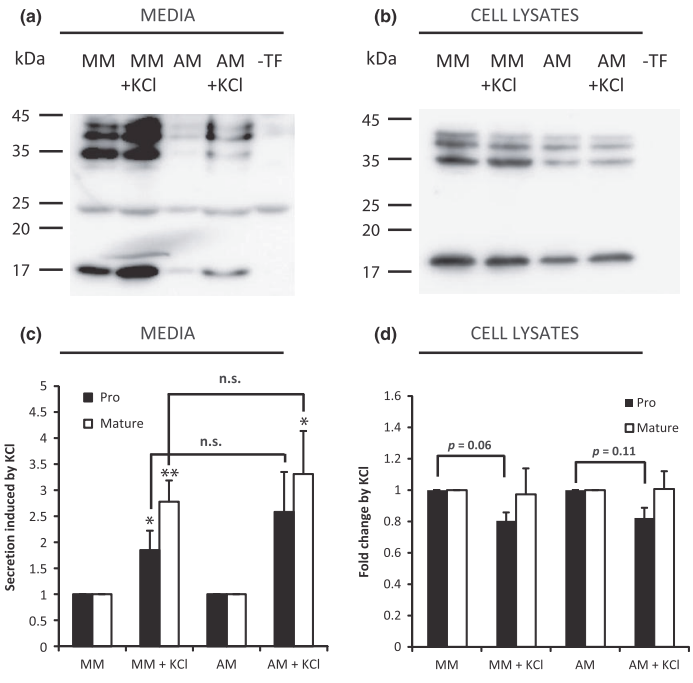

Fig. 5 Regulated secretion of alternative brain-derived neurotrophic factor (BDNF) isoforms in PC12 cells. PC12 cells transfected with MM and AM constructs were incubated for 30 min in fresh unsupplemented culture medium without or with KCl added to 50 mM (+KCl). Western blot analysis (anti-V5) of BDNF-V5 protein levels in culture media (a) and cell lysates (b). (c) Quantification of proBDNF-V5 and mature BDNF-V5 bands in culture media. Signals from different proBDNF-V5 bands were summed. Data are expressed as relative induction of secretion by KCl treatment. $*p < 0.05$ and $**p < 0.01$ between KCl-treated and untreated cells; n.s., not significant between KCl-treated groups (*t*-test); $n = 5$. (d) Quantification of proBDNF-V5 and mature BDNF-V5 bands in cell lysates (*p* values for *t*-tests are indicated); Signals from different proBDNF-V5 bands were summed. Data are expressed as relative induction of secretion by KCl treatment; $n = 4$. Error bars – SEM.

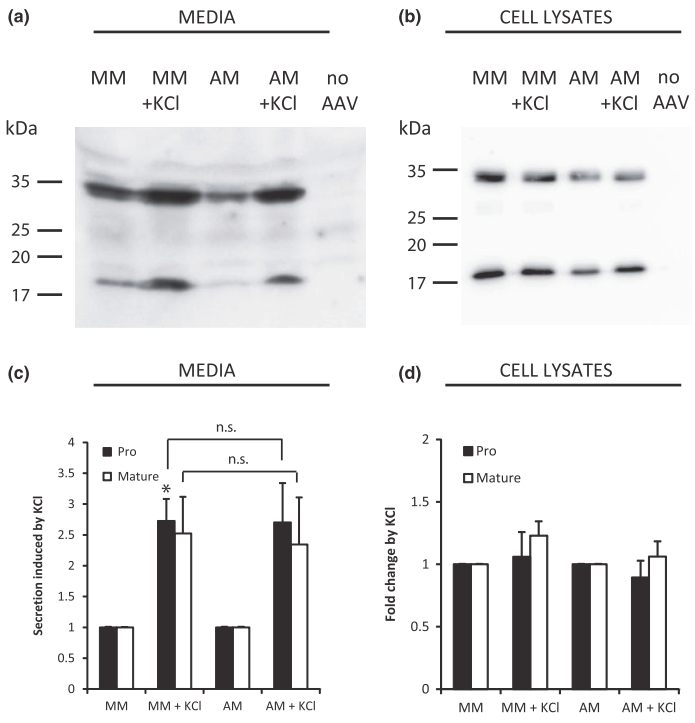

Fig. 6 Regulated secretion of alternative brain-derived neurotrophic factor (BDNF) isoforms in cultured neurons. Cultured cortical neurons were infected with adeno-associated viruses (AAV) encoding the MM/AM BDNF isoforms at 2 DIV. At 7 DIV, cells were incubated for 30 min in fresh unsupplemented culture medium or culture medium with KCl added to 25 mM (+KCl). Western blot analysis (anti-V5) of BDNF-V5 protein levels in culture media (a) and cell lysates (b). (c) Quantification of proBDNF-V5 and mature BDNF-V5 bands in culture media. Signals from proBDNF-V5 bands were summed. Data are expressed as relative induction of secretion by KCl treatment. *$p < 0.05$ between KCl-treated and untreated cells; n.s., not significant between respective KCl-treated groups (*t*-test); $n = 4$. (d) Quantification of proBDNF-V5 and mature BDNF-V5 bands in cell lysates; Signals from proBDNF-V5 bands were summed. Data are expressed as relative induction of secretion by KCl treatment; $n = 4$. Error bars – SEM.

revisited this topic, focusing on exon I-specific ATG, the only conserved alternative BDNF translation start site in vertebrates.

Use of exon I-specific ATG for translation initiation generates a preproBDNF isoform which differs from the canonical BDNF isoform only by an N-terminal 8 amino acid stretch extending the ER-targeting signal peptide. Although both of the two alternative signal peptides are rapidly cleaved to generate proBDNF with identical primary sequence, it is known that signal sequences can affect protein's fate in several ways. First, signal peptides (in the narrow sense – i.e., signals targeting to the secretory pathway) determine the efficiency of cotranslational peptide translocation to the ER, which in turn determines the amount of protein synthesized and eventually secreted. Second, signal peptides can also affect post-translational modifications and protein folding via differences in recruitment of chaperone proteins and post-translational processing proteins at the luminal side of the ER (Sakaguchi 1997; Hegde and Bernstein 2006; Braakman and Hebert 2013). In this study, we show that use of exon I-specific translation start codon results in higher levels of BDNF protein synthesis. This difference is independent of 5′-UTRs and can be attributed to either higher probability of translation initiation from exon I-specific ATG or enhanced translocation of BDNF protein across the ER membrane. Interestingly, in addition to revealing higher translation rate for the exon I-specific BDNF isoform, metabolic labeling experiments in PC12 cells showed isoform-specific banding pattern at 0.5 and 1 h time points, which could not be seen in the chase part of the analysis (1–6 h after labeling) or western blot analysis. These two faint bands, probably transient intermediates in BDNF biogenesis, suggest differential co- or pos-translational processing of these isoforms.

It is known that properties of signal sequences and posttranslational events can affect the efficiency of secretion (Sakaguchi 1997; Hegde and Bernstein 2006; Braakman and Hebert 2013). In a recent study, Jiang and colleagues reported enhanced regulated secretion of exon I- and exon VII-specific BDNF isoforms relative to the common BDNF isoform in mouse pituitary tumor AtT20 cells, indicating potential differences in sorting of BDNF isoforms to the regulated secretory pathway (Jiang *et al.* 2009). We undertook a similar analysis in pheochromocytoma PC12 cells and cultured cortical neurons, but failed to observe significant differences in either cell type, which contradicts the findings by Jiang *et al.* 2009. One explanation for this discrepancy is the use of PC12 cells or neurons in our study and AtT20 cells in Jiang *et al.* 2009. BDNF is secreted both constitutively and in a regulated manner (Goodman *et al.* 1996; Heymach *et al.* 1996; Canossa *et al.* 1997; Mowla *et al.* 1999) and partitioning of BDNF between constitutive and regulated secretion routes may be cell type-dependent. Alternatively,

levels of protein over-expression may affect the outcome of analyses addressing BDNF biosynthesis, as exemplified by a recent controversy related to the functions of proBDNF protein (Matsumoto *et al.* 2008; Yang *et al.* 2009). In this respect, both our study and that by Jiang *et al.* 2009 suffer from the same limitation of using protein over-expression, which most probably leads to production of unnaturally high levels of proBDNF, likely caused by clogging the cellular proteolytic processing machinery with excessive levels of proprotein substrate. Thus, to overcome this caveat of over-expression studies, it would be desirable to address the hypothesis of functional differences between alternative N-terminal BDNF isoforms *in vivo*, using transgenic mice carrying the mutations used in this study.

In this study, we show that an alternative translation start site in exon I can be used for efficient BDNF protein synthesis. In comparison to an earlier report by Jiang *et al.* (2009) addressing this subject, our results contribute several novel aspects. (i) Using mutagenesis, we show that ATG in exon I is indeed used for translation initiation. (ii) Higher protein levels synthesized from exon I-specific transcripts as compared to exon IV-specific transcripts are not dependent on the presence of 5′-UTRs. (iii) Metabolic labeling experiments showed that elevated protein levels derived from the exon I-specific construct are associated with higher translation efficiency and rule out differences in protein degradation and secretion. (iv) In contrast to the study by Jiang *et al.* (2009), we could not find differences in regulated secretion between the exon I-specific and the common BDNF isoform. In conclusion, our study suggests that exon I-containing BDNF transcripts may have a relatively large contribution to the total amount of synthesized BDNF protein. This finding proposes one explanation for the rather poorly understood biological function of BDNF alternative splicing.

Acknowledgments and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines. The authors have no conflict of interest to declare.

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Publication II

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Cellular/Molecular

AP-1 Transcription Factors Mediate BDNF-Positive Feedback Loop in Cortical Neurons

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Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, regulates both survival and differentiation of several neuronal populations in the nervous system during development, as well as synaptic plasticity in the adult brain. BDNF exerts its biological functions through its receptor TrkB. Although the regulation of *BDNF* transcription by neuronal activity has been widely studied, little is known about TrkB signaling-dependent expression of *BDNF*. Using rat primary cortical neuron cultures, we show that the *BDNF* gene is a subject to an extensive autoregulatory loop, where TrkB signaling upregulates the expression of all major *BDNF* transcripts, mainly through activating MAPK pathways. Investigating the mechanisms behind this autoregulation, we found that AP-1 transcription factors, comprising Jun and Fos family members, participate in the induction of *BDNF* exon I, III, and VI transcripts. AP-1 transcription factors directly upregulate the expression of exon I transcripts by binding two novel AP-1 *cis*-elements in promoter I. Moreover, our results show that the effect of AP-1 proteins on the activity of rat *BDNF* promoters III and VI is indirect, because AP-1 proteins were not detected to bind the respective promoter regions by chromatin immunoprecipitation (ChIP). Collectively, we describe an extensive positive feedback system in *BDNF* regulation, adding a new layer to the elaborate control of *BDNF* gene expression.

Key words: AP-1; BDNF autoregulation; BDNF-positive feedback loop; Fos; Jun; TrkB

Significance Statement

Here, we show for the first time that in rat primary cortical neurons the expression of all major *BDNF* transcripts (exon I, II, III, IV, VI, and IXa transcripts) is upregulated in response to TrkB signaling, and that AP-1 transcription factors participate in the induction of exon I, III, and VI transcripts. Moreover, we have described two novel functional AP-1 *cis*-elements in *BDNF* promoter I, responsible for the activation of the promoter in response to TrkB signaling. Our results indicate the existence of a positive feedback loop for obtaining sufficient BDNF levels necessary for various TrkB signaling-dependent physiological outcomes in neurons.

Introduction

Brain-derived neurotrophic factor (BDNF) is a secretory protein belonging to the neurotrophin family (Huang and Reichardt, 2001). BDNF binds to two distinctive classes of receptors, receptor tyrosine kinase subfamily member TrkB and tumor necrosis factor receptor superfamily member $p75^{NTR}$, with mature BDNF preferentially binding to the TrkB receptor and proBDNF to the $p75^{NTR}$ receptor (Lu et al., 2005). During development, BDNF functions as a differentiation and survival factor for various populations of neurons in the peripheral nervous system and CNS, strictly regulating the number of neurons needed for proper functioning of the nervous system (Bibel and Barde, 2000; Huang and Reichardt, 2001; Park and Poo, 2013). In the adult brain, BDNF has an important role in long-term potentiation (LTP) and memory formation (for review, see Park and Poo, 2013). Dysregulation of BDNF expression is implicated in various neuropsychiatric disorders, indicating the importance of BDNF in higher cognitive functions and mood regulation (for review, see Autry and Monteggia, 2012).

BDNF has a complex gene structure (Timmusk et al., 1993), with the rodent *BDNF* gene consisting of one protein-coding 3′ exon that is spliced together with one of the eight noncoding 5′

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The authors declare no competing financial interests.

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exons (Aid et al., 2007). In humans, two additional exons (Vh and VIIIh) have been described (Pruunsild et al., 2007). Each of the 5′ exons, except the human cassette exons VIII, VIIIh, and IXb, has its own promoter, allowing complex spatiotemporal regulation of *BDNF* expression in different brain regions and peripheral tissues throughout development (Aid et al., 2007; Pruunsild et al., 2007) and in response to various stimuli (Metsis et al., 1993; Karpova et al., 2010; Salerno et al., 2012; Baj et al., 2013).

AP-1 transcription factors, consisting of Fos and Jun family members, are immediate early genes, whose expression in the CNS is induced by various stimuli, including hypoxia, sensory stimulation, neurotransmitters, neurotrophins, and other growth factors (Sheng and Greenberg, 1990; Radler-Pohl et al., 1993; Karin et al., 1997; Herdegen and Leah, 1998). AP-1 proteins have a conserved basic leucine zipper domain, where the leucine zipper mediates protein dimerization and the basic region is responsible for binding to DNA (Eferl and Wagner, 2003). AP-1 transcription factors preferentially bind to the AP-1 element (also known as TRE, TPA-response element), with consensus sequence TGAC/GTCA (Eferl and Wagner, 2003). As with other basic leucine zipper domain-containing transcription factors, AP-1 proteins can bind DNA only as dimers, and binding to DNA greatly increases the dimer stability (Patel et al., 1994). Whereas Fos family proteins (c-Fos, FosB, Fra1, Fra2) are unable to form homodimers (Halazonetis et al., 1988; Shaulian and Karin, 2001), members of the Jun family (c-Jun, JunB, JunD) are able to form both homodimers and heterodimers within the family, and heterodimers with Fos family proteins or with other transcription factors, such as members of the ATF family, C/EBP, and others (Herdegen and Leah, 1998; Chinenov and Kerppola, 2001).

There is evidence suggesting that AP-1 proteins play a role in synaptic plasticity and memory (for review, see Alberini, 2009). For instance, AP-1 proteins have been implicated in both memory formation (Grimm et al., 1997) and consolidation of addictive behavior (Raivich and Behrens, 2006; Pérez-Cadahía et al., 2011). AP-1 proteins also regulate neuronal survival and apoptosis, with the outcome depending on the exact cellular context and stimulus (Herdegen and Leah, 1998).

Although the mechanisms of calcium- and neuronal activity-dependent *BDNF* expression have been widely studied (for review, see West et al., 2014), little is known about TrkB signaling-dependent regulation of *BDNF* gene expression, with the main focus having been on studying the regulation of exon IV transcripts (Yasuda et al., 2007; Zheng and Wang, 2009; Bambah-Mukku et al., 2014). Here, we show for the first time that in rat primary cortical neurons, the expression of all major *BDNF* transcripts is induced in response to TrkB signaling, and that part of this positive feedback loop is dependent on AP-1 family transcription factors.

Materials and Methods

Primary neuron cultures. All animal procedures were performed in compliance with the local ethics committee. Preparation of rat primary neuron cultures was performed as follows. Cortices and hippocampi of both male and female embryonic day (E)20–E21 Sprague Dawley rat pups were dissected, cells were dissociated using trypsin-EDTA (0.25% trypsin, 1 mM EDTA, Life Technologies) for 20 min at 37°C, washed once with 1× HBSS, and treated with 0.05% DNase I (Roche) solution in 1× HBSS (including 12 mM $MgSO_4$) for 5 min at 37°C. After washing with 1× HBSS again, solution of 0.1% trypsin inhibitor (AppliChem) and 1% BSA (Sigma-Aldrich) in 1× HBSS was added and cortices and hippocampi were triturated using flame-polished Pasteur pipette. Undissociated tissue clumps were allowed to sediment by gravity for 3–5 min, supernatant was moved to a new tube and centrifuged at 200 × *g* for 5 min. Neurons were resuspended in prewarmed Neurobasal A medium (Invitrogen) containing 1× B27 supplement (Invitrogen), 1 mM L-glutamine (PAA Laboratories), 100 U/ml penicillin (PAA Laboratories), and 0.1 mg/ml streptomycin (PAA Laboratories), and plated on culture plates previously coated with 0.2 mg/ml poly-L-lysine (Sigma-Aldrich) in borate buffer, pH 8.5. At 2 d *in vitro* (DIV), one-half of the medium was replaced with fresh medium, containing 5-fluoro-2′-deoxyuridine (10 μM final concentration, Sigma-Aldrich) to inhibit proliferation of glial cells.

Cloning and mutagenesis. Full-length coding sequences of AP-1 genes were amplified from either human genomic DNA or from human cDNA, for Jun family and Fos family members, respectively, using Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific). For expression in eukaryotic cells, the protein coding sequences of AP-1 genes were cloned into pQM vector backbone (Icosagen) after murine *phosphoglycerate kinase 1* (*PGK*) promoter (mouse genome assembly GRCm38/mm10, chromosome X region 106186727-106187231). The sequence coding for VP16 transactivation domain was obtained from pACT vector (Promega), the sequence coding for V5-tag was obtained from pcDNA3.1 vector (Invitrogen), to make vectors coding for VP16- or V5-tagged AP-1 proteins, respectively. Plasmid encoding A-Fos was obtained from Addgene (plasmid 33353).

The rat and human *BDNF* promoter I reporter constructs and the respective plasmids with mutated AP1-1 and PasRE site have been described previously (Pruunsild et al., 2011). For amplifying human and rat *BDNF* promoter III and VI regions from genomic DNA, Phusion Hot Start II High-Fidelity DNA polymerase (Thermo Scientific) was used. The respective promoter regions were cloned upstream of firefly luciferase-coding sequence into pGL4.15 [Luc2P/Hygro] vector (Promega).

Site-directed mutagenesis was performed using complementary primers containing the desired mutation and Phusion Hot Start II DNA polymerase. After PCR, sample was treated with 0.5 U/μl DpnI restriction enzyme (Thermo Scientific) for at least 1 h at 37°C to digest parental DNA template, and transformed into TOP10 competent cells (Invitrogen). All constructs were verified by sequencing.

Treatments and RT-qPCR. At 7–8 DIV, as indicated below each figure, primary neurons were treated with 50 ng/ml human recombinant BDNF (Peprotech). Where indicated, cells were pretreated with inhibitors of different signaling pathways for 30 min before adding BDNF to the medium. Final concentrations of the compounds were as follows: 0.1% DMSO (Sigma-Aldirch), 10 μM U0126 (Tocris Bioscience), 10 μM Bix02189 (Axon Medchem), 1 μM PD184352 (Sigma-Aldrich), 25 nM Az-23 (Axon Medchem), 10 μM Go6983 (Tocris Bioscience), 100 nM Wortmannin (Millipore), 1 μM U73122 (Cayman Europe). Total RNA was isolated using RNeasy Micro or Mini kit (Qiagen) according to the manufacturer's protocol.

For human embryonic kidney HEK293 cell-line, cells grown on six-well plate were transfected using LipoD293 DNA *In Vitro* Transfection Reagent (SignaGen) according to the manufacturer's instruction, using 3 μg of DNA and a ratio of 1:2 DNA–LipoD293 per well. Forty-eight hours after transfection, cells were lysed and RNA was isolated using RNeasy Mini kit (Qiagen).

First strand cDNA was synthesized from 500 to 2000 ng or 5 μg of RNA, for primary neurons or HEK293, respectively, using oligo$(dT)_{20}$ primer and Superscript III Reverse Transcriptase (Invitrogen) as recommended by the manufacturer. Quantitative PCR (qPCR) reactions were performed in triplicates using LightCycler 480 SYBR Green I Master qPCR mix (Roche) on LightCycler 480 II Real Time PCR System (Roche). Levels of *HPRT1* mRNA were used to normalize qPCR data. Before statistical analysis, normalized data were log-transformed and autoscaled using data of all time points for the respective transcripts (Fig. 1*A*), or using data of the respective transcripts in DMSO-treated neurons that were left untreated or treated with BDNF for 3 h (Fig. 1*B*,*C*). For graphical representation, data were backtransformed, with error bars representing upper and lower limits of backtransformed mean ± SEM.

Primer sequences used to amplify rat *HPRT1* and rat *BDNF* transcripts have been described previously (Kairisalo et al., 2009). For amplifying human *HPRT1* and human *BDNF* (h*BDNF*) transcripts, the following primers were used: human *HPRT1* (forward: GCCAGACTTTGTTGGATTTG, reverse: CTCTC ATCTTAGGCTTTGTATTTTG), h*BDNF* exon I (forward: AACAAGA

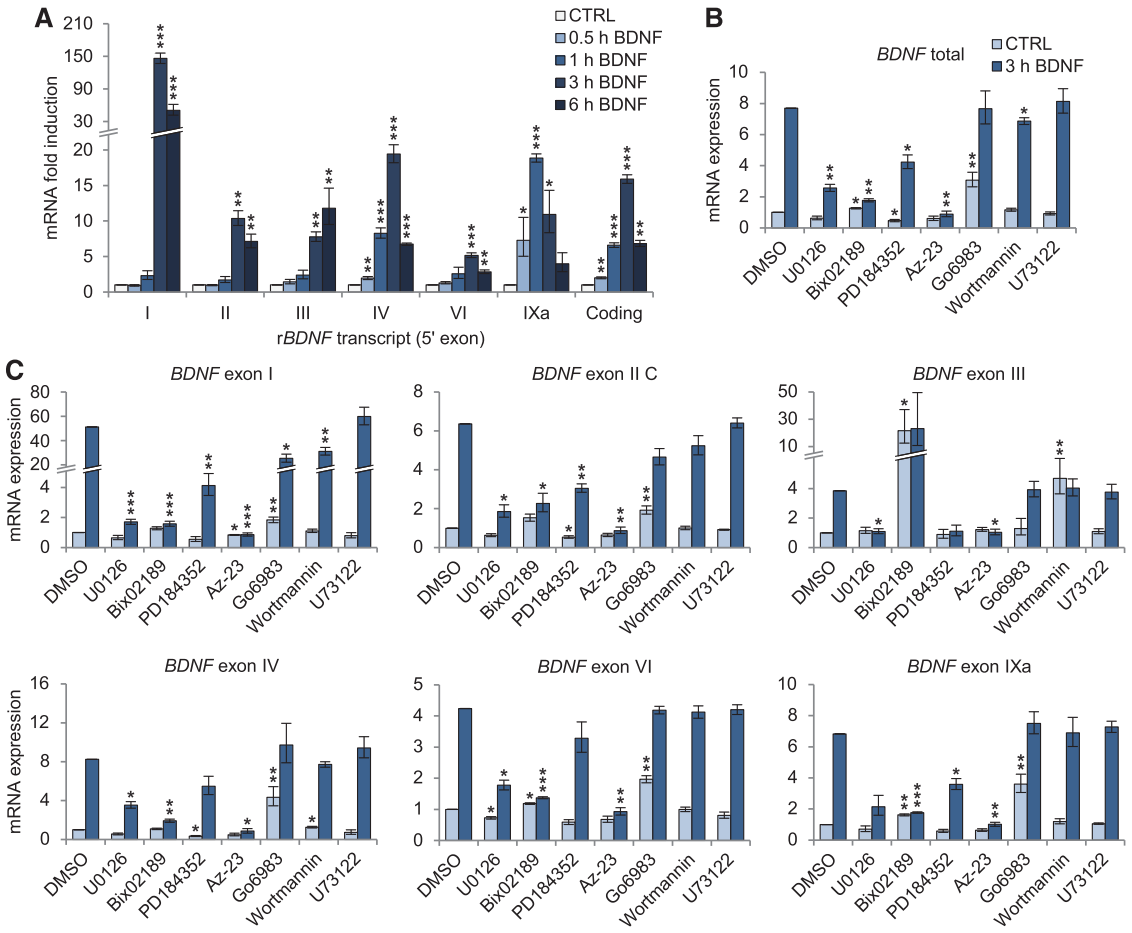

Figure 1. TrkB signaling induces r*BDNF* mRNA expression in primary cortical neurons. ***A***, Expression of all measured *BDNF* transcripts is induced by TrkB signaling. At 7 DIV, rat primary cortical neurons were treated with 50 ng/ml BDNF for the time indicated; endogenous *BDNF* mRNA levels were measured using RT-qPCR with primers specific for transcripts with the respective 5′ exons, or primers against coding region to measure total *BDNF* mRNA. mRNA levels are shown as fold induction relative to the levels of the respective transcripts in untreated cells (CTRL). ***B***, ***C***, *BDNF* induction in response to TrkB signaling is mediated mainly by the MAPK pathway. Inhibitors of different signaling pathways (10 μM U0126, 10 μM Bix02189, 1 μM PD184352, 25 nM Az-23, 10 μM Go6983, 100 nM Wortmannin, 1 μM U73122) or vehicle (0.1% DMSO) was added to the culture medium 30 min before treating primary neurons with 50 ng/ml BDNF for 3 h at 7 DIV. *BDNF* expression was measured using RT-qPCR. For each transcript, mRNA levels are shown relative to the respective transcript's mRNA expression in cells treated with vehicle and not treated with BDNF. Error bars represent SEM of at least three independent experiments in ***A***–***C***. Asterisks indicate statistical significance relative to the respective transcript's untreated control (***A***), or relative to the respective transcript levels in cells treated with DMSO and either left untreated or treated with BDNF for 3 h, respectively (***B***, ***C***). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; *t* test on log-transformed and autoscaled data.

CACATTACCTTCCAGCAT, reverse: CTCTTCTCACCTGGTGGAACATT), h*BDNF* exon III (forward: TGGCTTAGAGGGTTCCCGCT, reverse: ATGGGGGCAGCCTTCATGCA), h*BDNF* exon IV (forward: GAAGTC TTTCCCGGAGCAGCT, reverse: ATGGGGGCAGCCTTCATGCA), and h*BDNF* exon VI (forward: GGTTTGTGTGGACCCCGAGTTC, reverse ATGGGGGCAGCCTTCATGCA).

Western blot analysis. For Western blot analysis, primary neurons were lysed in 1× Laemmli buffer. Proteins were separated using SDS-PAGE and transferred to PVDF membrane (Millipore) using Trans-Blot SD SemiDry Transfer Cell (Bio-Rad). Membranes were blocked in 1× PBS containing 5% skimmed milk and 0.1% Tween 20 for 1 h at room temperature, followed by incubation with primary antibody in 1× PBS containing 2% skimmed milk and 0.1% Tween 20 for 1 h at room temperature or overnight at 4°C. Next, membranes were washed 3 × 5 min with 0.1% Tween 20 solution in 1× PBS. Blots were incubated with secondary antibody for 1 h at room temperature in 1× PBS containing 2% skimmed milk and 0.1% Tween 20. Membranes were washed 3 × 5 min and chemiluminescence signal was detected using SuperSignal West Femto Chemiluminescence Substrate (Thermo Scientific) and ImageQuant LAS 4000 bioimager (GE Healthcare).

Antibodies against AP-1 family proteins were obtained from Santa Cruz Biotechnology: c-Jun (1:5000, sc-1694x, H2808), JunB (1:5000, sc-8051x, B1611), JunD (1:5000, sc-74x, G1911), c-Fos (1:5000, sc-7202x, C3108), FosB (1:5000, sc-48x, K1811), Fra1 (1:5000, sc-605x, G1612), and Fra2 (1:1000, sc-604x, G1911). GAPDH antibody was from Millipore (1:5000, MAB374, lot 2506322). Stabilized Goat Anti-Mouse IgG (H+L) Peroxidase-Conjugated (32430) and Stabilized Goat Anti-Rabbit IgG (H+L) Peroxidase-Conjugated (32460) secondary antibodies (1:5000 dilution) used in Western blot analysis were purchased from Thermo Scientific.

Transduction using adeno associated virus vectors. Adeno associated virus (AAV) virions coding EGFP or A-Fos under the control of human *Synapsin I* promoter were prepared as described previously (Koppel et

al., 2015). Titer of infectious particles was assessed by immunocytochemistry using either anti-EGFP antibody (a kind gift from Andres Merits, Institute of Technology, University of Tartu, Tartu, Estonia) or anti-FLAG antibody (F1804, Sigma-Aldrich). Primary neurons were infected with two to three multiplicity of infection at 2 DIV, immunocytochemistry, and Western blot analysis or BDNF treatment and RNA extraction were performed at 8 DIV. RT-qPCR data were log-transformed and autoscaled using data of the respective transcripts in neurons infected with AAV-EGFP. Statistical analysis was performed on autoscaled data. For graphical representation, data were backtransformed to linear scale, with error bars indicating upper and lower limits of backtransformed mean ± SEM.

DNA transfection and luciferase reporter assay. For luciferase reporter assay, neurons grown on 48-well plates were transfected at 6 DIV using Lipofectamine 2000 (Invitrogen) reagent according to the manufacturer's recommendations. Lipofectamine–DNA ratio of 2:1 was used, with 0.5 μg of DNA per well. Where applicable, *BDNF* promoter construct and plasmids coding effector proteins or empty pQM vector were cotransfected at 1:1 ratio. For normalization, 20 ng p*PGK*/pGL4.83 plasmid, expressing *Renilla* luciferase under the control of murine *PGK* promoter, was cotransfected per well.

At 7 DIV, neurons were treated with 50 ng/ml human recombinant BDNF (Peprotech). Cells were lysed after 8 h of BDNF treatment using 1× Passive Lysis Buffer (Promega) and luciferase assays were performed using Dual-Glo Luciferase Assay System (Promega). Luminescence was measured in duplicate samples using GeniOS Pro (Tecan). For presenting data in relative luciferase units (RLUs), background signal from untransfected neurons was subtracted from both firefly luciferase (FFLuc) and *Renilla* luciferase (RLuc) signals. Background corrected FFLuc signals were normalized using RLuc signals. Normalized data were log-transformed and autoscaled using data of wild-type (WT) promoter construct (cotransfected with empty pQM vector, where applicable). For statistical analysis, autoscaled data were used. For graphical representation of results, means, and mean ± SEM of autoscaled data were calculated and backtransformed to linear scale. Error bars represent upper and lower limits of backtransformed mean ± SEM.

Electrophoretic mobility shift assay. For preparation of cell lysates, neurons grown for 7 DIV on a 10 cm dish were washed with 1× PBS and collected in 200 μl ice-cold sonication buffer containing 20 mM HEPES-KOH, pH 7.9, 25% glycerol, 500 mM KCl, 1.5 mM $MgCl_2$, 0.4 mM EDTA, 1 mM EGTA, 5 mM DTT, 0.5 mM PMSF, 1× cOmplete Protease Inhibitor Cocktail (Roche), and 1× PhosSTOP Phosphatase Inhibitor Cocktail (Roche). Lysates were then incubated on ice for 15 min, sonicated until no viscous matter remained, and centrifuged at 4°C and 16,100 × *g* for 10 min. Aliquots of supernatant were snap-frozen in liquid nitrogen and stored at −80°C.

Oligonucleotides (5 pmol per reaction) were labeled with T4 polynucleotide kinase (Thermo Scientific) using ATP γ-^{32}P (PerkinElmer). After labeling, sense and antisense oligonucleotides were annealed in annealing buffer (50 mM NaCl, 1 mM EDTA, 0.2× PNK buffer A, 100 μl total volume) by placing the tubes in a 95°C water bath and allowed to cool to room temperature overnight. Annealed oligonucleotides were separated from unincorporated label using Sephadex G-50 (Pharmacia Fine Chemicals) resin.

Electrophoretic mobility shift assay (EMSA) binding reaction contained ~10 μg (estimated using BCA Protein Assay Kit; Pierce) crude whole-cell protein extract (~2.25 μl lysate), 1 μg poly(dI-dC; Sigma-Aldrich), 0.1 mg/ml BSA (Thermo Scientific), and 1× binding buffer (10 mM HEPES-KOH, pH 7.5, 0.5 mM EDTA, 2 mM $MgCl_2$, 0.05% NP-40, 4% Ficoll-400) in a total volume of 20 μl. Binding reactions were equilibrated on ice for 10–15 min, followed by addition of 75 fmol radioactive probe and an additional 20 min incubation at room temperature. For competition experiments, tenfold excess of unlabeled competitor was added 5 min before adding the probe. For supershift experiments, 1 μg of antibody was added to the binding reaction and incubated for 1 h at room temperature before adding the probe. Electrophoresis was performed using 4–5% non-denaturing polyacrylamide gels containing 0.25× TBE and 0.01% NP-40, with 0.5× TBE as electrophoresis buffer.

ChIP. Neurons grown on 10 cm dishes were left untreated or treated with 50 ng/ml BDNF for 2 h at 7 DIV. Following treatment, chromatin was crosslinked for 10 min using 1% formaldehyde, crosslinking reaction was quenched by adding a final concentration of 200 mM glycine to the medium and incubating for 10 min. Cells were washed twice with 1× PBS and scraped together in ice-cold lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0, cOmplete Protease Inhibitor Cocktail, Roche). Lysates were kept on ice and sonicated to obtain DNA fragments of an average length <1 kbp. After sonication, lysate was centrifuged for 5 min at 16 100 g to remove insoluble material. Lysate protein content was measured using BCA Protein Assay kit (Pierce). Lysate (700–1000 μg protein per IP) was diluted 1:9 with dilution buffer (1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, cOmplete Protease Inhibitor Cocktail, Roche) and samples were rotated with 5 μg pan-Fos antibody (Santa Cruz Biotechnology, sc-253x, K0110), c-Jun antibody (Santa Cruz Biotechnology, sc-1694x, H2808), or no antibody overnight at +4°C. Then, 50 μl of 50% Protein A Sepharose CL-4B (GE Healthcare) slurry that had been preabsorbed with 200 μg/ml BSA and 10 μg/ml sheared salmon sperm DNA overnight at +4°C, was added to each sample and rotated an additional 2–4 h at +4°C. Sepharose-chromatin complexes were washed four times with wash buffer (1% Triton X-100, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, cOmplete Protease Inhibitor Cocktail, Roche), and once with final wash buffer (1% Triton X-100, 0.1% SDS, 500 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, cOmplete Protease Inhibitor Cocktail, Roche). Immune complexes were eluted three times using 50 μl elution buffer (1% SDS, 100 mM $NaHCO_3$); eluates from the same samples were combined. Chromatin was de-crosslinked by incubating samples with 250 mM NaCl at 65°C overnight. DNA was purified using QIAquick PCR Purification Kit (Qiagen). Abundance of target genomic regions was quantified using qPCR with the following primers: rat *BDNF* (r*BDNF*) promoter I (forward: ACGTCCGCTG GAGACCCTTAGT, reverse: GGCAGCCTCTCTGAGCCAGTTA), r*BDNF* promoter III (forward: TAGGTGAGAACCTGGGGCAA, reverse: CTTG AGCTTCCCCAACCTCG), rBDNF promoter IV (forward: ATGCAATGCC CTGGAACGGAA, reverse: CGGTGAATGGGAAAGTGGGTGG), r*BDNF* promoter VI (forward: CGCTGTCTGACCAATCGAAG, reverse: GTTTC CTTCTCCAAGCCGGG), rat *matrix metallopeptidase 9* (*MMP9*) promoter (forward: CTTTGGGCTGCCCAACACACA, reverse: GAAGCAGAATTT GCGGAGGTTTT), unrelated region (forward: TAGACCCAGGAGGGA GTTATTTAAGAG, reverse: TTGGGAATGCAATGCAGTGTGTAC). Data were calculated as a percentage of immunoprecipitated DNA relative to the respective target levels in input DNA. Data were log-transformed, means and mean ± SEM were calculated, and statistical analysis was performed. For graphical representation, data were backtransformed into linear scale with error bars representing backtransformed mean ± SEM.

For ChIP analysis in HEK293 cells, cells grown on 10 cm dishes were transfected with polyethylenimine (PEI), using 10 μg DNA per 10 cm dish and DNA:PEI ratio of 1:2. Twenty-four hours after transfection, chromatin was fixed and cells were lysed as with primary neurons. Lysate made from cells grown on one 10 cm dish was used per IP, 50 μl of anti-V5 agarose 50% slurry (Sigma-Aldrich) that had been preabsorbed with 200 μg/ml BSA and 10 μg/ml sheared salmon sperm DNA, was used per sample and rotated overnight at 4°C. Washes and elution were performed as with ChIP analysis from lysates of primary neurons. The following primers were used to quantify immunoprecipitated DNA regions with qPCR: h*BDNF* promoter I (forward: TCACGACCTCATCGGCTGGA, reverse: GACGACTAACCTCGCTGTTT), h*BDNF* promoter IV (forward: CTGGTAATTCGTGCACTAGAGT, reverse: CACGAGAGGGCTCCACG GT), human *metallothionein 2A* (*MT2A*) promoter (forward: GTTCGCT GGGACTTGGAGG, reverse: ACTCGTCCCGGCTCTTTCTA), unrelated region (forward: GTCATGAGGGCTCCACTCTTA, reverse: AAGGGCAAA-GAGGGCAACAGA). Data were normalized to the levels of the respective target in input DNA and calculated as fold induction relative to the respective levels in pQM transfected cells. Data were log-transformed and autoscaled, means and mean ± SEM were calculated, and statistical analysis was performed. For graphical representation, data were backtransformed into linear scale with error bars representing backtransformed mean ± SEM.

Statistical analysis. For statistical analysis, all data were log-transformed to obtain normal distribution of the data. Where noted in figure

legends, log-transformed data were autoscaled according to Willems et al. (2008) before statistical analysis, to account for variations between biological replicates. As the data does not meet ANOVA's requirement of homoscedasticity (due to normalization and autoscaling, the control groups have zero variance), two-tailed unequal variance *t* test (Welch's *t* test) was used on log-transformed data instead of ANOVA. Only hypothesis specified *a priori* were tested for statistical significance. To preserve the power of statistical analysis, *p* values were left uncorrected for multiple comparisons as recommended by Rothman (1990), Feise (2002), and Streiner and Norman (2011). Differences were considered statistically significant when $p < 0.05$. In all experiments, data are presented as mean ± SEM.

Results

Expression of *BDNF* mRNA in rat primary cortical neurons is induced in response to TrkB signaling

To investigate *BDNF* gene autoregulation and to determine the temporal pattern of TrkB signaling-induced *BDNF* mRNA transcription, we used rat primary cortical neuron cultures. At 7 DIV, neurons were either left untreated or treated with 50 ng/ml BDNF for 30 min to 6 h, after which the levels of different *BDNF* transcripts were measured using RT-qPCR (Fig. 1*A*).

Our results showed that the expression of *BDNF* mRNA was strongly induced in response to BDNF treatment of primary neurons (Fig. 1*A*), with a ~2-fold induction seen already at 30 min and a peak induction of ~16-fold at 3 h of BDNF treatment, after which the levels started to decline. Next, we measured the expression of different *BDNF* transcripts and found that the expression of exon I, II, III, IV, VI, and IXa transcripts was induced after treating primary neurons with BDNF. The expression of exon V transcripts was too low to measure reliably.

The induction of exon IV transcripts was similar to that of total *BDNF* mRNA, which is in accordance with the fact that exon IV-containing transcripts are the most abundant *BDNF* transcripts in the rat cerebral cortex (Timmusk et al., 1994; Aid et al., 2007), thus comprising the majority of total *BDNF* mRNA measured. A statistically significant ~2-fold increase in the expression of exon IV transcripts was seen at 30 min, and a maximum induction of ~19-fold was detected after 3 h of BDNF treatment.

The overall temporal pattern of induction for exon I, II (II C, longest splice variant), and VI transcripts was similar, with a slight (~2-fold) but statistically insignificant induction after 1 h of treatment with BDNF, and a statistically significant maximum induction at 3 h of stimulation. The highest induction in response to BDNF was seen for exon I-containing transcripts, with a peak of ~145-fold increase at 3 h. A moderate induction was seen for exon II transcripts (~10-fold) and a low induction for exon VI transcripts (~5-fold) at 3 h time point. Interestingly, the levels of exon III-containing transcripts continued to rise, albeit statistically insignificantly ($p = 0.18$ for 6 h vs 3 h time point) even after 3 h of treatment, reaching ~12-fold induction at 6 h.

Notably, the induction of exon IXa-containing transcripts showed a faster temporal pattern than other *BDNF* transcripts; a strong ~7-fold induction was seen already after 30 min of treatment, with a peak induction of ~19-fold at 1 h, after which the levels of exon IXa transcripts started to decline.

Next, we decided to check which signaling pathways were responsible for the TrkB signaling-dependent *BDNF* mRNA induction. It is known that TrkB signaling activates three major pathways: MAPK cascade, PI-3K and AKT pathway, and PLCγ1-dependent activation of PKC and intracellular calcium stores (Reichardt, 2006). Therefore, we applied inhibitors of these pathways 30 min before treating neurons for 3 h with BDNF. RNA was extracted and the expression levels of different *BDNF* transcripts were measured using RT-qPCR (Fig. 1*B*,*C*). We found that Az-23, a potent Trk inhibitor (Thress et al., 2009), abolished the BDNF-dependent *BDNF* mRNA induction, indicating that TrkB signaling, and not signaling through $p75^{NTR}$, was responsible for the induction.

Using MAPK cascade inhibitors U0126 (MEK1 and MEK2 inhibitor), Bix02189 (ERK5 inhibitor), and PD184352 (ERK1/2 inhibitor), we found that both ERK5 and ERK1/2 pathways contribute to the TrkB signaling-induced expression of *BDNF* mRNA. Pretreating neurons with U0126 decreased the BDNF-induced levels of all measured transcripts. Similar effect was seen for Bix02189, with the exception of exon III transcripts, for which Bix02189 strongly increased both the basal and the induced levels (both to ~30-fold compared with vehicle-treated neurons not stimulated with BDNF). The robust increase of exon III mRNA levels by the ERK5 inhibitor Bix02189 is possibly accountable to an off-target effect, as similar results were not seen when inhibiting both ERK1/2 and ERK5 with U0126. Similarly to U0126 treatment, PD184352, an ERK1/2-specific inhibitor, caused a statistically significant decrease in the BDNF-induced levels of exon I, II, and IXa transcripts.

Inhibiting PI-3K with Wortmannin decreased the BDNF-induced levels of total *BDNF* mRNA by ~10% (Fig. 1*B*). At the level of different transcripts, the BDNF-induced expression of exon I-containing transcripts was decreased by 40% compared with exon I mRNA levels in vehicle-treated neurons stimulated with BDNF (Fig. 1*C*). Interestingly, Wortmannin increased the basal, but not the BDNF-induced levels of exon III transcripts. The expression of other *BDNF* transcripts was not affected by inhibiting PI-3K. Together with the results obtained using Bix02189, this indicates that the regulation of exon III transcript basal expression is significantly different from that of other *BDNF* transcripts.

Applying PKC inhibitor Go6983 to the media did not have significant effect on the TrkB signaling-induced levels of total *BDNF* mRNA (Fig. 1*B*). Nevertheless, Go6983 decreased the induction of exon I-containing transcripts by ~50%, but did not have a statistically significant effect on the BDNF-induced levels of other transcripts (Fig. 1*C*). Notably, Go6983 increased the basal levels of all transcripts except exon III transcripts. However, U73122, a PLC inhibitor, did not have an effect on the expression of any *BDNF* transcripts neither at 1 μM (Fig. 1*C*) nor 2.5 μM concentration (data not shown). As inhibiting PLC upstream of PKC failed to reproduce the effect of Go6983 on the basal levels of *BDNF* expression, the upregulation by Go6983 can probably be attributed to an off-target effect.

Collectively, rat *BDNF* expression is strongly induced by applying BDNF to cultured primary neurons, indicating the existence of a positive feedback loop in the regulation of *BDNF* expression, and this feedback loop is mainly regulated by the activation of MAPK cascades downstream of TrkB receptor.

TrkB signaling-dependent induction of rat *BDNF* exon I, III, and VI transcripts requires AP-1 proteins

There is evidence that BDNF stimulates AP-1 binding and AP-1-dependent transcriptional activity in neurons (Gaiddon et al., 1996; Okamoto et al., 2003). Therefore, we decided to investigate the possible role of AP-1 transcription factors in the regulation of *BDNF* expression by TrkB signaling. By using RT-qPCR and Western blot analysis, we determined that the expression of mRNA and also protein of all AP-1 members was induced in response to TrkB signaling in our cortical neuron cultures (Fig. 2*A*,*B*).

To test whether AP-1 transcription factors play a role in the TrkB signaling-dependent *BDNF* induction, we used AAV-mediated overexpression of FLAG-tagged A-Fos (Fig. 3*A*,*B*), a dominant-negative form of AP-1, consisting of the c-Fos leucine

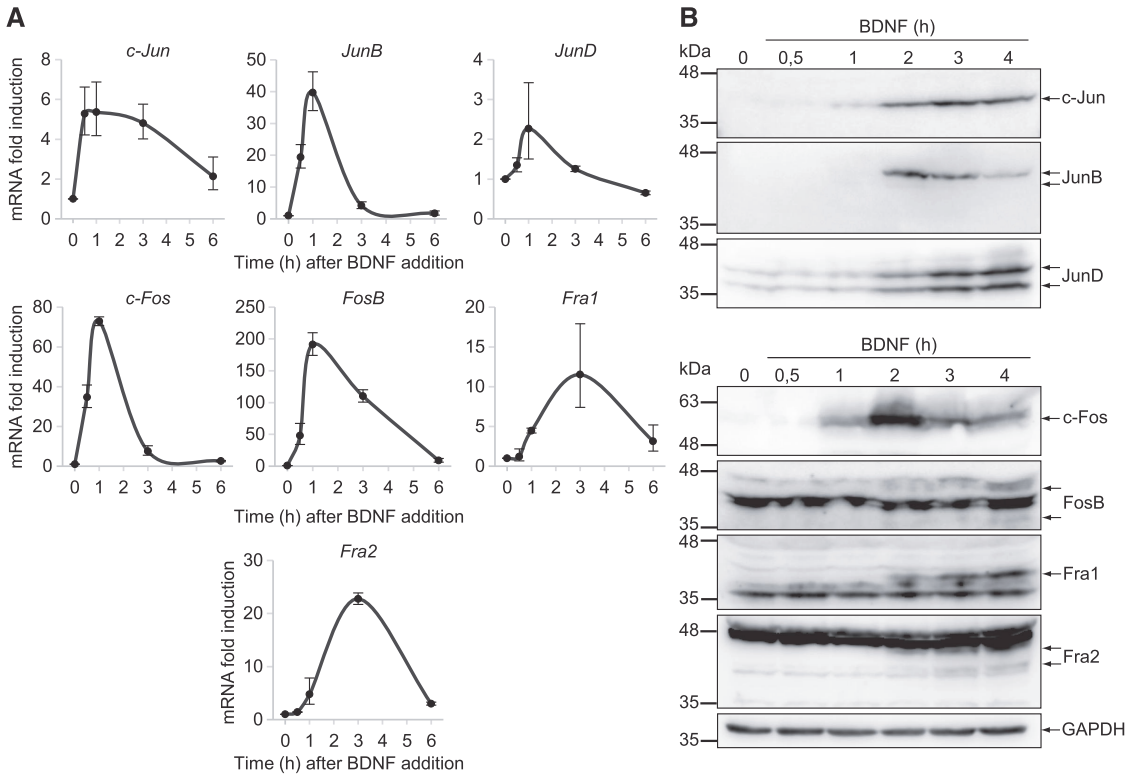

Figure 2. TrkB signaling induces expression of AP-1 family members in rat primary cortical neurons. ***A***, RT-qPCR analysis of AP-1 family mRNA levels after treating primary neurons with 50 ng/ml BDNF. mRNA levels are shown as fold induction relative to the levels of the respective transcripts in untreated cells. Error bars represent SEM of three independent experiments. ***B***, Western blot analysis of neurons treated with 50 ng/ml BDNF for the indicated time at 7 DIV showing that the expression of all the members of the AP-1 family is induced upon TrkB signaling. Bands corresponding to different AP-1 proteins or GAPDH are shown with arrows.

zipper domain with an acidic amphipathic extension appended to the N-terminus (Olive et al., 1997). Infected neurons were treated with BDNF at 8 DIV for different time periods, and *BDNF* transcript levels were measured using RT-qPCR. We found that overexpressing A-Fos did not change the basal level of *BDNF* mRNA expression (Fig. 3*C*). However, A-Fos overexpression decreased the induced level of total *BDNF* mRNA by ~30% at 3 h of BDNF treatment (from 9.8- to 6.9-fold induction compared with EGFP-expressing neurons not treated with BDNF).

At the level of alternative transcripts, A-Fos overexpression did not change the basal expression of any *BDNF* transcript, but effectively reduced the BDNF-dependent induction of exon I-containing transcripts by 86% and 73% at 3 and 6 h time point, respectively (Fig. 3*D*). However, the induction of exon I transcripts was not completely abolished by the overexpression of A-Fos, with a ~7.6-fold induction remaining at 3 h time point compared with untreated neurons overexpressing EGFP. This indicates that the induction of exon I transcripts might also be regulated by other transcription factors in addition to AP-1, or that the remaining induction of exon I transcripts occurs in the minority of cells (<10%) that were not infected. A-Fos also diminished the TrkB signaling-dependent induction of exon III-containing transcripts, with the induction being completely abolished at 3 h time point, and reduced by 36% at 6 h treatment with BDNF, and reduced the induced levels of transcript VI by ~18 and ~40% at 3 and 6 h BDNF treatment, respectively. The induction of exon II, IV, and IXa transcripts was not changed by overexpressing A-Fos, indicating that the integrity of TrkB signaling itself was not compromised. Collectively, these results indicate that AP-1 activity is needed for the TrkB signaling-dependent transcription of *BDNF* exon I, III, and VI transcripts, but not for their basal expression.

Induction of *BDNF* promoter I in response to TrkB signaling is mediated by AP-1 proteins

As *BDNF* exon I-containing transcripts showed the strongest induction in response to BDNF treatment, we first decided to elucidate the mechanism behind this induction. For this, we transfected plasmids containing the human or rat *BDNF* promoter I (pI) regions in front of luciferase coding sequence, together with either A-Fos, or c-Fos and c-Jun overexpression vectors into rat primary neurons. Neurons were treated with BDNF for 8 h to induce TrkB signaling, after which cells were lysed and luciferase activities were measured. According to our reporter assays, both rat (Fig. 4*A*) and human (Fig. 4*B*) *BDNF* pI activity were upregulated by BDNF treatment (~12.2- and ~10.9-fold induction, respectively). Cotransfecting A-Fos together with the reporter constructs effectively decreased the induced levels of promoter activity for both rat and human *BDNF* pI, by 88 and 83%, respectively. Overexpressing AP-1 proteins c-Jun and c-Fos increased the promoter activity of human and rat *BDNF* pI in both unstimulated and BDNF-treated cells (Fig. 4*A*,*B*).

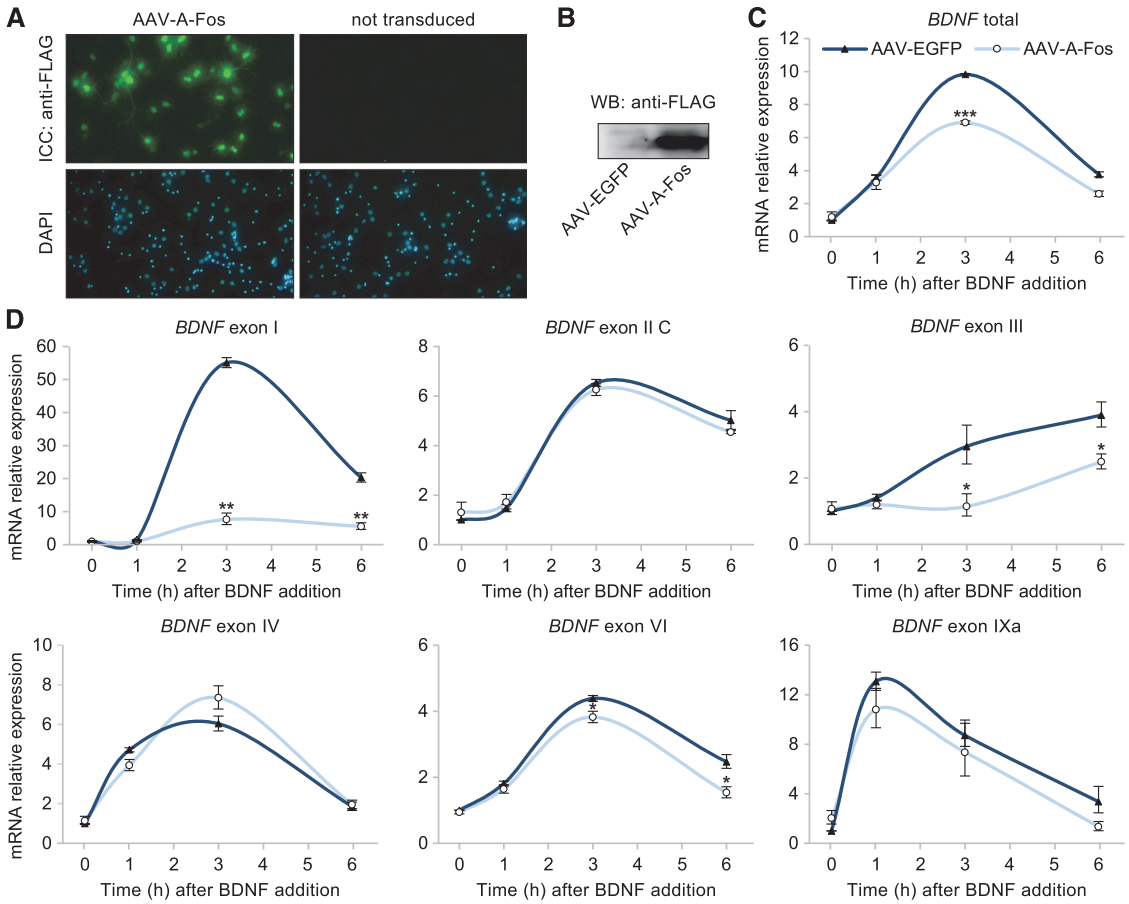

Figure 3. A-Fos, a dominant-negative form of AP-1, interferes with TrkB signaling-dependent induction of *BDNF* mRNA. ***A***, ***B***, Immunocytochemistry (ICC) (***A***) and Western blot (WB) analysis (***B***) showing FLAG-tagged A-Fos expression in 8 DIV neurons infected with AAV-A-Fos at 2 DIV. ***C***, ***D***, AP-1 activity is needed for the induction of *BDNF* mRNA (***C***) and exon I, III, and VI transcripts (***D***) upon TrkB signaling. Primary neurons were infected with AAVs encoding either EGFP or A-Fos at 2 DIV and treated with 50 ng/ml BDNF at 8 DIV. *BDNF* transcript levels were measured using RT-qPCR. mRNA levels are shown relative to the respective transcript levels in untreated neurons transduced with AAV-EGFP. Error bars indicate SEM of at least three independent experiments, and asterisks indicate statistical significance between the respective *BDNF* transcript levels in AAV-A-Fos and AAV-EGFP transduced neurons at the respective time points. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; *t* test on log-transformed and autoscaled data.

Next, we checked the effect of overexpressing different combinations of AP-1 transcription factors on human *BDNF* (h*BDNF*) pI activity. For that, rat primary neurons were cotransfected with the h*BDNF* pI luciferase construct and combinations of expression constructs encoding different AP-1 factors. The results of the luciferase assay (Fig. 4*C*) showed that overexpression of Fos family members alone was not able to elevate the basal or induced levels of h*BDNF* pI. This is possibly because of the fact that Fos family proteins can only bind DNA when heterodimerized with a member of the Jun family, ATFs, or other possible partner proteins (Eferl and Wagner, 2003). Interestingly, overexpression of FosB reduced the induced levels of h*BDNF* pI upon BDNF treatment. Overexpressing Jun family members alone increased the basal h*BDNF* pI activity ~1.5- to 2-fold, whereas only c-Jun was able to raise the pI-dependent luciferase levels in BDNF-treated cells (~2-fold). In combination with Fos family proteins, Jun family factors were more potent activators of h*BDNF* pI than Jun family members alone. Overexpression of c-Jun together with different Fos family proteins greatly increased the basal activity of pI (from ~7-fold for Fra1, up to ~15-fold for c-Fos), and also the BDNF-induced pI activity (~3-fold). JunB, on the other hand, was not a potent activator of h*BDNF* pI even together with Fos family proteins: although a modest increase (~3- to 4-fold) in the basal levels of pI expression was detected, only JunB in combination with c-Fos or Fra2 was able to slightly raise the induced levels of pI (~1.7-fold). Albeit with a less potent activation of h*BDNF* pI, overexpressing JunD together with Fos family transcription factors showed similar results as overexpressing c-Jun in combination with Fos family members.

Collectively, these results indicate that both rat and human *BDNF* pI are regulated by AP-1 proteins, and different combinations of AP-1 proteins can elicit distinct responses on the activity of h*BDNF* pI.

AP-1 proteins regulate human *BDNF* promoter I activity through two conserved AP-1 elements

To determine the location of *cis*-elements necessary for the BDNF-dependent h*BDNF* pI induction, we used deletion mutants of the pI reporter, where the promoter region was shortened

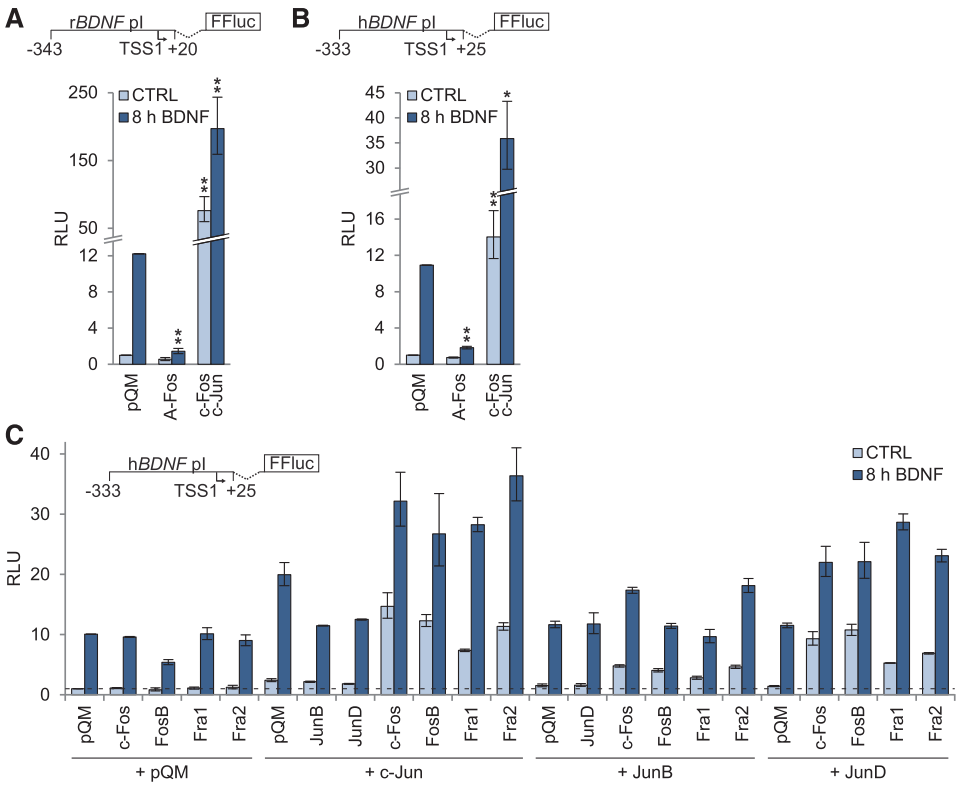

Figure 4. AP-1 proteins mediate *BDNF* promoter I activity in response to TrkB signaling. ***A***, ***B***, AP-1 proteins regulate both rat (***A***) and human (***B***) *BDNF* promoter I. At 6 DIV, rat primary neurons were cotransfected with *BDNF* pI-dependent luciferase reporter constructs (indicated above the graphs) together with constructs coding either A-Fos, or c-Fos, and c-Jun, or empty vector pQM. At 7 DIV, neurons were either treated with 50 ng/ml BDNF for 8 h or left untreated (CTRL), and luciferase activities were measured. ***C***, AP-1 complex combinations differentially regulate the activity of h*BDNF* pI. Rat primary neurons were cotransfected with the h*BDNF* pI construct together with different combinations of vectors encoding AP-1 proteins or empty vector (pQM) as indicated below the graph. Neurons were left untreated or treated with 50 ng/ml BDNF for 8 h at 7 DIV, after which luciferase activities were measured. ***A***–***C***, Luciferase activity in untreated cells transfected with pI reporter construct and empty pQM vector was arbitrarily set as 1 (***C***, dashed line). Numbers next to the schematic figures of promoter constructs indicate distance from the most 5′ transcription start site (TSS1; according to Pruunsild et al., 2007) of rat (***A***) or human (***B***, ***C***) *BDNF* exon I. Error bars represent SEM from three (***A***, ***B***) or two (***C***) independent experiments. Asterisks indicate statistical significance relative to luciferase activities in cells transfected with pI reporter construct and empty pQM vector that were either left untreated or treated with BDNF for 8 h, respectively (***A***, ***B***). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; *t* test on log-transformed and autoscaled data.

from the 5′ end (pI SphI and pI Eco31 constructs; Fig. 5*B*). We found that while the region between −333 and −242 was not needed for the BDNF-dependent promoter activation (pI SphI construct), removing the region −333 to −166 (pI Eco31 construct) decreased the fold induction by 69% compared with the pI WT reporter. Additionally, we determined that TrkB signaling also induced the activity of the −166 to +25 region (pI Eco31 construct) by ~4.4-fold. These results indicate that the *cis*-elements necessary for the TrkB signaling-dependent activation of h*BDNF* pI are located between −242 and +25 bp relative to the transcription start site, and that these elements reside both in the −242 to −166 bp region and the −166 to +25 bp region.

Using bioinformatic analysis of human and rat *BDNF* pI sequences, we found two putative AP-1 sites (TCACTCA, designated as AP1–1, and TTAGTCA, designated as AP1-2) in BDNF pI that are conserved between human and rat (Fig. 5*A*). According to Seldeen et al. (2009), *cis*-elements differing from the consensus AP-1 element (TGAC/GTCA) by any single nucleotide are capable of binding AP-1 transcription factors. Furthermore, functional AP-1 elements with the core sequence TTAGTCA have been described in the *SV40* promoter (Angel et al., 1987) and in the rat *follicle stimulating hormone receptor* (*FSHR*) promoter (Griswold et al., 2001).

To investigate the role of the identified AP1-1 and AP1-2 elements in h*BDNF* pI activation in response to TrkB signaling, we used reporter constructs where these putative AP-1 sites were mutated (Fig. 5*B*). Mutating the AP1-1 site (pI AP1–1m construct) decreased h*BDNF* pI induction upon BDNF treatment from 14.0-fold to 7.6-fold, whereas mutation of the AP1-2 site (pI AP1-2m construct) lowered the induction to 4.1-fold. Mutating both the AP1-1 and the AP1-2 site (pI AP1–1m2m construct) did not further decrease the fold induction compared with mutating only the AP1-2 site. It has been reported that the AP1-1 site overlaps with a functional PasRE *cis*-element necessary for h*BDNF* pI induction in response to neuronal activity (Pruunsild et al., 2011). Interestingly, mutation of the PasRE (mutation according to Pruunsild et al., 2011) enhanced the induction of h*BDNF* pI in response to TrkB signaling by 2.3-fold, indicating a possible competitive regulation of pI activity by AP-1 and bHLH-PAS proteins. Together, these results show that the AP1-1 and

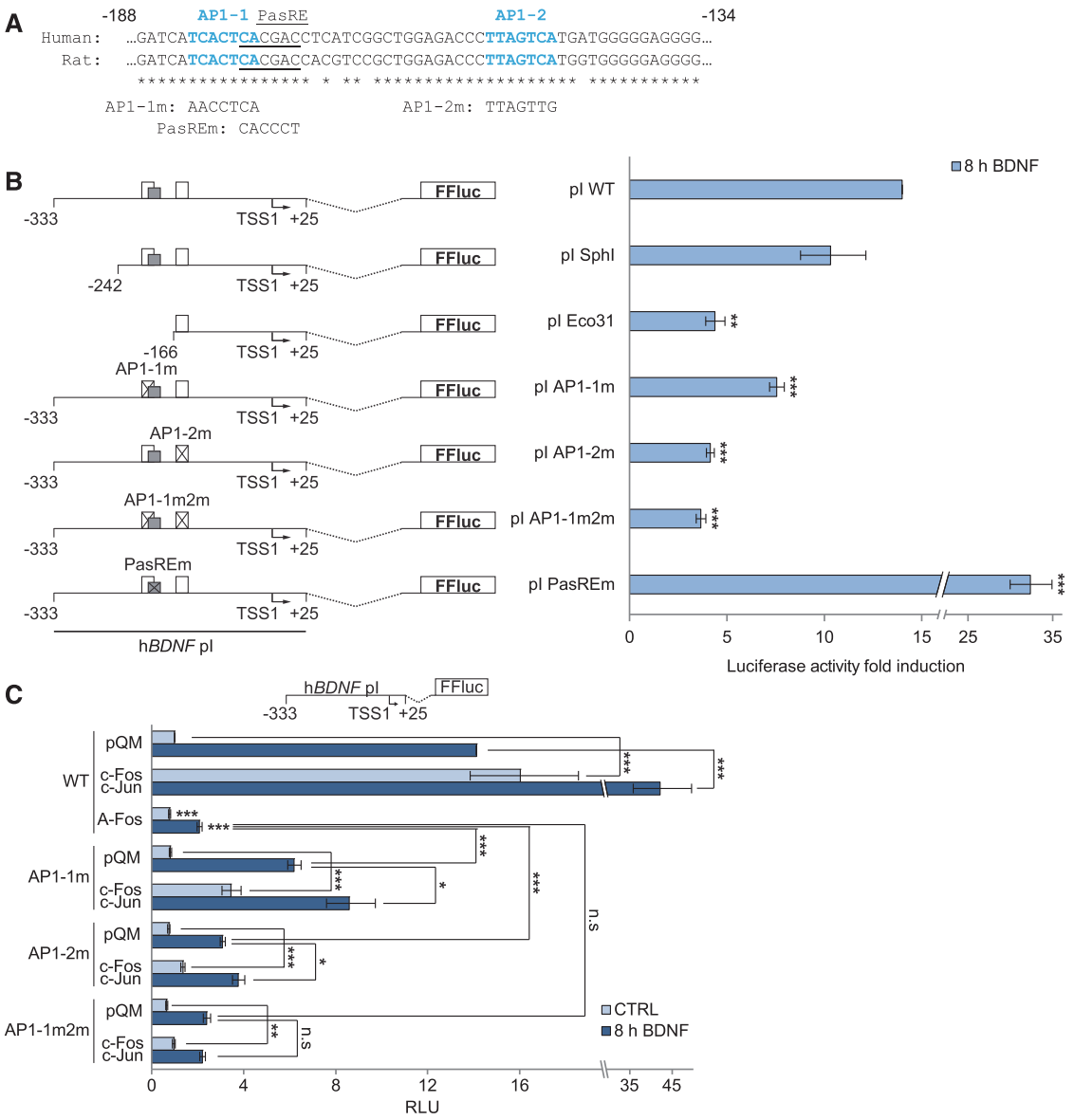

Figure 5. The effect of AP-1 proteins on h*BDNF* promoter I activity is mediated by two AP-1 *cis*-elements. ***A***, Alignment of the region in *BDNF* pI containing two putative AP-1 sites, designated here AP1-1 and AP1-2. The AP-1 sites are shown in blue and bold, the PasRE site overlapping the AP1-1 site is underlined. Mutations used in promoter constructs in this study are shown below the alignment. Numbers next to the alignment or schematic figures of promoter constructs indicate distance from the most 5′ transcription start site (TSS1; according to Pruunsild et al., 2007) of h*BDNF* exon I (***A***–***C***). ***B***, Schematic representation of the h*BDNF* pI constructs used in the current study (left), with luciferase signal induction in neurons transfected with the respective promoter constructs in response to BDNF treatment for 8 h (right). White boxes depict the AP-1 sites; gray boxes represent the PasRE site. ***C***, AP1-1 and AP1-2 sites mediate h*BDNF* pI response to TrkB signaling. At 6 DIV, neurons were cotransfected with the designated h*BDNF* pI luciferase reporter constructs together with different expression constructs or empty pQM vector. At 7 DIV, neurons were left untreated (CTRL) or treated with 50 ng/ml BDNF for 8 h, followed by measurement of luciferase activities. Luciferase activity in untreated cells transfected with the pI WT reporter construct and pQM expression construct was arbitrarily set as 1. Error bars represent SEM of at least three (***B***) or at least five (***C***) independent experiments. Asterisks indicate statistical significance relative to luciferase fold induction in cells transfected with WT promoter construct (***B***), or relative to luciferase activities in cells cotransfected with WT promoter construct and empty pQM vector that were either left untreated or treated with BDNF for 8 h, respectively, or between indicated groups (***C***). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; *t* test on log-transformed and autoscaled data.

AP1-2 sites are both needed for the induction of h*BDNF* pI by TrkB signaling, with the AP1-2 site having the predominant role.

As the combination of c-Fos and c-Jun overexpression was one of the most efficient combinations of AP-1 proteins in inducing transcription from h*BDNF* pI in both untreated and BDNF-treated neurons (Fig. 4*C*), we decided to use this combination to investigate the role of the AP1-1 and AP1-2 sites in the regulation of h*BDNF* pI by AP-1 proteins. For this, we used the WT h*BDNF*

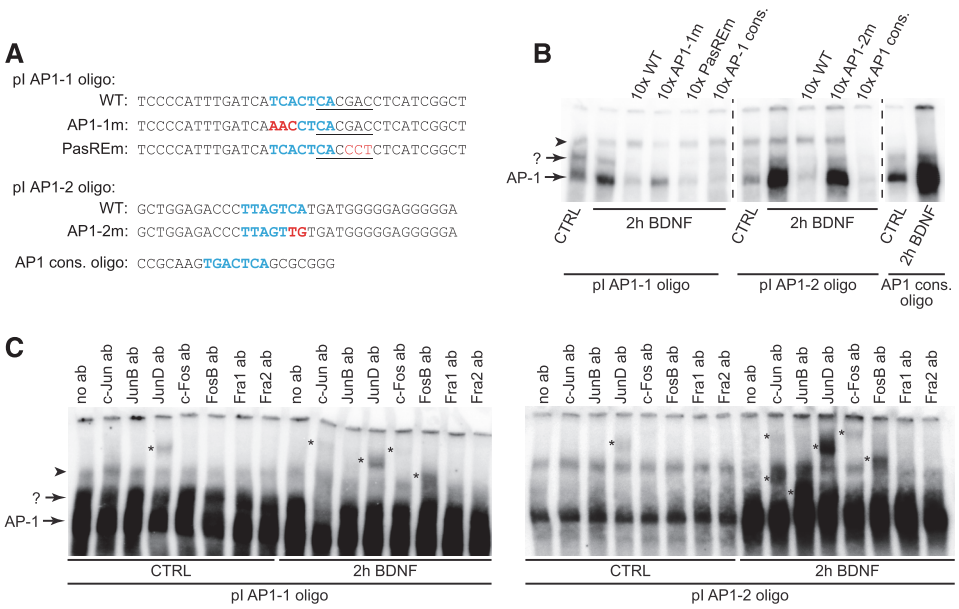

Figure 6. h*BDNF* promoter I binds AP-1 family members *in vitro*. ***A***, Oligonucleotides used in EMSA experiments. The sequence of the sense oligo is shown for simplicity; double-stranded oligos were used in all EMSA experiments. The AP-1 sites are shown in blue and bold, the PasRE site is underlined. Mutated nucleotides are shown in red. AP-1 site from the human *MT2A* promoter was used as an AP-1 consensus oligo. ***B***, EMSA showing AP-1 protein complex binding to h*BDNF* pI oligos. The probes and lysates used are shown below the panel. CTRL and 2 h BDNF denote lysates of untreated neurons and lysates of neurons treated with 50 ng/ml BDNF for 2 h, respectively. Where indicated above the panel, 10-fold excess of unlabeled oligo was added to the binding reaction before adding the probe. ***C***, Supershift experiments showing the composition of the AP-1 complex binding to h*BDNF* pI. Where indicated above the panel, 1 μg of the respective antibody (ab) was added to the binding reaction and incubated for 1 h at room temperature before adding the probe. Supershifted complexes are indicated with asterisks. Probes and lysates used are shown below the panel, as in ***B***. ***B***, ***C***, Arrows show the position of the main AP-1 complex (AP-1), or a complex of unknown composition (denoted with ?). Arrowhead indicates unspecific binding. ***B***, ***C***, Representative images of three independent experiments. ***C***, Gamma has been altered for clearer visualization of supershifted complexes.

pI promoter construct, or constructs with mutated AP-1 sites, together with overexpression of c-Fos and c-Jun in rat primary neurons (Fig. 5*C*). Overexpressing c-Fos/c-Jun together with the WT h*BDNF* pI construct raised both the basal (~16-fold) and the BDNF-induced expression levels (~3-fold) of h*BDNF* pI (Fig. 5*C*). Mutating either of the AP1-1 or AP1-2 elements decreased the effect of c-Fos/c-Jun overexpression on pI activity upon TrkB signaling, but did not completely remove it. When both sites were mutated, the BDNF-induced levels of pI activity were not changed by AP-1 protein overexpression. However, mutating both the AP1-1 and the AP1-2 site did not completely abolish the effect of AP-1 protein overexpression on the basal promoter activity levels, as AP-1 protein overexpression was able to raise the pI AP1–1m2m reporter activity ~1.5-fold. Mutating both the AP1-1 and the AP1-2 site together, but not either of the sites alone, decreased the BDNF-induced activity of pI to the same level as in the case of overexpressing A-Fos together with the WT h*BDNF* pI construct (~2.1 and ~2.4-fold induction, respectively, compared with the WT promoter activity in untreated cells cotransfected with empty pQM vector), indicating that the two AP-1 sites described in this study are the main *cis*-acting elements responsible for the AP-1-mediated activation of h*BDNF* pI upon TrkB signaling.

Fos and Jun family proteins bind human *BDNF* promoter I *in vitro*

Next, we decided to investigate whether h*BDNF* pI is able to bind AP-1 proteins *in vitro*. For that, we conducted EMSA using oligonucleotides containing either the AP1-1 or the AP1-2 sequence from h*BDNF* pI, denoted as pI AP1-1 oligo and pI AP1-2 oligo, respectively (Fig. 6*A*). As a positive control, we used an oligo containing the AP-1 consensus site from the human *MT2A* promoter (Angel et al., 1987; Lee et al., 1987).

Using lysates from 7 DIV neurons treated with BDNF for 2 h or left untreated, we found that, whereas the increase in AP-1 binding activity to AP1-1 oligo in response to 2 h of BDNF treatment remained modest, AP1-2 oligo displayed a substantial induction of AP-1 binding activity upon BDNF treatment, comparable to that of the *MT2A* promoter oligo with the AP-1 consensus site (Fig. 6*B*). Using EMSA with excess unlabeled WT oligos or oligos with mutations in the AP1–1, PasRE or AP1-2 sites, we found the major binding complexes (Fig. 6*B*, arrow with AP-1) to be specific to both the AP1-1 and the AP1-2 site.

To determine the composition of the AP-1 complex, we preincubated lysates with antibodies against different Jun and Fos family proteins and performed EMSA (Fig. 6*C*). We detected that with lysates of untreated neurons, both the AP1-1 and the AP1-2 site bound only JunD. As revealed by the formation of supershifted complexes with lysates from neurons treated with BDNF for 2 h, the AP1-1 was bound by c-Jun, JunD, c-Fos, and FosB; and the AP1-2 was bound by c-Jun, JunB, JunD, c-Fos, and FosB. We did not detect binding of Fra1 and Fra2 to either of the pI oligos (Fig. 6*C*). It is possible that our assay was not sensitive enough to detect JunB supershift using h*BDNF* pI AP1-1 oligo, as the supershifted complex of JunB has similar mobility as another complex seen even without the addition of antibody (Fig. 6*C*, denoted with?).

When using pI oligos in EMSA, we noticed a complex with slower mobility (Fig. 6*B*, *C*, denoted with ?) than that of the main

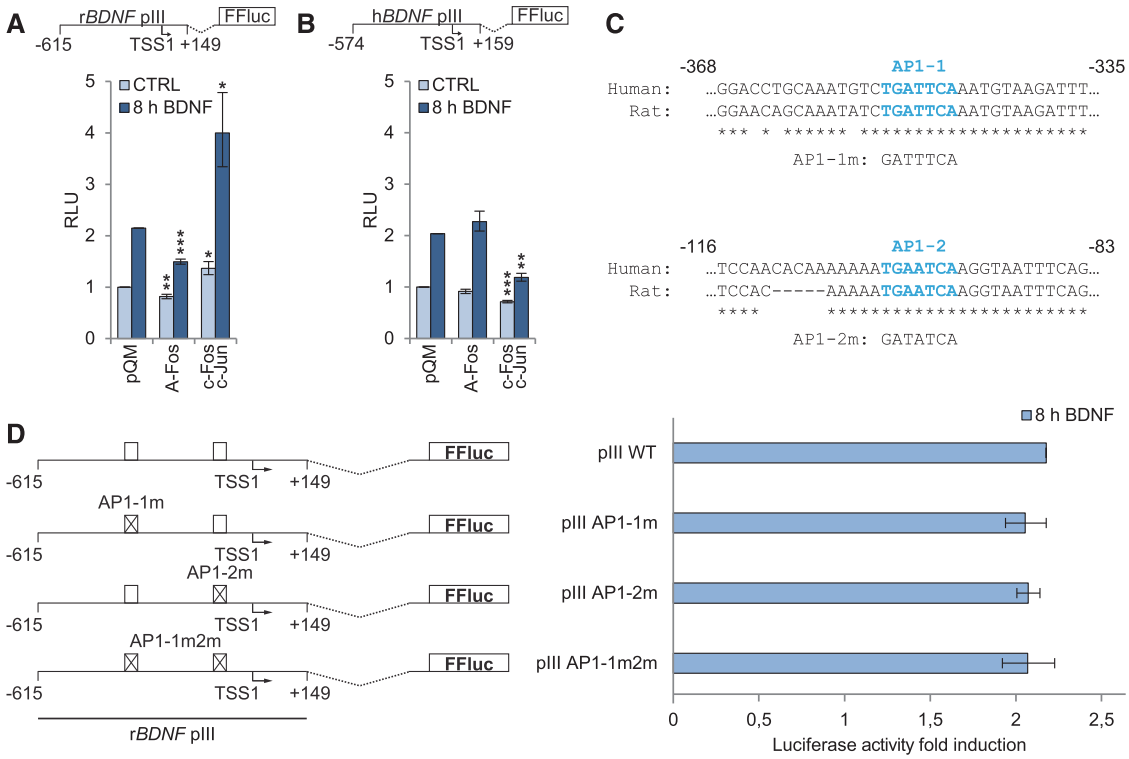

Figure 7. TrkB signaling-dependent induction of r*BDNF* pIII but not of h*BDNF* pIII is positively regulated by AP-1 proteins. ***A***, ***B***, At 6 DIV, rat primary neurons were cotransfected with rat (***A***) or human (***B***) *BDNF* promoter III-dependent luciferase reporter constructs (indicated above the graphs) together with different expression constructs or empty vector pQM. At 7 DIV, neurons were left untreated (CTRL) or treated with 50 ng/ml BDNF for 8 h, after which luciferase activities were measured. Luciferase activity in untreated cells cotransfected with pQM was arbitrarily set as 1. ***C***, Alignment of the regions in *BDNF* pIII containing two putative AP-1 sites, designated AP1-1 and AP1-2. The AP-1 sites are shown in blue and bold. The mutations used in the promoter constructs are shown below the alignment. Numbers next to the alignments or schematic figures of promoter constructs indicate distance from the most 5′ transcription start site (TSS1; according to Pruunsild et al., 2007) of rat (***A***, ***D***) or human (***B***, ***C***) *BDNF* exon III. ***D***, Schematic representation of the r*BDNF* pIII constructs (left), and luciferase signal induction in neurons transfected with the respective promoter constructs in response to 50 ng/ml BDNF treatment for 8 h (right). White boxes depict putative AP-1 elements. Error bars represent SEM from eight (***A***) or five (***B***, ***D***) independent experiments. Asterisks indicate statistical significance relative to luciferase activity in cells transfected with the respective pIII luciferase reporter construct and empty pQM vector either left untreated or treated with BDNF, respectively (***A***, ***B***), or relative to luciferase fold induction in cells transfected with WT promoter construct (***D***). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; *t* test on log-transformed and autoscaled data.

AP-1 complex. Furthermore, a complex with similar mobility was also seen when using AP-1 consensus oligo as the probe. Competition with unlabeled oligos indicated that this complex binds to the AP1-2 site, but is not specific for the AP1-1 site (Fig. 6*B*). Of note, we saw a decrease in binding of this complex in supershift experiments using pI AP1-1 oligo when the untreated neuronal lysate was incubated with c-Jun and JunD antibody, and when lysate from BDNF-treated neurons was incubated with c-Jun antibody (Fig. 6*C*). Even though according to the competition experiments the complex was not AP1–1-specific, this indicates that Jun family proteins might be involved. When using pI AP1-2 oligo as the probe, we could not determine a clear pattern in the decrease in binding of the slower-mobility complex in supershift experiments. We propose that the complex consists of Jun family homodimers, Jun family proteins together with ATFs, or other possible Jun heterodimerization partners.

TrkB signaling-dependent induction of rat *BDNF* promoter III but not human *BDNF* promoter III is positively regulated by AP-1 proteins

As abolishing AP-1 activity reduced the induction of r*BDNF* exon III transcripts upon TrkB signaling (Fig. 3*D*), we decided to study whether the exon III proximal promoter region contains the necessary elements for TrkB signaling-dependent induction. For that, we cloned both the rat and human *BDNF* promoter III (pIII) regions upstream of luciferase reporter gene, and transfected these constructs into rat primary neurons together with plasmids encoding different effector proteins. Neurons were then treated with BDNF and luciferase activities were measured.

Our results showed that both rat *BDNF* pIII (Fig. 7*A*) and human *BDNF* pIII (Fig. 7*B*) were induced ~2-fold upon TrkB signaling. Overexpressing A-Fos decreased both the basal and the BDNF-induced activity of rat *BDNF* (r*BDNF*) pIII, by 18 and 30%, respectively, indicating that AP-1 activity is involved in the induction of r*BDNF* pIII. Moreover, overexpressing c-Fos and c-Jun increased both the basal and the induced levels of r*BDNF* pIII, by 36 and 86%, respectively (Fig. 7*A*). In contrast, the activity of human *BDNF* (h*BDNF*) pIII was not affected by A-Fos, and overexpressing c-Fos and c-Jun rather decreased the activity of the promoter, suggesting that AP-1 proteins do not participate in the TrkB signaling-dependent regulation of h*BDNF* pIII (Fig. 7*B*).

Using bioinformatic analysis, we identified two putative AP-1 elements in the rat *BDNF* pIII region, that we named AP1-1 and

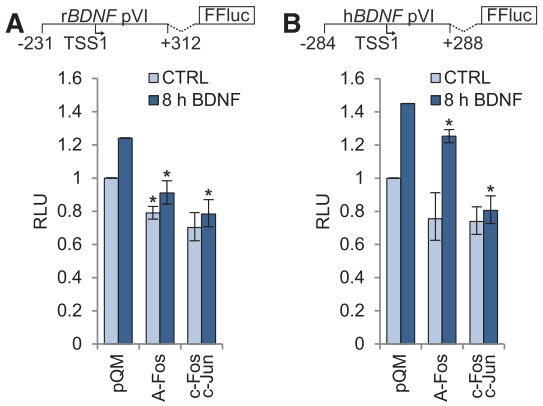

Figure 8. AP-1 activity is needed for the induction of both rat and human *BDNF* pVI in response to TrkB signaling. ***A***, ***B***, At 6 DIV, rat primary neurons were cotransfected with either rat (***A***) or human (***B***) *BDNF* promoter VI-dependent luciferase reporter constructs, together with different expression constructs or empty pQM vector. At 7 DIV, neurons were left untreated (CTRL) or treated with 50 ng/ml BDNF for 8 h, followed by measuring luciferase activities. Luciferase activity in untreated cells cotransfected with pQM was set as 1. Numbers next to the schematic figures of promoter constructs indicate distance from the most 5′ transcription start site (TSS1; according to Pruunsild et al., 2007) of rat (***A***) or human (***B***) *BDNF* exon VI. Error bars represent SEM from four (***A***) or three (***B***) independent experiments. Statistical significance is relative to luciferase activity in cells transfected with the respective pVI reporter construct and empty pQM vector left either untreated or treated with BDNF for 8 h, respectively. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; *t* test on log-transformed and autoscaled data.

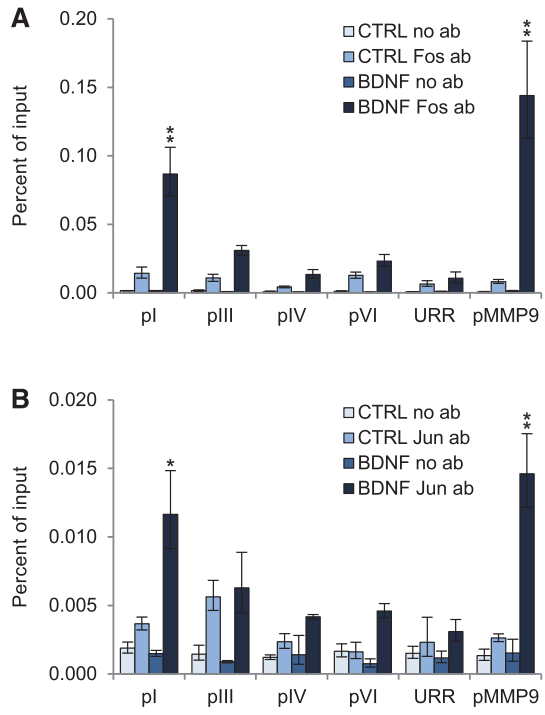

Figure 9. AP-1 proteins bind to endogenous r*BDNF* pI in rat primary neurons upon TrkB signaling. At 7 DIV, rat primary neurons were either left untreated (CTRL) or treated with 50 ng/ml BDNF for 2 h (BDNF). ChIP analysis was performed using either pan-Fos antibody (ab) (***A***) or c-Jun ab (***B***), together with no ab control in immunoprecipitation. DNA quantity of the respective regions was measured using qPCR. Data are shown as a fraction of immunoprecipitated DNA to total DNA quantity of the respective region (percent of input). pI, pIII, pIV, and pVI denote the respective r*BDNF* promoter regions. The *MMP9* promoter region (pMMP9) was used as a positive control, an unrelated region in chromosome 1 (URR) was used as a negative control. Statistical significance is relative to the respective group's binding to URR. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; *t* test on log-transformed data.

AP1-2 (Fig. 7*C*). Of note, both of these sites are conserved between human and rat. To check whether these AP-1 elements are responsible for the TrkB signaling-dependent induction of r*BDNF* pIII, we used luciferase reporter constructs where these sites alone (pIII AP1–1m and pIII AP1-2m constructs) or together (pIII AP1–1m2m construct) were mutated (Fig. 7*D*). Using reporter assay, we found that neither mutating these sites alone nor in combination had effect on the induction of r*BDNF* pIII in response to TrkB signaling (Fig. 7*D*).

Together, these results indicate that rat and human *BDNF* pIII are differentially regulated in response to TrkB signaling, and that r*BDNF* pIII does not seem to contain functional AP-1 sites, suggesting that the effect of AP-1 proteins on r*BDNF* pIII activity is indirect.

Induction of *BDNF* promoter VI in response to TrkB signaling needs AP-1 activity

To elucidate the mechanism behind the TrkB signaling-induced expression of *BDNF* exon VI transcripts, we cloned the rat and human *BDNF* promoter VI (pVI) regions into luciferase reporter plasmid and transfected them into rat primary neurons together with either A-Fos, or c-Fos and c-Jun coding plasmids, treated the cells with BDNF and measured luciferase activity (Fig. 8*A*,*B*). The activities of r*BDNF* pVI and h*BDNF* pVI were only slighty induced, 1.2- and 1.4-fold, respectively, upon TrkB signaling. Overexpressing A-Fos together with r*BDNF* pVI reporter construct decreased both the basal and the induced activity of the promoter, by 21 and 26%, respectively. However, overexpressing c-Jun and c-Fos also reduced the promoter activity, by 30 and 37% for the basal and the induced levels, respectively. Comparable effects were seen for h*BDNF* pVI, indicating that the regulation of both rat and human *BDNF* pVI is similar. Bioinformatic analysis failed to find AP-1 *cis*-elements conserved between rat and human in *BDNF* pVI, suggesting that the effect of AP-1 proteins on *BDNF* pVI activity is indirect.

AP-1 proteins bind endogenous *BDNF* promoter I in rat primary neurons upon TrkB signaling

Next, we analyzed how the binding of AP-1 proteins to different r*BDNF* promoter regions changed in response to TrkB signaling in primary neurons. For this, we treated primary neurons with BDNF for 2 h at 7 DIV, and performed ChIP analysis using pan-Fos and c-Jun antibodies. The *MMP9* promoter region, which has previously been shown to bind c-Fos in primary neurons in response to TrkB signaling (Kuzniewska et al., 2013), was used as a positive control. As transduction with AAV-A-Fos did not change the inducibility of r*BDNF* exon IV-containing transcripts in response to TrkB signaling (Fig. 3*C*), we used the r*BDNF* promoter IV region as an internal negative control for ChIP analysis.

Our results showed that in unstimulated neurons, neither Fos family proteins (Fig. 9*A*) nor c-Jun (Fig. 9*B*) bound to r*BDNF* promoters I, III, IV, and VI. This is in accordance with the fact that the basal expression and DNA binding activity of Fos family proteins and c-Jun is very low in unstimulated neurons (Figs. 2*B*, 6*C*). However, when the cells were treated with BDNF for 2 h, binding of the Fos family members and c-Jun was detected at the

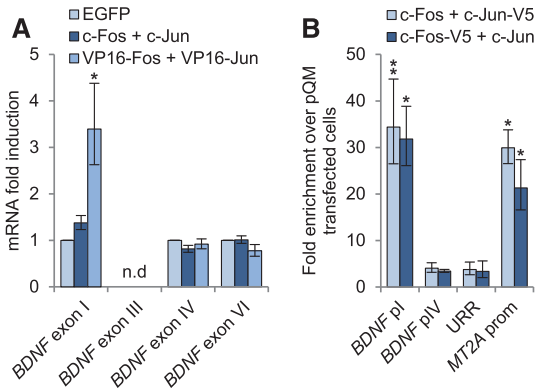

Figure 10. AP-1 proteins regulate the levels of h*BDNF* exon I transcripts and bind h*BDNF* pI in HEK293 cells. ***A***, RT-qPCR analysis of different h*BDNF* transcripts in HEK293 cells transfected with either EGFP coding vector, c-Fos and c-Jun overexpression constructs, or the respective AP-1 VP16-fusion protein overexpression constructs. The expression of h*BDNF* exon III transcripts could not be detected (n.d., Not detectable). mRNA expression is shown relative to the expression levels of the respective transcripts in EGFP overexpressing cells. ***B***, ChIP analysis showing binding of AP-1 proteins to endogenous h*BDNF* pI in cells overexpressing V5-tagged c-Fos and c-Jun proteins. HEK293 cells were transfected with combination of V5-tagged Fos and Jun expression constructs or empty pQM vector. Immunoprecipitation was performed using anti-V5 agarose. Binding of overexpressed Fos-V5 and Jun-V5 to h*BDNF* pI and pIV was measured using qPCR, the human *MT2A* promoter (*MT2A* prom) region and an unrelated region in chromosome 11 (URR) were used as positive and negative controls, respectively. Data are shown as fold enrichment of immunoprecipitated DNA normalized to input relative to the respective DNA levels in cells transfected with empty pQM vector. Error bars represent SEM of at least three independent experiments (***A***, ***B***). Statistical significance is relative to the respective transcript levels in EGFP transfected cells (***A***), or relative to the respective fold enrichment of binding at URR (***B***). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; *t* test on log-transformed (***A***), and on log-transformed and autoscaled data (***B***).

r*BDNF* pI region (Fig. 9). The extent of this binding was comparable to that of the *MMP9* promoter region used as a positive control. No significant binding of AP-1 proteins was seen for r*BDNF* promoters III, IV, and VI.

Collectively, these results indicate that AP-1 proteins directly regulate the activity of r*BDNF* pI, whereas the effect of AP-1 proteins on r*BDNF* pIII and pVI activity is indirect, possibly acting through different transcription factors that are induced by AP-1 proteins.

AP-1 proteins upregulate human *BDNF* exon I but not exon III or exon VI transcripts in HEK293 cells

To assess, whether AP-1 proteins regulate h*BDNF* transcription in the endogenous context, we overexpressed c-Fos and c-Jun, or their constitutively active VP16-fusion counterparts in HEK293 cells and measured the expression of different h*BDNF* transcripts using RT-qPCR (Fig. 10*A*). Whereas overexpressing c-Fos and c-Jun did not significantly increase the expression of h*BDNF* transcripts, VP16-Fos and VP16-Jun induced the expression of h*BDNF* exon I-containing transcripts ~3.4-fold, indicating that AP-1 proteins are able to upregulate the expression of endogenous h*BDNF* exon I transcripts. mRNA levels of exon IV and exon VI transcripts were not changed in response to overexpressing AP-1 proteins or their VP16-chimeric counterparts. The expression of h*BDNF* exon III transcripts was not detectable in HEK293 cells.

Next, we investigated whether AP-1 proteins bind endogenous h*BDNF* pI using ChIP assay in HEK293 cells overexpressing V5-tagged AP-1 proteins (Fig. 10*B*). We found that the overexpressed AP-1 proteins bound the h*BDNF* pI region and the *MT2A* promoter, used as a positive control, but not the h*BDNF* pIV region, which was used as a negative control. As AP-1 proteins did not regulate the expression of h*BDNF* exon III and exon VI transcripts, we did not check AP-1 binding to these promoters. Together, our results indicate that the expression of endogenous h*BDNF* exon I transcripts can be directly regulated by AP-1 proteins.

Discussion

The role of BDNF as an autocrine or paracrine survival factor has been well established (Miranda et al., 1993; Ghosh et al., 1994; Acheson et al., 1995; Davies and Wright, 1995; Hansen et al., 2001; Jiang et al., 2005; Kuribara et al., 2011; Wang et al., 2015). Autocrine functions of BDNF have also been implicated in axon development (Cheng et al., 2011) and dendritogenesis (Wirth et al., 2003). Here, we show that the expression of *BDNF* mRNA is extensively upregulated by TrkB signaling. We propose that this positive feedback loop is important in providing sufficient levels of BDNF in the aforementioned processes. Furthermore, as TrkB signaling can induce BDNF secretion (Krüttgen et al., 1998), the positive feedback mechanism of *BDNF* expression described in the current study could result in a local self-amplifying autocrine loop.

Autocrine activation of BDNF-TrkB axis is also associated with an unfavorable outcome in neuroblastomas, with TrkB signaling stimulating both growth and survival of tumor cells (Ho et al., 2002; Girgert et al., 2003). Although it has not been shown that the activation of TrkB induces *BDNF* transcription in neuroblastomas, it is plausible that such positive feedback loop exists and contributes to the survival of tumor cells. Furthermore, dysregulation of BDNF expression is associated with the pathophysiology of various psychiatric disorders (Autry and Monteggia, 2012). Therefore, elucidating the mechanism behind BDNF autoregulation could lead to better therapeutic means for both treating cancer and psychiatric disorders where the regulation of BDNF expression is defective.

So far, two studies have assessed the BDNF-TrkB-induced positive feedback loop in *BDNF* transcriptional regulation in primary cortical neurons, focusing on the regulation of exon IV transcripts (Yasuda et al., 2007; Zheng and Wang, 2009). The study by Yasuda et al. (2007) showed that *BDNF* exon IV transcripts are upregulated in response to TrkB activation by BDNF in cultured rat primary cortical neurons. However, the regulation of other *BDNF* transcripts was not studied. Similar observation about the induction of *BDNF* exon IV transcripts was made by Zheng and Wang (2009). In their study, the effect of BDNF treatment on the induction of *BDNF* exon I, II, and VI transcripts was addressed in addition to exon IV transcripts, and it was found that the levels of these transcripts are not induced in response to TrkB signaling. In the present study, we show that not only the expression of exon IV transcripts, but also of exon I, II, III, VI, and IXa transcripts is upregulated by TrkB signaling in primary neurons, with the fastest induction for exon IV and exon IXa transcripts, and the strongest induction for exon I transcripts, indicating that different *BDNF* promoters are used to control the exact temporal pattern of total *BDNF* mRNA expression after TrkB activation. It should be noted that in the study by Zheng and Wang (2009), *BDNF* transcript levels were measured only at 1 h after TrkB activation. Thus, their results are in accord with our study, because we show that the expression of exon I, II, and VI transcripts was not significantly induced after 1 h of BDNF treat-

ment, whereas exon IV transcripts displayed a moderate ~8-fold induction. Furthermore, we show that at later time points the levels of exon I, II, and VI transcripts are also increased in response to TrkB signaling. As it has been reported that the expression of *BDNF* exon IV transcripts can be induced as an immediate-early gene in response to neuronal activity (Lauterborn et al., 1996; Tao et al., 1998), it is possible that the fast induction of exon IV and IXa transcripts upon TrkB signaling could be independent of protein synthesis. It is plausible that *in vivo*, the *BDNF* transcripts that are induced first in response to TrkB signaling provide enough BDNF to start and sustain the BDNF autocrine loop, which in turn would induce the expression of other, more slowly inducible *BDNF* transcripts (eg, exon I, II, III transcripts), further prolonging the TrkB signaling necessary for various physiological outcomes.

The effect of TrkB signaling on the regulation of *BDNF* transcription has also been shown *in vivo*: infusion of BDNF into the dentate gyrus of adult anesthetized rats triggers stable LTP, an activity-dependent synaptic plasticity, and upregulates both *BDNF* exon IV-containing transcripts and total *BDNF* mRNA levels (Wibrand et al., 2006). Furthermore, it has been reported that C/EBPβ-mediated BDNF-positive feedback loop regulating *BDNF* exon IV mRNA expression in the hippocampus is important for memory consolidation *in vivo* (Bambah-Mukku et al., 2014), indicating that BDNF autoregulation is critical for hippocampus-dependent memory functions. We propose, in the light of our results obtained using primary cortical neuron cultures, that not only the induction of exon IV transcripts, but also of other *BDNF* transcripts could be important for memory-related functions. Moreover, our results suggest that in addition to the role of BDNF-positive feedback loop in the hippocampus, such regulatory mechanisms might be involved in proper functioning and/or development of the cortex.

It is well proven that BDNF potentiates synaptic transmission that is connected to neuronal membrane depolarization (Lu, 2003; Park and Poo, 2013). As the expression of *BDNF* is also activated by membrane depolarization and calcium influx into neurons (Park and Poo, 2013), one might argue that the effects seen in this study are a result of increased synaptic activity, and not caused by direct regulation of *BDNF* gene expression by TrkB signaling. However, it has been reported that TrkB signaling but not neuronal activity activates ERK5 pathway in neurons (Cavanaugh et al., 2001). By using pharmacological inhibitors, we show that the BDNF-positive feedback loop in primary cortical neurons is conveyed through both ERK1/2 and ERK5 pathways, suggesting that the induction of *BDNF* is at least partially conveyed through different pathways upon TrkB signaling and neuronal activity. Furthermore, it has been described that upon neuronal activity, h*BDNF* pI is activated by the depolarization-induced NPAS4 transcription factor through a conserved PasRE element in *BDNF* pI (Pruunsild et al., 2011). Here, we show that this PasRE element is not involved in the TrkB signaling-mediated activation of h*BDNF* pI. Moreover, it has been reported that in primary culture of cortical neurons, synapses are not yet functional at 7 DIV (Basarsky et al., 1994; Chiappalone et al., 2006; Cohen et al., 2008). Therefore, it is highly unlikely that the induction of *BDNF* in response to TrkB signaling in our study was due to an increase in neuronal activity.

Although the *cis*-elements responsible for the neuronal depolarization-dependent *BDNF* transcription have been widely studied (West et al., 2001; Greer and Greenberg, 2008; Pruunsild et al., 2011), the *cis*-elements and *trans*-acting factors regulating *BDNF* expression in response to TrkB signaling have been poorly characterized. Here, we show that AP-1 family transcription factors are necessary for the upregulation of *BDNF* exon I, III, and VI transcripts in response to TrkB signaling. Moreover, we have identified two functional AP-1 *cis*-elements in h*BDNF* pI, that are conserved between human and rat. Importantly, we have previously shown that the AP1-1 *cis*-element in h*BDNF* pI is not necessary for h*BDNF* pI activation in response to depolarization (Pruunsild et al., 2011). Here, we show that the AP1-1 element mediates TrkB signaling-dependent activation of h*BDNF* pI. Furthermore, as stated previously, the neuronal activity-responsive PasRE element in h*BDNF* pI (Pruunsild et al., 2011) does not contribute to the BDNF-dependent promoter activation. Rather, mutating the PasRE site increased the promoter's inducibility in response to TrkB signaling, possibly indicating complex interplay and competitive inhibition between TrkB signaling and neuronal activity-dependent induction of h*BDNF* pI. Together, these results indicate that the regulation of *BDNF* expression by neuronal activity and TrkB signaling can be conveyed through different *cis*-elements and *trans*-acting factors, further adding complexity to the elaborate regulation of *BDNF* gene expression.

AP-1 transcription factors have been implicated in the regulation of *BDNF* transcription by other groups. Compared with wild-type mice, the induction of BDNF in response to kainate-evoked neuronal activity was reduced in the hippocampi of postnatal forebrain neuron conditional *c-Fos* knock-out mice both at mRNA (Dong et al., 2006) and protein levels (Zhang et al., 2002). No change in basal BDNF levels was seen in those knock-out mice (Zhang et al., 2002). It is plausible that the lower BDNF inducibility by kainate treatment seen in the *c-Fos* knock-out mice is due to faulty BDNF autoregulation, since our results indicate that AP-1 activity is needed for the TrkB signaling-dependent induction of *BDNF* exon I, III, and VI transcripts. The dysregulation of BDNF expression in the c-Fos knock-out mice could partially be caused by subeffective use of the two novel AP-1 sites in *BDNF* pI identified in our study. Moreover, in agreement with our results, overexpressing constitutively active c-Fos in cultured hippocampal neurons increases *BDNF* mRNA levels (Benito et al., 2011), further indicating that AP-1 proteins are involved in the regulation of *BDNF* expression.

In conclusion, we have shown that in primary cultured cortical neurons, the *BDNF* gene is a subject to an extensive autoregulatory-positive feedback loop, where TrkB signaling induces the expression of all major *BDNF* transcripts. Elucidating the mechanism behind this phenomenon, we found that the upregulation of exon I, III, and VI transcripts is dependent on AP-1 family transcription factors, with a direct effect on *BDNF* promoter I and an indirect effect on promoters III and VI. Finally, we have identified two conserved functional AP-1 sites in the *BDNF* promoter I region necessary for the induction of exon I transcripts in response to TrkB signaling. This novel knowledge of BDNF autoregulation and its mechanisms provides new insights into the complex regulation of *BDNF* gene expression.

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Publication III

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RESEARCH ARTICLE

Dopamine cross-reacts with adrenoreceptors in cortical astrocytes to induce BDNF expression, CREB signaling and morphological transformation

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Abstract

Expression of brain-derived neurotrophic factor (BDNF) is induced in cultured rat cortical astrocytes by catecholamines norepinephrine and dopamine as well as selective α1 and β adrenergic agonists. However, it has remained unknown which receptors mediate dopamine-induced BDNF upregulation in astrocytes. Here, we demonstrate that β adrenoreceptors are the main mediators of this effect in cultured cortical astrocytes, while α1 adrenoreceptors and D1 dopamine receptors contribute to a lesser extent. We show that in cortical astrocytes BDNF exon IV and exon VI containing mRNAs are induced by dopamine and norepinephrine via CREB-dependent signaling and that this regulation is mediated by a mechanism that is distinct from activity-dependent CREB-mediated activation of BDNF transcription in neurons. We also show that regulation of BDNF promoters IV and VI by catecholamines requires a distal regulatory element in the BDNF locus. Finally, we demonstrate that dopamine-induced astrocyte stellation and induction of CREB signaling are mediated by cross-reaction of dopamine with β adrenoreceptors.

KEYWORDS
BAC, BDNF, catecholamine, CREB

1 | INTRODUCTION

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family that promotes neuronal survival and differentiation in mammalian development (Bibel & Barde, 2000; Binder & Scharfman, 2004) and regulates activity-dependent synaptic plasticity in the adult nervous system (Bramham & Panja, 2013). In addition to neurons, BDNF is expressed in other central nervous system cells such as microglia (Elkabes, DiCicco-Bloom, & Black, 1996), oligodendrocytes (Dai, Qu, & Dreyfus, 2001) and astrocytes (Dougherty, Dreyfus, & Black, 2000; Schwartz & Nishiyama, 1994; Zafra, Lindholm, Castrén, Hartikka, & Thoenen, 1992) as well as in several non-neural tissues such as heart and lung (Maisonpierre et al., 1990; Timmusk et al., 1993). Interestingly, a recent RNA-seq study of mouse cerebral cortex cell types on postnatal day 7 (www.brainrnaseq.org) reported higher BDNF mRNA levels in mouse cortical astrocytes than in cortical neurons (Zhang et al., 2014). In addition, a more recent study using the same approach to analyze cells of human cerebral cortex found comparable BDNF mRNA levels in neurons and astrocytes (Zhang et al., 2016).

BDNF transcription has been extensively studied in cultured hippocampal and cortical neurons, where a neuronal activity-dependent regulation mechanism has been described to a great detail (West, Pruunsild, & Timmusk, 2014). In contrast, relatively little is known about regulation of BDNF in astrocytes, the predominant cell type of the nervous system with several important roles in CNS development

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and homeostasis (Barres, 2008). In an early study on BDNF regulation, Zafra and colleagues reported (as data not shown) robust induction of BDNF mRNA levels in cultured astrocytes by norepinephrine and to a lesser extent by dopamine (Zafra et al., 1992). Isoproterenol, a nonselective β adrenoreceptor agonist upregulates BDNF mRNA levels in cortical and striatal astrocytes (Schwartz & Nishiyama, 1994). BDNF protein levels have been shown to be induced in cultured astrocytes by norepinephrine (Juric, Miklic, & Carman-Krzan, 2006), α1 and β adrenoreceptor agonists (Juric, Loncar, & Carman-Krzan, 2008) and dopamine (Inoue, Susukida, Ikeda, Murase, & Hayashi, 1997; Juric et al., 2006). However, receptors mediating BDNF induction by dopamine in astrocytes have remained unknown.

Here we show that upregulation of BDNF mRNA in cultured cortical astrocytes by dopamine is mainly mediated by adrenoreceptors of β and α1 subtypes and to a lesser extent by D1 dopamine receptors. Using lentivirus-mediated delivery of dominant-negative CREB, we show that BDNF upregulation by dopamine is mediated by the cAMP/CREB signaling pathway. Promoter and BDNF-BAC luciferase assays revealed that this regulation relies on a distal regulatory element in the BDNF locus. Next, we show that morphological transformation of astrocytes upon dopamine treatment—a well-known phenomenon in astrocytes associated with elevated cAMP/PKA signaling (Moonen, Cam, Sensenbrenner, & Mandel, 1975)—was blocked by the nonselective β adrenoreceptor antagonist propranolol. In addition, propranolol pretreatment prevented induction of cAMP/CREB reporter (CRE-luc) activity by dopamine, showing that β adrenoreceptors are critical for dopamine-cAMP/CREB signaling axis in cortical astrocytes.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Primary cortical astrocytes were cultured from prenatal (E21) Sprague Dawley rats using a modified McCarthy and DeVellis method (McCarthy & de Vellis, 1980). 2–4 cortices were dissected and digested with trypsin-EDTA 0.25% (Thermo Fisher) for 20 min. 0.5 mg/mL DNase I (Roche) was added at 10 min of digestion to reduce viscosity. Cortices were mechanically dissociated using 1 mL pipette tips in HBSS containing 1% bovine serum albumin (low endotoxin, Sigma) and 1 mg/mL trypsin inhibitor (>7000 BAEE, AppliChem). Dissociated cells were collected by 5 min centrifugation at 200g and seeded in 75 cm^2 flasks in culturing medium (DMEM medium supplemented with 10% Fetal Bovine Serum Gold, 100 units/mL penicillin and 100 μg/mL streptomycin—all from PAA Laboratories). On the following day, loosely attached cells and debris were removed by washing with a jet of medium using a 10 mL pipette. At 7 DIV confluent cells were shaken at 180 rpm in the culture incubator to remove nonastroglial cells growing on the astrocyte monolayer. Astrocytes were washed 2x with PBS, lifted with trypsin-EDTA and seeded on 6-well plates for RNA analysis, 24-well plates for immunocytochemistry and 48-well plates for luciferase assays. Immunocytochemical analysis showed these cultures contained >95% of GFAP-positive cells. Cultured primary cortical neurons were prepared from embryonic day 21 rats and transfected as described previously (Pruunsild, Sepp, Orav, Koppel, & Timmusk, 2011). For qRT-PCR experiments, RNA was isolated from cortical neurons at 9 days *in vitro*.

2.2 | Drug treatments

Confluent cortical astrocytes (11–14 days *in vitro*) were treated with catecholamine receptor agonists for 3 hr (qRT-PCR analysis and immunocytochemistry) or 6 hr (luciferase assays) in serum-free DMEM. For pretreatments, catecholaminergic antagonists were added to full growth medium for 30 min, followed by treatments in serum-free DMEM (antagonists were included in the treatment medium). Clonidine, rauwolscine and prazosin were purchased from Cayman Chemical, norepinephrine bitartrate was purchased from Enzo Life Sciences and all other drugs were purchased from Tocris Bioscience. All drugs were dissolved in DMSO and added to cultures at a final DMSO concentration of 0.1%.

2.3 | Quantitative RT-PCR analysis

RNA was isolated with RNeasy Mini kit (Qiagen) and treated on-column with DNase (Qiagen) according to manufacturer's instructions. cDNA was synthesized from 500 to 1,000 ng of RNA with Superscript III (lentivirus experiments) or RevertAid (all other experiments) reverse transcriptases (both from Thermo Scientific) using oligo$(dT)_{20}$ 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 cyclophilin B mRNA levels. All amplicons were cloned into pTZ57R/T (InsTAclone PCR cloning kit, Thermo Fisher Scientific) and verified by sequencing. Expression levels of BDNF transcripts and catecholamine receptor transcripts were calculated using standard curves generated from plasmid dilution series of amplicons cloned into pTZ57R/T vector (Thermo Fischer Scientific). Levels of BDNF alternative transcripts were normalized with total BDNF mRNA (primers detecting a common BDNF exon IX).

The following primers were used in this study: BDNF_IX_F (detects all alternative transcripts), GGCCCAACGAAGAAAACCAT; BDNF_IX_R, AGCATCACCCGGGAAGTGT; BDNF_I_F, AACAAGACACATTACCTTCCAGCAT; BDNF_I_R, CTCTTCTCACCTGGTGGAACATT; BDNF_II_F, TGGTATACTGGGTTAACTTTGGGAAA; BDNF_II_R, CACTCTTCTCACCTGGTGGAACTT; BDNF_IV_F, GCTGCCTTGATGTTTACTTTGA; BDNF_IV_R, GCAACCGAAGTATGAAATAACC; BDNF_VI_F, GAACAAACGATTGCTGAAAATG; BDNF_VI_F GAACAAACGATTGCTGAAAATG; BDNF_VI_R, TTCTCACCTGGTGGAACTTTATG; cyclophilinB_F, AGATCGAAGTGGAGAAACCCTTTG; cyclophilinB_R, TAAAAATCAGGCCTGTGGAATGTG; ADRA1A_F, AGAAGAAAGCTGCCAAGACG; ADRA1A_R, GAAATCCGGGAAGAAAGACC; ADRA1B_F, TCTTATGTTGGCTCCCCTTC; ADRA1B_R, ACGGGTAGATGATGGGATTG; ADRA1D_F, TGAGGCTGCTCAAGTTTTCC; ADRA1D_R, GCCAGAAGATGACCTTGAAGAC; ADRA2A_F, TTCCTGAGAGGGAAGGGATT; ADRA2A_R, AGTTACTGGGGCAAGTGGTG; ADRA2B_F, AATTCTCTGAACCCCCAAGC; ADRA2B_R, CAAGTTGGGAAGACAACCAG; AD

RA2C_F, GGGTTTCCTCATCGTTTTCA; ADRA2C_R, GAAAAGGGCATGACCAGTGT; ADRB1_F, GCTCTGGACTTCGGTAGACG; ADRB1_R, ACTTGGGGTCGTTGTAGCAG;

ADRB2_F, AGCCACCTACGGTCTCTGAA; ADRB2_R, GTCCCGTTCCTGAGTGATGT; ADRB3_F, TCGTCTTCTGTGCAGCTACG; ADRB3_R, ATGGTCCTTCATGTGGGAAA; DRD1_F, AGCAGGACGTATGCCATTTC; DRD1_R, ATTCTTGGCATGGACTGCTG; DRD2_F, TCCCAGCAGAAGGAGAAGAA; DRD2_R, GTTGACGGCACTGTTGACAT; DRD3_F, ACCCTGGATGTCATGATGTG; DRD3_R, TGATCATGAGTGCCACACG; DRD4_F, GTCTTCCCGCAGAAAGAGAG; DRD4_R, AGAAAGGCGTCCAACACATC; DRD5_F, ACCAAGACACGGTCTTCCAC; DRD5_R, CACAGTCAAGCTCCCAGACA

2.4 | Western blot

Cells grown in 6-well dishes were washed with PBS and lysed directly in Laemmli buffer. Proteins were separated by 15% PAGE and transferred to PVDF membrane (Millipore). Five percent of skimmed milk in PBST buffer (0.1% Tween-20) was used for blocking membranes and 2% milk-PBST was used for incubations with primary and secondary antibodies. Membranes were incubated overnight at 4°C with anti-BDNF antibody (clone 3C11, Icosagen) at 1:1,000 dilution. This antibody has been previously characterized by Y.A. Barde group (Chacón-Fernández et al., 2016). The membrane was washed 3 x 5 min with PBST, and incubated with secondary antibody (1:5,000, Goat Anti-Mouse IgG (H + L) Peroxidase-Conjugated, 32430, Thermo Scientific) overnight at 4 C. The next day, the membrane was washed again 3 × 5 min. Chemiluminescence was detected by SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific) and quantified using ImageQuant LAS 4000 imager and ImageQuant TL software (GE Healthcare). For normalization, membranes were stained with Coomassie Brilliant Blue and quantified using ImageQuant.

2.5 | Immunocytochemistry

Cells grown on 24-well plates were pretreated as indicated in culturing medium for 30 min, treated with indicated compounds in serum-free DMEM and fixed directly in culturing dishes in 4% paraformaldehyde/PBS for 15 min. Fixed cells were treated with 50 mM NH_4Cl in PBS for 10 min, permeabilized in 0.5% Triton X-100 in PBS for 15 min, blocked with 2% BSA in PBS, incubated with 1:800 dilution of GFAP antibody (clone GA5, Millipore) in 0.2% BSA 0.1% Tween 20 in PBS at room temperature for 1.5 hr, washed and incubated with or Alexa 488-conjugated anti-mouse IgG 1:2,000 (Molecular Probes) in 0.2% BSA 0.1% Tween 20 in PBS at room temperature for 1 hr. Cells were counterstained with DAPI and analyzed by epifluorescence microscopy (Axiovert 200M, Zeiss).

2.6 | Lentivirus experiments

HEK293FT cells (Invitrogen) were cultured on 145 mm dishes coated with 0.1% gelatin, in DMEM with high glucose and stable glutamine (Pan Biotech), supplemented with 10% SeraPlus fetal bovine serum (Pan Biotech), 100 U/mL penicillin (Gibco) and 100 μg/mL streptomycin (Gibco). 80–90% confluent cells were transfected with PEI reagent (Sigma) in lentivirus production media (DMEM, supplemented with 10% FBS, 1x MEM Nonessential Amino Acids (Capricorn Scientific), 1 mM sodium-pyruvate, 20 mM HEPES pH 7.4). The following plasmids were used for transfection: transfer vector pRRL-hPGK-EGFP or pRRL-hPGK-ACREB, encoding EGFP or A-CREB, respectively, psPAX2 (Addgene #12260), and pLP/VSVG (Invitrogen) at a ratio of 4:3:2. 18 hr post transfection, medium was replaced and virus-containing medium was harvested twice with 24 hr intervals. To purify lentiviral particles, medium was centrifuged at 4,500g for 5 min at +4°C and supernatant was filtered using Stericup-HV 0.45 μm PVDF filter unit (Millipore). Lentiviral particles were concentrated 100-fold using Speedy Lentivirus Purification solution (Applied Biological Materials Inc.). Astrocytes were transduced at 9–10 DIV and treated with 100 μM dopamine at 14 DIV.

2.7 | Plasmids and BACs

BDNF promoter constructs were generated by amplifying the corresponding sequences from rat genomic DNA using the Expand High Fidelity PCR System (Roche) and cloning into pGL4.15[luc2P/Hygro] (Promega) in front of the firefly luciferase coding sequence. Rat and human BDNF promoter IV and VI constructs have been described previously: rat IV (Koppel & Timmusk, 2013), human IV (Pruunsild et al., 2011), rat and human VI (Tuvikene, Pruunsild, Orav, Esvald, & Timmusk, 2016). Genome Browser coordinates for the used genomic fragments are as follows: rat IV chr3:100,786,659-100,787,183/rn6, rat VI chr3:100,787,725-100,788,266/rn6, human IV chr11:27,701,313-27,701,837/hg38, human VI chr11:27,700,200-27,700,772/hg38. The rat and human IV-VI constructs are minigenes carrying a larger genomic fragment spanning exons IV-VI (-203 bp relative to exon IV transcription start site, 455 bp 3′ of exon VI splice site in rat; −204 bp relative to exon IV transcription start site, 507 bp 3′ of exon VI splice site in human) and a splicing acceptor site from exon IX (–236 to +6 relative to exon IX 5′ end in rat; −217 to +6 relative to exon IX 5′ end in human). Rat BDNF BAC construct was derived from BAC clone CH230-106M15 (AC108236). Human BDNF BAC construct was derived from BAC clone RP11–651M4 (AC087446). For both BACs Red/ET homologous recombination in *E. Coli* (Counter-Selection BAC Modification Kit, Gene Bridges GmbH) was used to replace BDNF coding region with coding sequence of *Firefly* luciferase (luc2P), amplified from pGL4.15 (Promega). The following primers were used for generating homologous recombination cassette for human BDNF coding region: CTG TCT TGT TTC TGC TTT CTC CCT ACA GTT CCA CCA GGT GAG AAG AGT GAT GGA AGA TGC CAA AAA CAT TAA G and AAT AGA TAA TTT TTG TCT CAA TAT AAT CTA ATC TAT ACA ACA TAA ATC CAT TAG ACG TTG ATC CTG GCG CTG GCG C. The following primers were used for generating homologous recombination cassette for rat BDNF coding region: TGT CTG TCT CTG CTT CCT TCC CAC AGT TCC ACC AGG TGA GAA GAG TGA TGG AAG ATG CCA AAA ACA TTA AG and ATA CAA ATA GAT AAT TTT TGT CTC AAT ATA ATC TAT ACA ACA TAA ATC CAT TAG ACG TTG ATC CTG

GCG CTG GCG C. BAC DNA used for transfection was purified using NucleoBond Xtra BAC Kit (Macherey-Nagel).

2.8 | Luciferase assays

Confluent astrocytes grown in 48-well plates were transfected at 10–11 DIV with 3xCRE-luc (pGL4.29, Promega), BDNF promoter or BDNF-BAC constructs. pGL4.83 (Promega) containing SRα promoter in front of *Renilla* luciferase coding sequence was used for normalization. For plasmid transfections, 190 ng of plasmid and 10 ng of normalization plasmid per well were transfected using Lipofectamine 2000 (Thermo Fisher) at 1:3 w/v ratio. For BAC transfections, 200 ng of BAC DNA per well was transfected using Lipofectamine 2000 at 1:3 w/v ratio. Transfection complexes (in 20 μL unsupplemented DMEM) were added to cells in 100 μL of unsupplemented DMEM and incubated while shaking at 250 rpm for 1 hr, after which transfection medium was removed and changed for fresh growth medium. Drug treatments were performed 3 days after transfection, cells were lysed in Passive Lysis Buffer (Promega) and luciferase activity was measured using Dual-Glo Luciferase Assay System (Promega). Relative luciferase activity was calculated by dividing firefly luciferase signals with *Renilla* luciferase signals.

Rat cortical neurons were grown in 48-well plates and transfected at 7 DIV using Lipofectamine 2000 at 1:3 w/v ratio with 200 ng BAC DNA per well. Transfection complexes (20 μL in Neurobasal-A medium) were added to cells in 100 μL unsupplemented Neurobasal-A and incubated with shaking at 250 rpm for 2 hr at 37°C. Transfection medium was removed and replaced with 200 μL 1:1 mixture of old growth medium and fresh growth medium (Neurobasal-A, 100 units/mL penicillin and 100 μg/mL streptomycin, B27 Supplement (all from Thermo Scientific), and 10 μM 5-fluoro-2′-deoxyuridine (Sigma). Two days after transfection neurons were depolarized for 8 hr with 25 mM KCl. Cells were lysed in Passive Lysis Buffer and luciferase activity was measured using Dual-Glo Luciferase Assay System.

3 | RESULTS

3.1 | Dopamine induces BDNF expression in cultured cortical astrocytes mainly via adrenoreceptors

To study regulation of BDNF by catecholamines in astrocytes, we treated confluent cultures of rat cortical astrocytes with dopamine- and adrenoreceptor agonists for 3 hr and measured BDNF mRNA levels (pan-BDNF mRNA with primers in exon IX) by qRT-PCR. Our analysis showed that 150 μM dopamine (concentration used by Inoue et al., 1997; Miklic, Juric, Carman-Krzan, & Caman-Krzan, 2004) and 25 μM norepinephrine (concentration used by Zafra et al., 1992) robustly induced BDNF mRNA (Figure 1A) and protein (Figure 1B,C and Supporting Information Figure S1). However, selective dopamine receptor agonists showed little or no effect: D1-like receptor agonist SKF-38393 and D1/2 heteromer agonist SKF-83959 (all treatments 10 μM unless otherwise indicated) only weakly induced BDNF mRNA and D2-like receptor agonist quinpirole did not affect BDNF mRNA levels. In contrast, α1 adrenoreceptor agonist phenylephrine and β adrenoreceptor agonist isoproterenol significantly induced BDNF mRNA expression. Treatment with α2 adrenoreceptor agonist clonidine did not have an effect on BDNF mRNA levels.

Next, we studied which receptors mediate the induction of BDNF by dopamine, pretreating cortical astrocyte cultures for 30 min with selective catecholamine receptor antagonists (all 10 μM) before 3 hr treatment with 150 μM dopamine in combination with indicated antagonists (Figure 2A). We observed that BDNF mRNA induction by dopamine was decreased by D1-like dopamine receptor antagonist SCH22390 (mean decrease by 40%) and to a larger extent by α1 adrenoreceptor antagonist prazosin and β adrenoreceptor antagonist propranolol (mean decreases by 68% and 67%, respectively). D2-like receptor antagonist sulpiride and α2 adrenoreceptor antagonist rauwolscine did not affect BDNF induction by dopamine. As the 150 μM dopamine concentration used here and previously (Inoue et al., 1997; Miklic et al., 2004) may be higher than physiologically relevant concentrations, we performed a dose-response analysis with dopamine concentrations ranging from 1 to 100 μM with or without prazosin and propranolol pretreatments (Figure 2B). Interestingly, β adrenoreceptor blockade abolished BDNF upregulation by dopamine at concentrations up to 100 μM. α1 adrenoreceptor antagonist prazosin decreased, but did not abolish BDNF increases by 30 μM and 100 μM dopamine treatment. These results suggest that β adrenoreceptors are main mediators of BDNF regulation by dopamine in cortical astrocytes, whereas α1 adrenoreceptors and D1-like receptors contribute to a lesser extent.

The inefficiency of selective dopaminergic agonists to induce BDNF expression prompted us to study catecholamine receptor expression in cultured astrocytes. Expression of adrenoreceptor subtypes in astrocytes acutely isolated from from 10–12-week-old or P7 mouse cerebral cortex has been recently shown using microarray (Hertz, Lovatt, Goldman, & Nedergaard, 2010) or RNAseq analyses (Zhang et al., 2014), respectively. However, it is unknown to which extent these expression profiles are mirrored in astrocyte cultures prepared according to the McCarthy-deVellis method (McCarthy & de Vellis, 1980). Moreover, to the best of our knowledge expression of dopamine receptors in cultured cortical astrocytes has not been comparatively studied. Here we quantified expression levels of adrenergic and dopaminergic receptors by qRT-PCR (Figure 2C). Our analysis showed that α2A adrenoreceptors were expressed at highest levels, followed by β1 adrenoreceptors with threefold lower levels and α1D, α2C, β2, and D1 receptors with about 10-fold lower levels. Other analysed receptor subtypes were expressed at significantly lower levels.

3.2 | CREB-dependent signaling mediates BDNF induction by dopamine

CREB (cAMP-responsive element-binding protein) has a central role in neuronal activity-dependent induction of BDNF mRNA in cultured cortical neurons (Hong, McCord, & Greenberg, 2008; Pruunsild et al., 2011; Shieh, Hu, Bobb, Timmusk, & Ghosh, 1998; Tao, Finkbeiner, Arnold, Shaywitz, & Greenberg, 1998). To study the contribution of CREB-mediated signaling to BDNF induction by dopamine, we

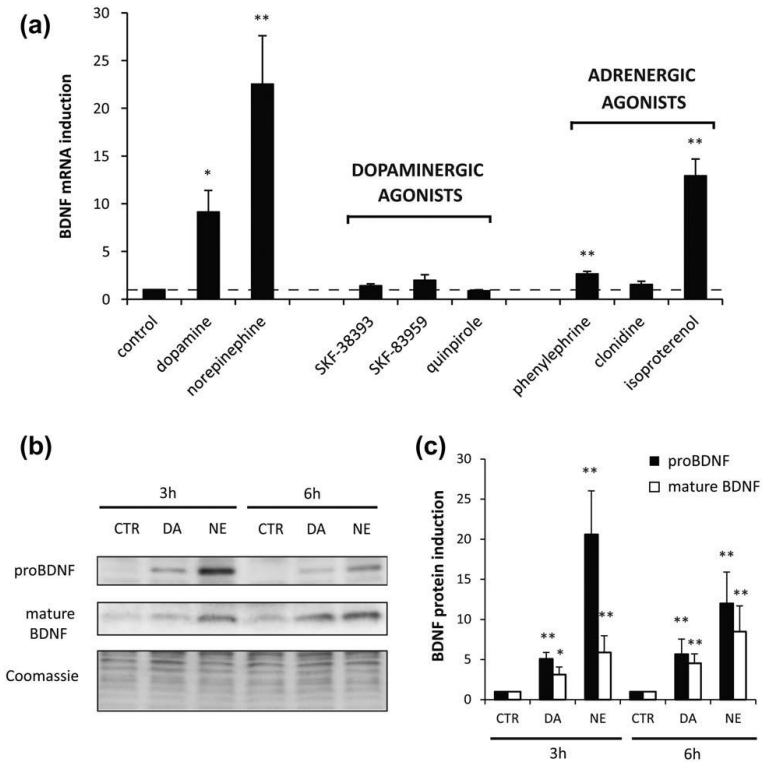

FIGURE 1 Regulation of BDNF expression in cultured cortical astrocytes by catecholamine receptor ligands. (A) Confluent cultures of rat cortical astrocytes were treated for 3 hr with 150 μM dopamine, 25 μM norepinephrine and 10 μM of other indicated compounds and BDNF mRNA levels were analyzed by qRT-PCR. BDNF mRNA levels were normalized to cyclophilin B (cycB) and are expressed relative to DMSO-treated controls (indicated by dashed line) * - $p < .05$ ** - $p < .01$ relative to control; n.s - not significant (*t*-test); $n = 3–5$. (B) Western blot analysis of proBDNF and mature BDNF in cortical astrocytes treated with 150 μM dopamine, 25 μM norepinephrine or DMSO (CTR). Coomassie staining for a part of the membrane is shown. (C) Quantification of proBDNF and mature BDNF shown in (B) from five biological replicates. * - $p < .05$ ** - $p < .01$ relative to control (*t*-test). BDNF signal was normalized to Coomassie signal (Supporting Information Figure S1). Error bars – *SEM*

transduced astrocytes with lentiviruses expressing either GFP or A-CREB, a dominant negative inhibitor of the CREB family (Ahn et al., 1998) at 9–10 DIV. At 14 DIV, cells were treated with DMSO or 100 μM dopamine for 3 hr and BDNF mRNA induction was measured by qRT-PCR. In addition to quantifying total BDNF mRNA, we also analyzed the effect of dopamine and perturbation of CREB signaling on alternative BDNF transcripts. In contrast to cultured cortical neurons expressing exon IV-containing transcript as the most abundant BDNF mRNA species, cortical astrocytes predominantly expressed exon VI-containing mRNA and barely detectable levels of other major 5′ exon-specific transcripts (Figure 3A). Exon IV mRNA levels, however, were robustly (about 110-fold) induced by dopamine treatment (Figure 3B), suggesting that BDNF promoter IV that is virtually silent under basal conditions can be activated by catecholamine-dependent signaling. Exon VI mRNA levels were also increased by dopamine treatment, but to a lesser extent. Dominant negative CREB significantly decreased induction of total BDNF mRNA and both BDNF exon IV and exon VI transcripts, suggesting that the CREB family of transcription factors is a master regulator of BDNF transcriptional response to dopamine (Figure 3B). It must be noted, however, that transduction with A-CREB lentivirus did not completely abolish BDNF induction by dopamine (the following residual inductions were observed, expressed relative to untransduced cells: 26% for total BDNF mRNA, 5% for BDNF IV mRNA and 33% for BDNF VI mRNA) indicating possible contribution of CREB-independent mechanisms.

3.3 | Activation of BDNF promoters IV and VI by catecholamines depends on a distal regulatory element in the BDNF locus

Next, we investigated catecholamine-mediated regulation of BDNF promoters IV and VI in astrocytes. In addition to dopamine, we treated astrocytes with norepinephrine that induced BDNF mRNA to a higher extent (Figure 1A). We transfected confluent cortical astrocytes with luciferase reporter constructs (Figure 4A) and 3 days after transfection

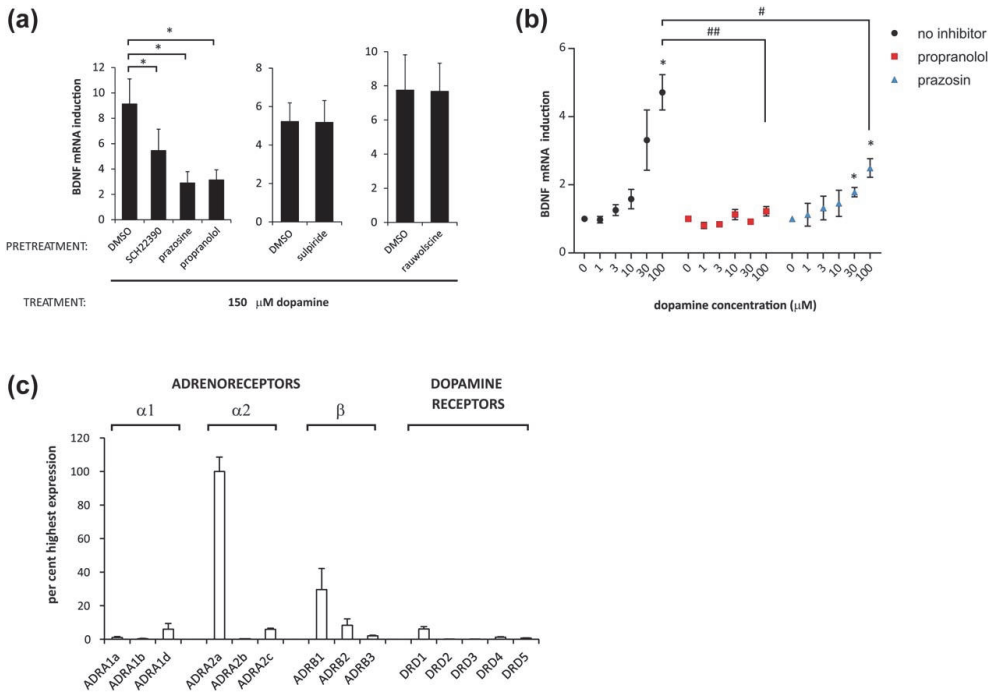

FIGURE 2 Dopamine induces BDNF expression in astrocytes mainly via β adrenoreceptors. (A) Confluent astrocytes were pretreated with indicated antagonists (10 μM) or DMSO (0.1%) for 30 min and treated with 150 μM dopamine with or without antagonists for 3 hr. BDNF mRNA levels were determined by qRT-PCR and normalized with cycB. Data are expressed relative to DMSO or respective antagonist treatments without dopamine. $n = 5$ (SCH22390, prazosine and propranolol); $n = 3$ (sulpiride and rauwolscine). * - $p < .05$ (*t*-test). (B) Astrocytes were pretreated with α1 adrenoreceptor antagonist prazosin (10 μM) or β adrenoreceptor antagonist propranolol (10 μM) or DMSO for 30 min and treated with indicated concentrations of dopamine with or without antagonists for 3 hr. * $p < .05$: dopamine vs DMSO and dopamine + prazosin vs DMSO + prazosin; # $p < .05$: 100 μM dopamine vs 100 μM dopamine + prazosin; ## $p < .01$: 100 μM dopamine vs 100 μM dopamine + propranolol, (*t*-test); $n = 3$. (C) Catecholamine receptor mRNA levels were quantified by qRT-PCR from three independent cultures. To compare relative levels of different mRNAs, amplicons were cloned and quantified in astrocyte cDNA using the standard curve method. Here, expression levels are shown relative to these of α2A adrenoreceptor (Adra2a). As a reference point, the measured mean raw Cp value for Adra2a mRNA was 24.8 ± 0.13). $n = 3$; Error bars – *SEM* [Color figure can be viewed at wileyonlinelibrary.com]

treated the cells with dopamine or norepinephrine for 6 hr. 25 μM norepinephrine (NE) and 150 μM dopamine (DA) treatment robustly induced the activity of the the cAMP reporter CRE-luciferase (Figure 4B), but failed to induce luciferase reporters with rat or human BDNF promoters IV and VI (Figure 4C,D). In addition, no induction was seen with BDNF minigenes carrying larger rat and human genomic fragments spanning exons IV and VI, splicing donor site from exon VI and splicing acceptor site from exon IX (Figure 4E). The used promoter constructs, except the minigenes, have previously been used by us for mapping BDNF and KCl responsive elements in primary cortical neurons (Koppel & Timmusk, 2013; Pruunsild et al., 2011; Tuvikene et al., 2016). As dopamine treatment robustly induced BDNF exon IV- and VI-containing mRNA levels (Figure 3B), these results suggest that regulation of corresponding promoters depends on interplay with a distal regulatory region. To test this hypothesis, we transfected cortical astrocytes with rat and human BDNF-BAC constructs harboring about 207 and 169 kb of BDNF locus, respectively, and carrying a luciferase reporter instead of the BDNF ORF (Figure 4A). These BDNF-BAC clones have previously been shown to recapitulate BDNF expression in transgenic mice (Koppel et al., 2009, 2010) and HeLa cell lines (Jaanson, Sepp, Aid-Pavlidis, & Timmusk, 2014). Both BDNF-BAC luciferase reporters were induced by catecholamine treatments, showing that both constructs carry distal regulatory regions necessary for catecholamine-dependent activation of BDNF transcription (Figure 4F, G). The cAMP response element (CRE) in BDNF promoter IV is a well-characterized regulation hub for activity-dependent BDNF transcription in neurons (reviewed in Lyons & West, 2011). We hypothesized that this element may contribute to regulation of BDNF expression via a long-distance interaction between transcriptional complexes on promoters IV and VI. However, induction of the human BDNF-BAC luciferase was not affected by mutation of the CRE element in BDNF promoter IV (Figure 4G). In contrast, in cultured cortical neurons this

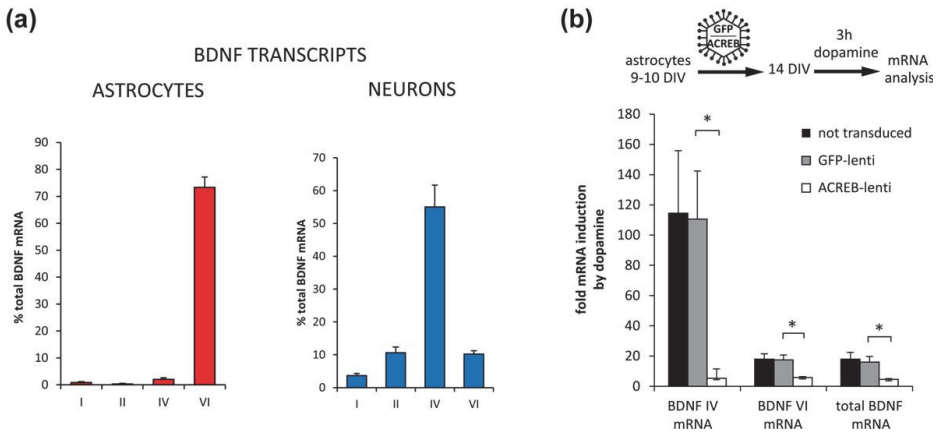

FIGURE 3 Dopamine induces expression of BDNF transcripts IV and VI in astrocytes in a CREB family-dependent manner. (A) qRT-PCR quantification of major BDNF transcripts in cultured rat cortical astrocytes (2 weeks *in vitro*) and cultured cortical neurons (9 days *in vitro*). Levels of alternative BDNF transcripts are expressed relative to total BDNF mRNA; $n = 4$. (B) At 9–10 DIV cultured astrocytes were transduced with lentiviruses expressing either GFP or A-CREB, a dominant negative inhibitor of the CREB family transcription factors. At 14 DIV, cells were treated with DMSO or 100 μM dopamine for 3 hr. Total BDNF mRNA and BDNF exon IV- and VI-containing mRNAs were quantified by qRT-PCR. BDNF expression levels are expressed relative to DMSO-treated controls.* $p < .05$ (*t*-test); $n = 3$. Error bars – *SEM* [Color figure can be viewed at wileyonlinelibrary.com]

mutation significantly dampened induction of human BDNF-BAC luciferase activity by KCl-mediated depolarization (Figure 4H). These results suggest that the CRE element in promoter IV is not involved in catecholamine-dependent regulation of BDNF in astrocytes.

3.4 | Dopamine-induced morphological transformation in cortical astrocytes and activation of cAMP/CREB signaling is abolished by β adrenoreceptor blockade

Agents elevating cAMP levels in astrocytes trigger a morphological transformation of cultured astrocytes, rapidly reorganizing their architecture from flat epithelial-like to stellated process-bearing cells (Moonen et al., 1975). Dopamine treatment induced clear morphological transformation of single cells in the culture at 1 μM, a subset of cells at 10 μM and almost all cells at 100 μM (Figure 5A), suggesting cell-to-cell variations in receptor expression levels or downstream effectors. Dopamine-induced transformation was completely blocked by propranolol, indicating that β adrenoreceptors are main mediators of cytoskeletal changes induced by dopamine (Figure 5A). Both D1-like dopamine receptors and β adrenoreceptors are Gs-coupled GPCRs and activate adenylate cyclases (De Blasi, 1990; Missale, Nash, Robinson, Jaber, & Caron, 1998). To investigate the contribution of β adrenoreceptors to dopamine-mediated cAMP/PKA signaling, we used a cAMP-responsive element (CRE)-containing luciferase reporter transfected into cultured astrocytes. We found that 10 and 100 μM dopamine treatments (6 hr) robustly induced luciferase activity (Figure 5B). This effect was completely abolished by propranolol for 10 μM dopamine treatment and almost completely abolished for 100 μM dopamine indicating that β adrenoreceptors are principal mediators of dopamine-induced cAMP signaling in cortical astrocytes.

4 | DISCUSSION

In this study, we show that in cultured cortical astrocytes dopamine induces BDNF expression, morphological transformation and cAMP/PKA signaling mainly via β adrenoreceptors. It has been shown that dopamine can bind and activate β2 adrenoreceptors as a full agonist, although with a 100-fold lower potency than norepinephrine (Swaminath et al., 2004). In HEK293 cells expressing β2 adrenoreceptors the EC_{50} of cAMP accumulation by dopamine was approximately 10 μM (Swaminath et al., 2004), which agrees with the effective dopamine concentrations observed here. Peak dopamine concentration in the synaptic cleft may reach the 10 μM range (Fisher, Morris, Alpert, & Fischman, 1995), suggesting that dopamine could transiently activate β adrenoreceptors in perisynaptic astrocytic domains of dopaminergic synapses. β adrenoreceptors have been implicated in facilitating LTP induction, memory acquisition and retrieval (O'Dell, Connor, Guglietta, & Nguyen, 2015), but their importance is usually discussed in the context of their neuronal function. Astrocytic β adrenoreceptors—despite being highly expressed in these cells (Hertz, Chen, Gibbs, Zang, & Peng, 2004)—have received relatively little attention as players in normal and pathological CNS function. Only recently it has been shown that hippocampal astrocytes, but not neurons are critical for β adrenoreceptor-dependent long-term memory consolidation (Gao et al., 2016). In addition, astrocytic β adrenoreceptors have been found to have a role in neuroprotection (Juncker, 2002) and promotion of neuritic outgrowth via neurotrophin signaling (Day et al., 2014). Considering the roles of BDNF in promoting neuron survival and differentiation (Bibel & Barde, 2000; Binder & Scharfman, 2004) as well as in synaptic plasticity (Bramham & Panja, 2013), it is possible that BDNF released from astrocytes as a response to β adrenoreceptor stimulation may

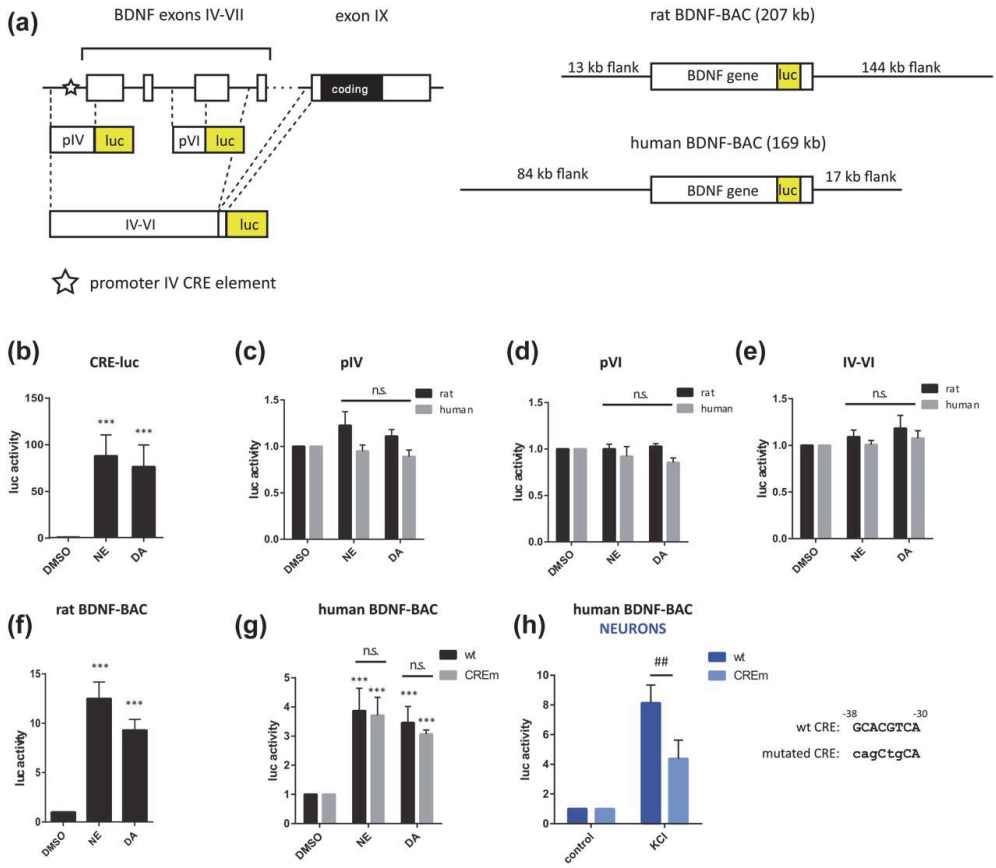

FIGURE 4 Regulation of BDNF promoters IV and VI by catecholamines is dependent on a distal regulatory element. (A) Schematic of BDNF luciferase reporter constructs. pIV and pVI are promoter constructs, IV-VI is a minigene carrying exon VI splice donor site and exon IX splice acceptor site. BDNF-BAC constructs carry the whole rat or human BDNF genes, where the coding sequence has been replaced by the luciferase ORF. Genomic fragments 5′ and 3′ of the BDNF gene included in the BACs are indicated with lines. (B) Induction of 3xCRE-luciferase reporter activity in cultured cortical astrocytes by 6 hr treatments with 25 μM norepinephrine (NE) or 150 μM dopamine (DA). Luciferase activity is expressed relative to DMSO-treated controls, *** $p < .001$ (*t*-test), $n = 9$. (C-E) Luciferase activity in astrocytes transfected with indicated rat BDNF promoter constructs and treated for 6 hr with norepinephrine, dopamine or DMSO; n.s. —not significant between DA/NE-treated and DMSO-treated (*t*-test); $n = 3–4$. (F) Cortical astrocytes were transfected with the rat BDNF-BAC, treated and analyzed as in (B-E). *** $p < .001$ compared with DMSO-treated controls (*t*-test); $n = 5$. (G,H) Cultured cortical astrocytes (G) or neurons (H) were transfected with unmodified human BDNF-BAC (wt), or human BDNF-BAC carrying mutated CRE element in exon IV promoter (CREm). Astrocytes were treated as in (B-F), neurons were treated for 8 hr with 25 mM KCl. *** $p < .001$ compared with DMSO-treated controls. ## $p < .01$ wt vs CREm; $n = 3–9$ (astrocytes), $n = 6$ (neurons). Error bars – *SEM* [Color figure can be viewed at wileyonlinelibrary.com]

contribute to these processes. The results of the present study suggest that dopamine may act as an additional β adrenoreceptor ligand, upregulating BDNF synthesis and release in cortical astrocytes. Studies on the roles of BDNF in astrocytes have been complicated by its relatively low expression levels in these cells. In the present study, we show (to the best of our knowledge, for the first time) expression of BDNF protein in cultured astrocytes by Western blot analysis and its regulation by dopamine and norepinephrine.

Regulation of BDNF expression has been thoroughly investigated in neurons, where it is considered a classical example of a gene that is dynamically regulated by neuronal activity (Lyons & West, 2011; West et al., 2014). In non-neuronal cells, however, regulation of BDNF has been very poorly studied and it is not well known to which extent the neuronal regulation machinery operates in other cell types. We show that dopamine acting via β adrenoreceptors activated CRE-luciferase which carries the cAMP/Ca^{2+}-response element (CRE). Several studies have demonstrated that CRE and CREB (the CRE-binding protein) are critical for BDNF promoter IV regulation by neuronal activity (Hong et al., 2008; Pruunsild et al., 2011; Shieh et al., 1998; Tao et al., 1998). Overexpression of dominant-negative A-CREB in astrocytes significantly

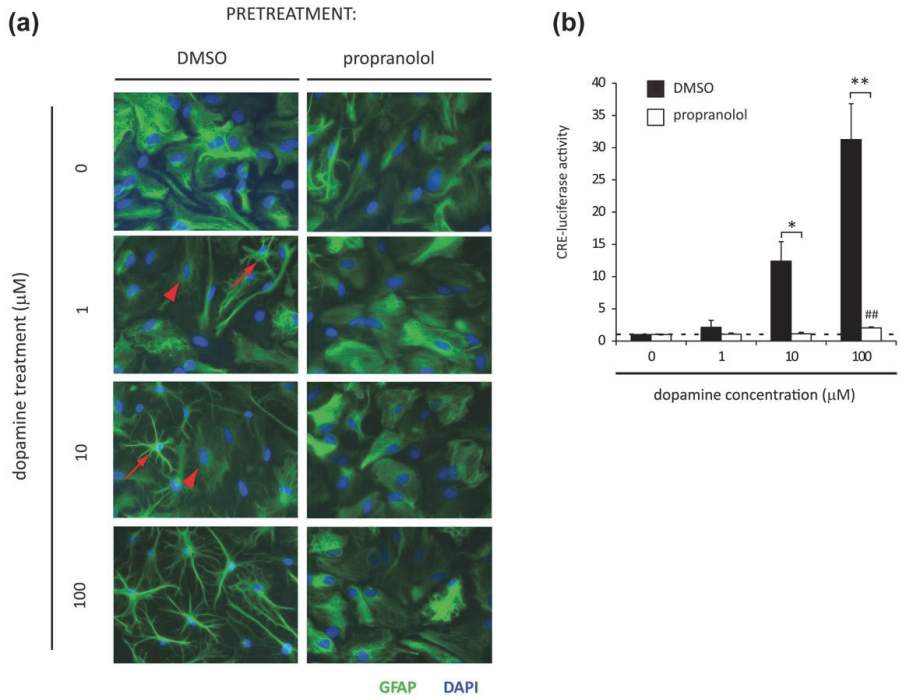

FIGURE 5 β adrenoreceptors mediate dopamine-induced morphological transformation and induction of cAMP/CREB signaling in cortical astrocytes. (A) GFAP staining of cultured astrocytes treated with indicated concentrations of dopamine for 3 hr with 30 min pretreatment with 10 μM propranolol or vehicle (DMSO). Arrows indicate astrocytes with process-bearing morphology and large arrowheads indicate astrocytes that have retained flat morphology. (B) CRE-luciferase assay in cortical astrocytes pretreated with propranolol or DMSO and treated with indicated concentrations of dopamine for 6 hr. Luciferase activity is expressed relative to respective controls without dopamine (dashed line). * $p < .05$, ** $p < .01$ (*t*-test); ## $p < .01$ 100 μM dopamine + propranolol vs propranolol alone; $n = 3$. Error bars – *SEM* [Color figure can be viewed at wileyonlinelibrary.com]

decreased induction of both exon IV- and exon VI-containing BDNF mRNAs by dopamine, suggesting that CRE/CREB-mediated signaling is also critical for regulation of BDNF in astrocytes. Surprisingly, neither BDNF proximal promoters IV and VI nor the larger genomic fragment containing both of these promoters were induced by catecholamines in luciferase reporter assays, despite the promoter IV CRE element being contained in both promoter IV and IV-VI minigene constructs. These results suggested that BDNF regulation in astrocytes involves an enhancer element, a hypothesis that was supported by luciferase assays with BAC constructs carrying the whole BDNF locus. As the rat and human BDNF-BAC constructs used in our experiments both contain the entire BDNF gene, but different lengths of 5′ and 3′ flanking genomic regions, it is likely that the enhancer element is contained within or near the BDNF gene. The higher induction observed for rat BDNF-BAC-luciferase, however, may indicate the presence of additional distal regulatory elements which may be important for driving BDNF expression in non-neuronal cells. Interestingly, we have previously shown that the rat BDNF-BAC fragment can drive BDNF expression in the heart (Koppel et al., 2010), whereas the human BDNF-BAC cannot (Koppel et al., 2009). Further studies will be needed to delineate the exact locations and modes of operation of the distal regulatory elements for non-neuronal BDNF expression.

It is long known that α1, α2 and β adrenoreceptors are expressed in cultured cortical astrocytes (Ebersolt, Perez, & Bockaert, 1981; Harden & McCarthy, 1982). In addition, expression of D2 receptors in cortical astrocytes has been inferred by immunocytochemistry and radioligand binding experiments (Khan, Koulen, Rubinstein, Grandy, & Goldman-Rakic, 2001). However, to the best of our knowledge comparative quantitative mRNA expression analysis of catecholamine receptors in cultured astrocytes has not been performed. Here, we show that in cortical astrocytes dopamine receptors are expressed at low levels (D1 receptors) or are essentially absent (D2–5 receptors) whereas adrenoreceptors are more highly expressed, with the most prominent subtypes being β1 and α2A receptors. These results are in good agreement with a recent RNA-seq study of P7 mouse cortical cell types showing that all dopamine receptors are expressed at very low levels in cortical astrocytes, whereas β1 receptors and α2A adrenoreceptors receptors are highly expressed (Zhang et al., 2014). In addition, mRNAs of these adrenoreceptor subtypes were consistently found in astrocytes acutely isolated from adult mouse brain (Hertz et al., 2010).

However, the latter study also reported consistent expression of α1A adrenoreceptor mRNA, which we found to be expressed at negligible levels in our astrocyte cultures. This suggests α1A receptor expression may be upregulated in development as astrocyte cultures used in the current study, prepared from cortices of prenatal (E21) mice, represent a relatively immature state of these cells.

In conclusion, by studying regulation of BDNF expression in astrocytes we have identified a dopamine-adrenoreceptor crosstalk mechanism, implying the possibility of cortical astrocytes "listening" to midbrain dopaminergic neurons via β adrenoreceptors. Second, our results suggest that a distal regulatory element—a potential enhancer—has a critical role in catecholamine-dependent regulation of BDNF expression in astrocytes. Using BDNF-BAC constructs, we have limited the localization of this element to the BDNF locus. The exact localization of this element has to be determined in future efforts.

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SUPPORTING INFORMATION

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TITLE PAGE

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ABSTRACT

Brain-derived neurotrophic factor (BDNF) controls the survival, growth, and function of neurons both during the development and in the adult nervous system. BDNF gene is transcribed from several distinct promoters generating transcripts with alternative 5' exons. BDNF transcripts initiated at the first cluster of exons have been associated with the regulation of body weight and various aspects of social behavior, but the mechanisms driving the expression of these transcripts have remained poorly understood. Here, we identify an evolutionarily conserved intronic enhancer region inside the BDNF gene that regulates both basal and stimulus-dependent expression of the BDNF transcripts starting from the first cluster of 5' exons in neurons. We further uncover a functional E-box element in the enhancer region, linking the expression of BDNF and various pro-neural basic helix-loop-helix transcription factors. Collectively, our results shed new light on the cell-type- and stimulus-specific regulation of the important neurotrophic factor BDNF.

INTRODUCTION

Brain-derived neurotrophic factor (BDNF) is a secreted protein of the neurotrophin family (Park and Poo, 2013). During the development, BDNF promotes the survival of various sensory neuron populations (Ernfors et al., 1994; Jones et al., 1994). In the adult organism, BDNF is also required for the proper maturation of synaptic connections and regulation of synaptic plasticity (Korte et al., 1995; Park and Poo, 2013). Defects in BDNF expression and signaling have been implicated in various neuropsychiatric and neurodegenerative diseases, including major depression, schizophrenia, Alzheimer's disease and Huntington's disease (Autry and Monteggia, 2012; Burbach et al., 2004; Jiang and Salton, 2013; Murray et al., 1994; Ray et al., 2014; Wong et al., 2010; Zuccato et al., 2001; Zuccato and Cattaneo, 2009).

Murine BDNF gene contains eight independently regulated non-coding 5' exons (exons I-VIII) followed by a single protein-coding 3' exon (exon IX). Splicing of one of the alternative exons I-VIII with the constitutive exon IX gives rise to different BDNF transcripts (Aid et al., 2007). Additionally, transcription can start from an intronic position upstream of the coding exon producing an unspliced 5' extended variant of the coding exon (exon IXa-containing transcript) (Aid et al., 2007). The usage of multiple promoters enables complex cell-type and stimulus-specific BDNF expression (reviewed in West et al., 2014). For instance, BDNF exon I, II, and III-containing transcripts show mainly nervous system-specific expression patterns, whereas BDNF exon IV and VI-containing transcripts are expressed in both neural and non-neural tissues (Aid et al., 2007; Timmusk et al., 1993). Similar expression patterns for different BDNF transcripts are also observed in human (Pruunsild et al., 2007). Notably, different BDNF transcripts have distinct contribution to various aspects of neural circuit functions and behavior (Hallock et al., 2019; Hill et al., 2016; Maynard et al., 2018, 2016; McAllan et al., 2018; Sakata et al., 2009).

In addition to proximal promoter regions, the complex regulation of gene expression is often controlled by distal regulatory elements called enhancers (reviewed in Buecker and Wysocka, 2012). Enhancers are usually active in a tissue- and cell type-specific manner (reviewed in Heinz et al., 2015; Wu et al., 2014), and can be located inside or outside, upstream or downstream of the target gene, within another gene or even on a different chromosome (Banerji et al., 1981; Lettice et al., 2003; reviewed in Ong and Corces, 2011). Many enhancers are activated only after specific stimuli which cause enrichment of active enhancer-associated histone modifications and increased chromatin accessibility (Su et al., 2017). Genome-wide analysis has proposed approximately 12000 neuronal activity-regulated enhancers in cortical neurons (Kim et al., 2010). Importantly, dysregulation of enhancers or mutations in proteins that participate in the formation of enhancer-promoter complexes are associated with a variety of disorders, including neurodegenerative diseases (reviewed in Carullo and Day, 2019).

Previous studies from our laboratory have suggested that the expression of the BDNF gene is also regulated via distal regulatory regions. Notably, the induction of BDNF mRNA after BDNF-TrkB signaling in neurons seems to depend on unknown distal regulatory regions (Esvald et al., 2020). Furthermore, dopamine-induced expression of BDNF in astrocytes is controlled by an unknown regulatory region within the BDNF gene locus (Koppel et al., 2018). Here, we identify a novel enhancer region in the BDNF gene located downstream of the BDNF exon III and show that this regulatory element selectively activates basal and stimulus-dependent expression of the exon I, II and III-containing BDNF transcripts in neurons.

RESULTS

1. BDNF +3 kb region shows enhancer-associated characteristics in mouse and human brain tissue

To uncover novel enhancer regions regulating BDNF expression in the central nervous system, we started with bioinformatic analysis of the enhancer-associated characteristics. Active enhancers are characterized by nucleosome-free DNA that is accessible to transcription factors and other DNA binding proteins. Chromatin at active enhancer regions typically has distinct histone modifications – H3K4me1, a hallmark of enhancer regions, and H3K27ac, usually associated with active regulatory regions. Active enhancers also bind RNA polymerase II and are bidirectionally transcribed from the regions marked by enhancer-associated histone modifications giving rise to non-coding enhancer RNAs (eRNAs) (Nord and West, 2020). Based on the mouse brain tissue ChIP-seq data from the ENCODE project and transcription start site (TSS) data from the FANTOM5 consortium, a region ~3 kb downstream of BDNF exon I TSS has prominent enhancer-associated features (Figure 1A). First, the +3 kb region is hypersensitive to DNaseI, indicative of an open chromatin structure. Second, ChIP-seq data shows that this region is enriched for H3K4me1, H3K4me3 and H3K27ac modifications. Third, the +3 kb region interacts with RNA polymerase II, with a strong evidence for bidirectional transcription according to the FANTOM5 CAGE database. Finally, the region is conserved between mammals, pointing at its possible functional importance.

We next used H3K27ac ChIP-seq data from Nord et al (Nord et al., 2013) to determine the activity of the potential enhancer region in different tissues throughout the mouse development. We found that the +3 kb region shows H3K27ac mark in the mouse forebrain, with the highest signal from embryonic day 14 to postnatal day 7, but not in the heart or liver (Supplementary Figure 1). This suggests that the +3 kb enhancer region might be active mainly in neural tissues in late prenatal and early postnatal life.

To further investigate which cell-types the +3 kb region could be active in human *in vivo*, we used data from a recently published human brain cell-type-specific ATAC-seq and ChIP-seq experiments (Nott et al., 2019) (Figure 1B). We found that the +3 kb region shows remarkable neuron specificity, as evident from open chromatin identified using ATAC-seq, and H3K27ac histone mark, which are missing in microglia, oligodendrocytes, and astrocytes. To further elucidate which human tissues and cell types the +3 kb region could be active in, we used the Slidebase tool (Ienasescu et al., 2016) that gathers data of transcription start sites from FANTOM5 consortia and summarizes eRNA transcription levels based on various tissue and cell types. We found that in human the +3 kb region shows the strongest eRNA expression in the brain, spinal cord, and olfactory region (Figure 1C). When grouped by cell type, the strongest expression of +3 kb eRNAs is in hair follicle cells, neurons, and trabecular meshwork cells (Figure 1D).

Collectively, this data suggests that the +3 kb region is an evolutionarily conserved nervous system-specific enhancer that is active mostly in neural tissues and predominantly in neurons but not in other major brain cell types.

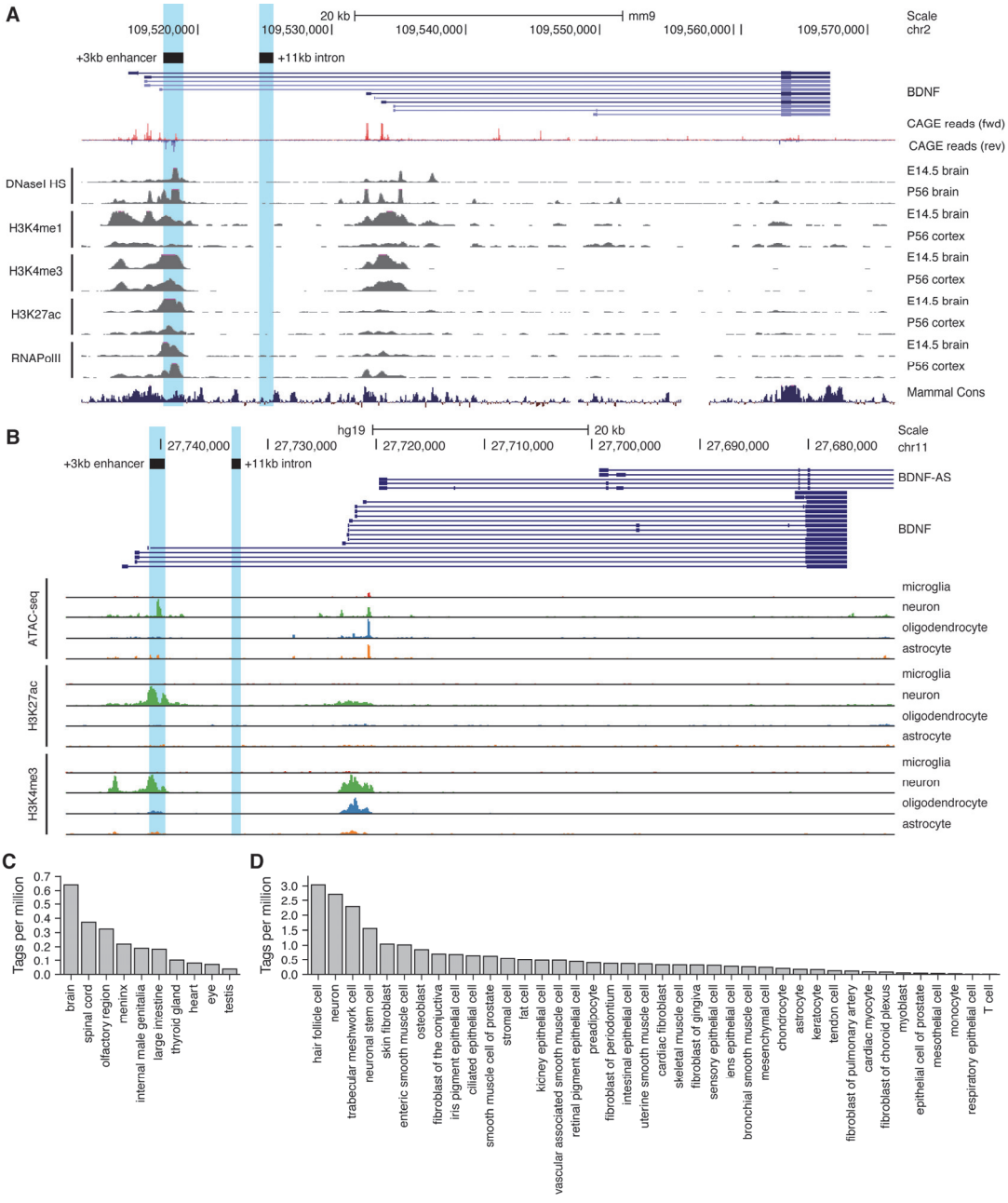

Figure 1. ***Region downstream of BDNF exon III shows enhancer-associated characteristics in mouse and human neural tissues.*** *UCSC Genome browser was used to visualize (A) DNaseI hypersensitivity sites and ChIP-seq data from the ENCODE project in mouse brain tissue, CAGE data of transcription start sites from the FANTOM5 consortium (all tissues and cell types), and (B) open chromatin (ATAC-seq) and ChIP-seq in different human brain cell-types by (Nott et al., 2019). E indicates embryonic day, P*

postnatal day. Signal clipping outside the visualization range is indicated with purple color. The +3 kb region, a potential enhancer of the BDNF gene, and +11 kb intronic region, a negative control region used in the present study were converted from rat genome to mouse or human genome using UCSC Liftover tool and are shown as light blue. The names of the regions represent the distance of the respective region from rat BDNF exon I transcription start site. (C, D) +3 kb enhancer region (chr11:27693843-27694020, hg19 genome build) eRNA expression levels based on CAGE sequencing data from the FANTOM5 project obtained from the Slidebase tool (Ienasescu et al., 2016, http://slidebase.binf.ku.dk). eRNA expression levels were grouped by different tissue types (C) or different cell types (D). Only tissue and cell types with non-zero eRNA expression are shown.

2. +3 kb enhancer region shows bidirectional transcription in luciferase reporter assay in rat cultured cortical neurons and astrocytes

Based on the enhancer-associated characteristics of the +3 kb enhancer region, we hypothesized that the region could function as an enhancer region for BDNF gene in neural cells. It has been reported that the bidirectional transcription of enhancer RNAs (eRNAs) from the enhancer region is correlated with the expression of nearby genes, indicating that the transcription from an enhancer region is a proxy to enhancer's activity (Kim et al., 2010). Therefore, we first investigated whether the +3 kb enhancer region shows bidirectional transcription in rat cultured cortical neurons and astrocytes, the two major cell types in the brain, in a heterologous context using reporter assays. We cloned a ~1.4 kb fragment of the +3 kb enhancer and a similarly sized control (+11 kb) intronic sequence lacking enhancer-associated characteristics in either forward or reverse orientation upstream of the firefly luciferase gene (Figure 2A) and performed luciferase reporter assays.

In rat cortical neurons the +3 kb enhancer region showed very strong transcriptional activity (~300-fold higher compared to the promoterless luciferase reporter vector) that was independent of the orientation of the +3 kb region (Figure 2B). As expected, the +11 kb negative control reporter showed very low luciferase activity in cortical neurons. To determine whether the enhancer region is responsive to different stimuli in neurons and could be involved in stimulus-dependent regulation of the BDNF gene, we used two treatments shown to induce BDNF gene expression – KCl treatment to chronically depolarize the cells and mimic neuronal activity (Ghosh et al., 1994; Pruunsild et al., 2011), and BDNF treatment to activate TrkB signaling and mimic BDNF autoregulation (Esvald et al., 2020; Tuvikene et al., 2016; Yasuda et al., 2007). Our results indicate that the activity of the +3 kb region is upregulated ~2-3-fold in response to both stimuli, suggesting that the region could be a stimulus-dependent enhancer in neurons.

In rat cultured cortical astrocytes, the +3 kb enhancer construct showed modest transcriptional activity (depending on the orientation ~6-23-fold higher compared to the promoterless vector control, Figure 2C). We have previously shown that in cultured cortical astrocytes, BDNF is induced in response to dopamine treatment, and the induction is regulated by an unknown enhancer region within BDNF gene locus (Koppel et al., 2018). However, dopamine treatment had no significant effect on the activity of the +3 kb construct in cultured cortical astrocytes, suggesting that this region is not a dopamine-activated enhancer in astrocytes.

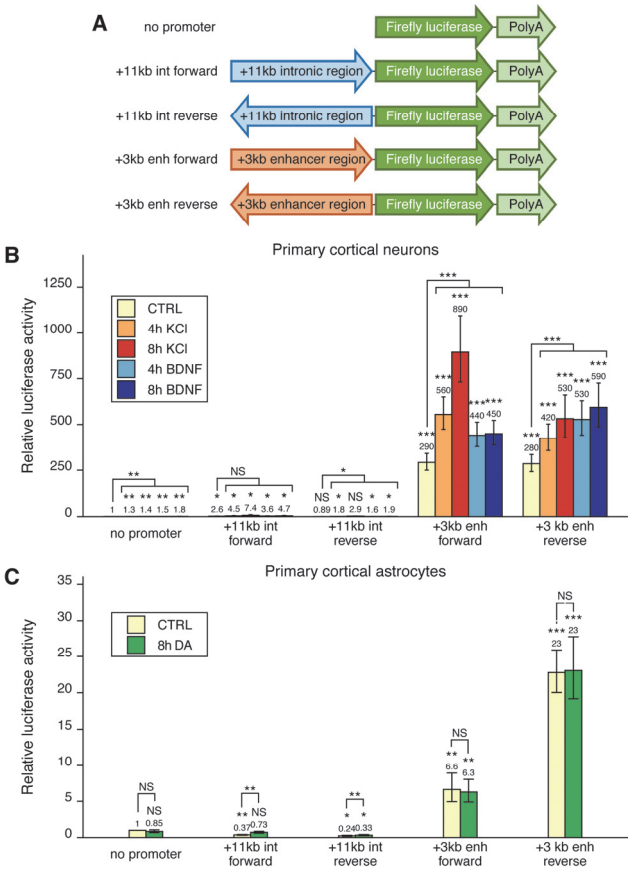

Figure 2. ***+3 kb enhancer region shows bidirectional transcription in luciferase reporter assay in rat cultured cortical neurons and astrocytes.*** *(A) Reporter constructs used in the luciferase reporter assay where the +3 kb enhancer region and the +11 kb control region were cloned in either forward or reverse orientation (respective to the rat BDNF gene) in front of the luciferase expression cassette. (B, C) Rat cortical neurons (B) or astrocytes (C) were transfected with the indicated reporter constructs at 6 and 13 DIV, respectively. 2 days post transfection, neurons were left untreated (CTRL) or treated with 25 mM KCl (with 5 μM D-APV) or 50 ng/ml BDNF for the indicated time (B); astrocytes were treated with 150 μM dopamine (DA) or respective volume of vehicle (CTRL) for the indicated time (C), after which luciferase activity was measured. Luciferase activity in cells transfected with a vector containing no promoter and treated with vehicle or left untreated was set as 1. The average luciferase activity of independent experiments is depicted above the columns. Error bars indicate SEM (n = 7 (B, +3 kb enhancer constructs and no promoter construct), n = 3 (B, intron constructs), and n = 4 (C) independent experiments). Asterisks above the columns indicate statistical significance relative to luciferase activity in untreated cells transfected with the reporter vector containing no promoter, or between indicated groups. NS – not significant, * p<0.05, ** p<0.01, *** p<0.001 (paired two-tailed t-test).*

3. The +3 kb enhancer region potentiates the activity of BDNF promoters I and IV in luciferase reporter assay in rat cultured cortical neurons

To find out whether the newly identified +3 kb enhancer could control the activity of BDNF promoters in a heterologous context, we cloned the +3 kb region or the +11 kb negative control sequence in forward or reverse orientation (respective to the rat BDNF gene) downstream of BDNF promoter-driven luciferase expression cassette (Figure 3A).

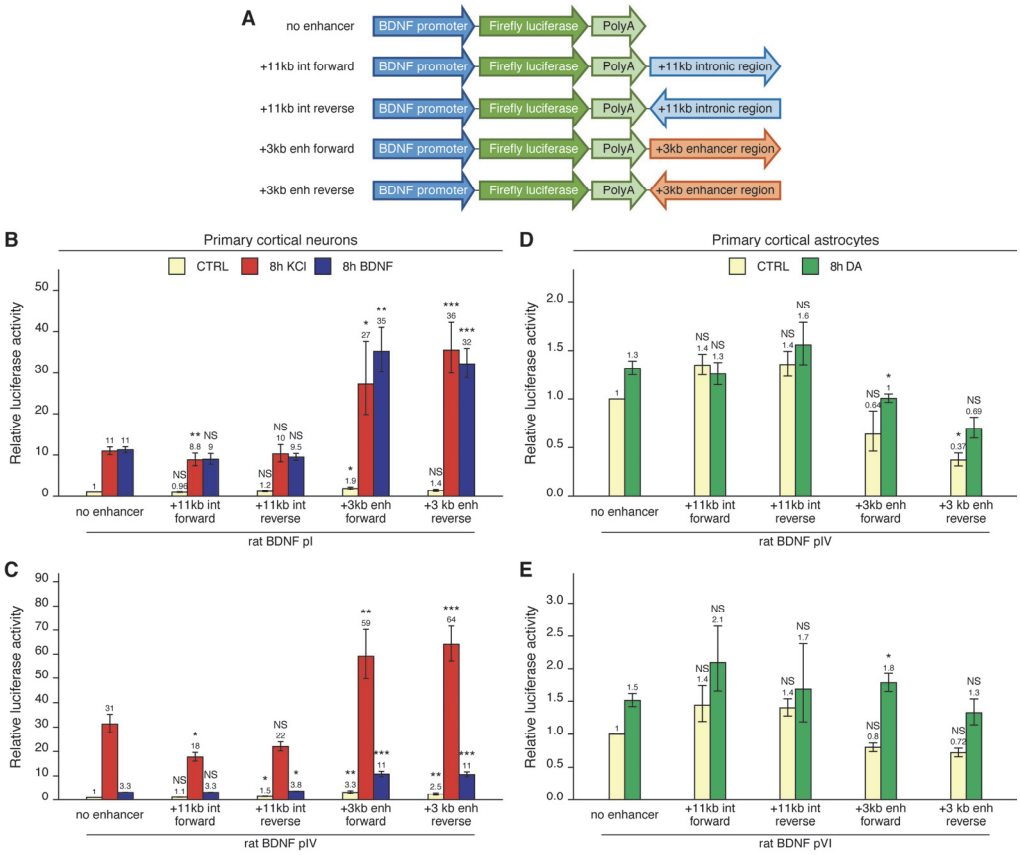

Figure 3. ***+3 kb enhancer region potentiates the activity of BDNF promoters in luciferase reporter assay in rat cortical neurons but not in astrocytes.*** *(A) A diagram of the used luciferase reporter constructs used in this experiment, with a BDNF promoter in front of the firefly luciferase coding sequence and the +3 kb enhancer or +11 kb intronic region in either forward or reverse orientation (respective to the rat BDNF gene) downstream of the luciferase expression cassette. Rat cortical neurons (B, C) or astrocytes (D, E) were transfected with the indicated reporter constructs at 6 and 13 DIV, respectively. Two days post transfection, neurons were left untreated (CTRL) or treated with 25 mM KCl (with 5 µM D-APV) or 50 ng/ml BDNF for 8 hours (B, C); astrocytes were treated with 150 µM dopamine (DA) or respective volume of vehicle (CTRL) for 8 hours (D, E), followed by luciferase activity assay. Luciferase activity is depicted relative to the luciferase activity in untreated or vehicle-treated (CTRL) cells transfected with respective BDNF promoter construct without an enhancer region. The average luciferase activity of independent experiments is shown above the columns. Error bars represent SEM (n = 6 (B, +3 kb enhancer-containing constructs and no enhancer construct), n = 3 (B, + 11 kb intron constructs), n = 4 (C) and n = 3 (D-E) independent experiments). Statistical significance was calculated compared to the activity of the respective BDNF promoter regions without the enhancer region after the respective treatment. NS – not significant, * p<0.05, ** p<0.01, *** p<0.001 (paired two-tailed t-test).*

First, we transfected rat cultured cortical neurons with constructs containing BDNF promoter I and IV, as these promoters are the most widely studied in neurons (West et al., 2014). We treated neurons with either KCl or BDNF, and used luciferase reporter assay to measure the activity of the BDNF promoter region. For BDNF promoter I (Figure 3B), the addition of the +3 kb enhancer region slightly increased the basal activity of the promoter region (~1.5-2-fold). The +3 kb enhancer region also potentiated the KCl and BDNF-induced activity of this promoter ~3-fold in an orientation-independent manner. Similar effects

were observed for BDNF promoter IV (Figure 3C), where the addition of the +3 kb enhancer region potentiated the basal activity of the promoter ~3-fold and KCl and BDNF-induced activity levels ~2-fold. The +11 kb intronic region failed to potentiate the activity of BDNF promoters I and IV.

Cortical astrocytes preferentially express BDNF transcripts containing exons IV and VI (Koppel et al., 2018). Therefore, we studied whether the +3 kb enhancer region could promote the activity of BDNF promoters IV and VI in rat cultured cortical astrocytes. The +3 kb enhancer did not significantly increase the activity of BDNF promoters IV and VI in unstimulated astrocytes and had virtually no effect on the response of these promoters to dopamine treatment (Figure 3D, 3E).

Overall, we found that in the heterologous context the +3 kb enhancer region could potentiate transcription from BDNF promoters in cultured cortical neurons, but not in cortical astrocytes. These results imply that the +3 kb enhancer could be important for BDNF gene expression in neurons, but not in astrocytes.

4. +3 kb enhancer region is a positive regulator of the first cluster of BDNF transcripts in rat cortical neurons but is in an inactive state in rat cortical astrocytes.

To investigate the functionality of the +3 kb region in its endogenous context, we used CRISPR interference (CRISPRi) and activator (CRISPRa) systems. Our system comprised of catalytically inactive Cas9 (dCas9) fused with Krüppel associated box domain (dCas9-KRAB, CRISPRi), or 8 copies of VP16 domain (VP64-dCas9-VP64, CRISPRa) to repress or activate the target region, respectively. dCas9 without effector domains was used to control for potential steric effects (Qi et al., 2013) on BDNF transcription when targeting CRISPR complex inside the BDNF gene. To direct the dCas9 and its variants to the desired location, we used four different gRNAs per region targeting either the +3 kb enhancer region or the +11 kb intronic control region, with all gRNAs targeting the template strand to minimize the potential inhibitory effect of dCas9 binding on transcription elongation (as suggested by Qi et al., 2013). The +11 kb intronic control was used to rule out the possibility of CRISPRi and CRISPRa-induced passive spreading of chromatin modifications within the BDNF gene locus. As a negative control, we used a gRNA not corresponding to any sequence in the rat genome.

We first examined the functionality of the +3 kb enhancer region in cultured cortical neurons. Targeting the +3 kb enhancer or +11 kb intronic region with dCas9 without an effector domain had no major effect on the expression of any of the BDNF transcripts, indicating that targeting CRISPR complex to an intragenic region in BDNF gene does not itself affect BDNF gene expression (Figure 4, left panel). Repressing the +3 kb enhancer region using CRISPRi decreased the basal expression levels of BDNF exon I, IIc, and III-containing transcripts by 2.2, 11, and 2.4-fold, respectively (Figure 4A, 4B, 4C, middle panel). In contrast, no significant effect was seen for basal levels of BDNF exon IV, VI, and IXa-containing transcripts (Figure 4D, 4E, 4F, middle panel). Repressing the +3 kb enhancer region also decreased the KCl and BDNF-induced levels of transcripts starting from the first three 5’ exons ~4-7-fold, but not of other BDNF transcripts (Figure 4A-F, middle panel). These effects correlated with subtle changes in total BDNF expression levels (Figure 4G, middle panel). Targeting CRISPRi to the +11 kb intronic region had no significant effect on any of the BDNF transcripts (Figure 4A-F, middle panel).

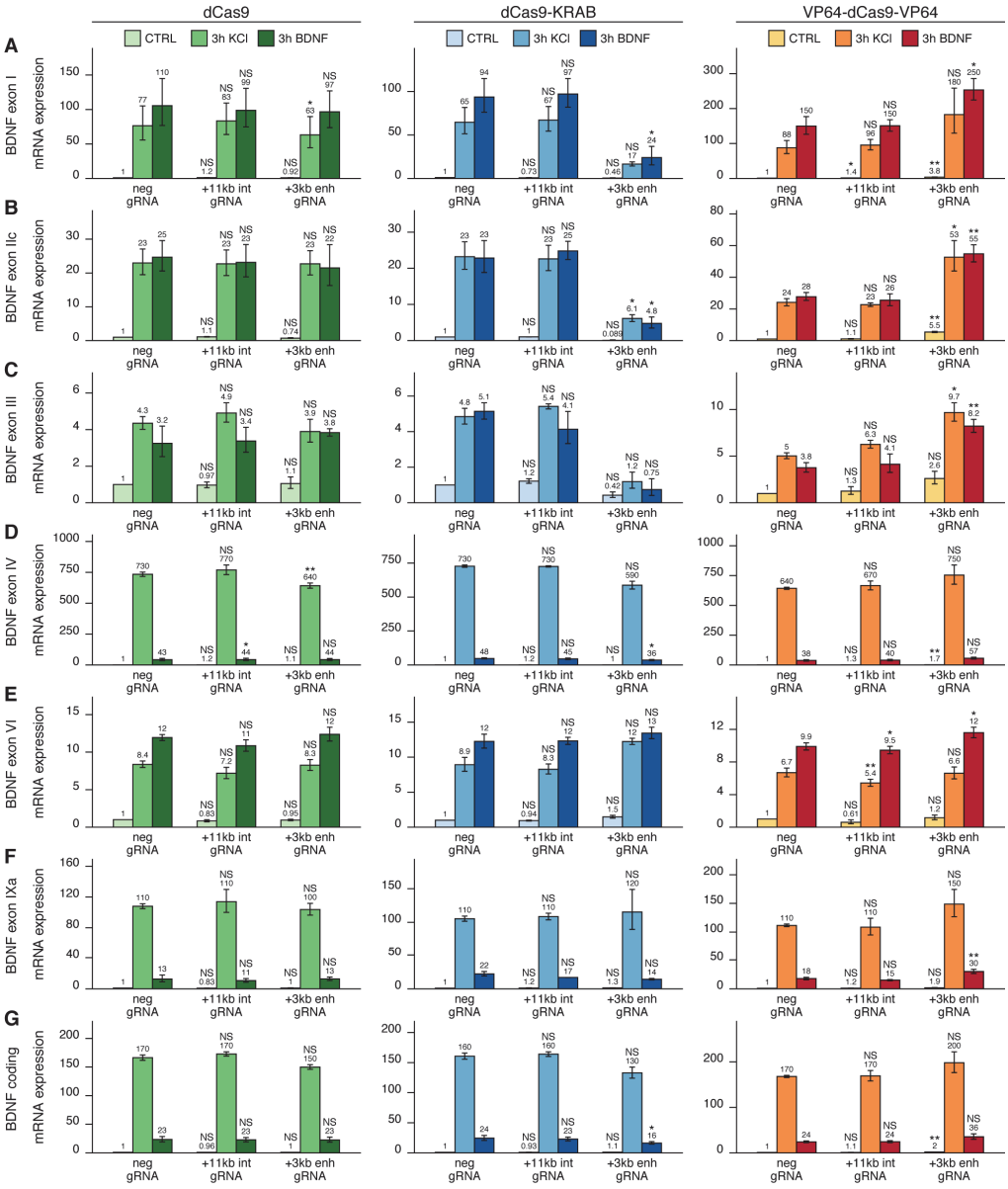

Figure 4. ***+3 kb enhancer is a positive regulator of BDNF exon I, IIc, and III-containing transcripts in rat cortical neurons.*** *Rat cultured cortical neurons were transduced at 0 DIV with lentiviral particles encoding either catalytically inactive Cas9 (dCas9, left panel, green), dCas9 fused with Krüppel associated box domain (dCas9-KRAB, middle panel, blue) or 8 copies of VP16 domain (VP64-dCas9-VP64, right panel, orange) together with lentiviruses encoding either guide RNA that has no corresponding target sequence in the rat genome (neg gRNA), a mixture of four gRNAs directed to the putative +3 kb BDNF enhancer (+3 kb enh gRNA) or a mixture of four guide RNAs directed to +11 kb intronic region (+11 kb int gRNA). Transduced neurons were left untreated (CTRL) or treated with 50 ng/ml BDNF or 25 mM KCl (with 5 μM D-APV) for 3 hours at 8 DIV. Expression levels of different BDNF*

*transcripts were measured with RT-qPCR. mRNA expression levels are depicted relative to the expression of the respective transcript in untreated (CTRL) neurons transduced with negative guide RNA within each set (dCas9, dCas9-KRAB, or VP64-dCas9-VP64). The average mRNA expression of independent experiments is depicted above the columns. Error bars represent SEM (n = 3 independent experiments). Statistical significance was calculated between the respective mRNA expression levels in respectively treated neurons transduced with neg gRNA within each set (dCas9, Cas9-KRAB, or VP64-dCas9-VP64). NS – not significant, * p<0.05, ** p<0.01, *** p<0.001 (paired two-tailed t-test).*

Activating the +3 kb enhancer region with CRISPRa in cultured cortical neurons increased the expression levels of BDNF transcripts of the first cluster (Figure 4A-C, right panel) both in unstimulated neurons (~3-5-fold) and after KCl or BDNF treatment (~2-fold). Slight effect of the activation was also seen for BDNF exon IV and IXa-containing transcripts (Figure 4D, 4F, right panel). Total BDNF basal levels also increased ~2-fold with CRISPR activation of the +3 kb enhancer region, whereas a slightly weaker effect was seen in stimulated neurons. As with CRISPRi, targeting CRISPRa to the +11 kb intronic region had no significant effect on the expression of any of the BDNF transcripts (Figure 4A-F, right panel).

Next, we carried out CRISPRi and CRISPRa experiments in cultured cortical astrocytes. Targeting dCas9 to the BDNF locus did not affect the expression of any of the BDNF transcripts (Figure 5A-F, left panel). Similarly, targeting CRISPRi to the +3 kb enhancer region in astrocytes did not affect the basal expression levels of any BDNF transcript (Figure 5, middle panel). In cells treated with dopamine, repressing +3 kb enhancer region completely abolished the induction of exon IIc-containing transcripts (Figure 5B, middle panel), but did not affect the expression of any of the other transcripts. In contrast, activating the +3 kb region with CRISPRa greatly increased both basal and dopamine-induced levels of all measured BDNF transcripts (Figure 5A-F, right panel). Targeting CRISPRi or CRISPRa to the +11 kb intronic control region did not have a noteworthy effect on any of the measured BDNF transcripts.

As bioinformatic analysis showed bidirectional transcription from the +3 kb enhancer (Figure 1) and our luciferase reporter assays also indicated this (Figure 2), we next decided to directly measure eRNAs from the +3 kb enhancer region in our cortical neurons and astrocytes. Since the sense eRNA is transcribed in the same direction as BDNF pre-mRNA, we could only reliably measure eRNAs from the antisense orientation from the +3 kb enhancer region using antisense eRNA-specific cDNA priming followed by qPCR (Supplementary figure 2A). We found that the +3 kb enhancer antisense eRNA was expressed in cultured neurons and the expression level of the eRNA was induced ~3.5- and ~6-fold upon BDNF and KCl treatment, respectively. Furthermore, repressing the +3 kb enhancer region using CRISPRi decreased the expression of the eRNA ~3-fold. However, activating the +3 kb enhancer region using CRISPRa did not change the expression level of the eRNA. When comparing +3 kb enhancer eRNA expression levels in neurons and astrocytes, the astrocytes showed ~6-fold lower eRNA transcription from the +3 kb enhancer region than neurons (Supplementary figure 2B), also indicating that the +3 kb region is in a more active state in our cultured neurons than in astrocytes.

The main findings of the CRISPRi and CRISPRa experiments are summarized in Figure 6. Taken together, our results suggest that the +3 kb enhancer region is an active BDNF enhancer in rat cultured cortical neurons and regulates the basal and stimulus-induced expression of BDNF transcripts of the first cluster of exons (exons I, II and III). In contrast, the +3 kb region is mostly inactive in rat cultured cortical astrocytes.

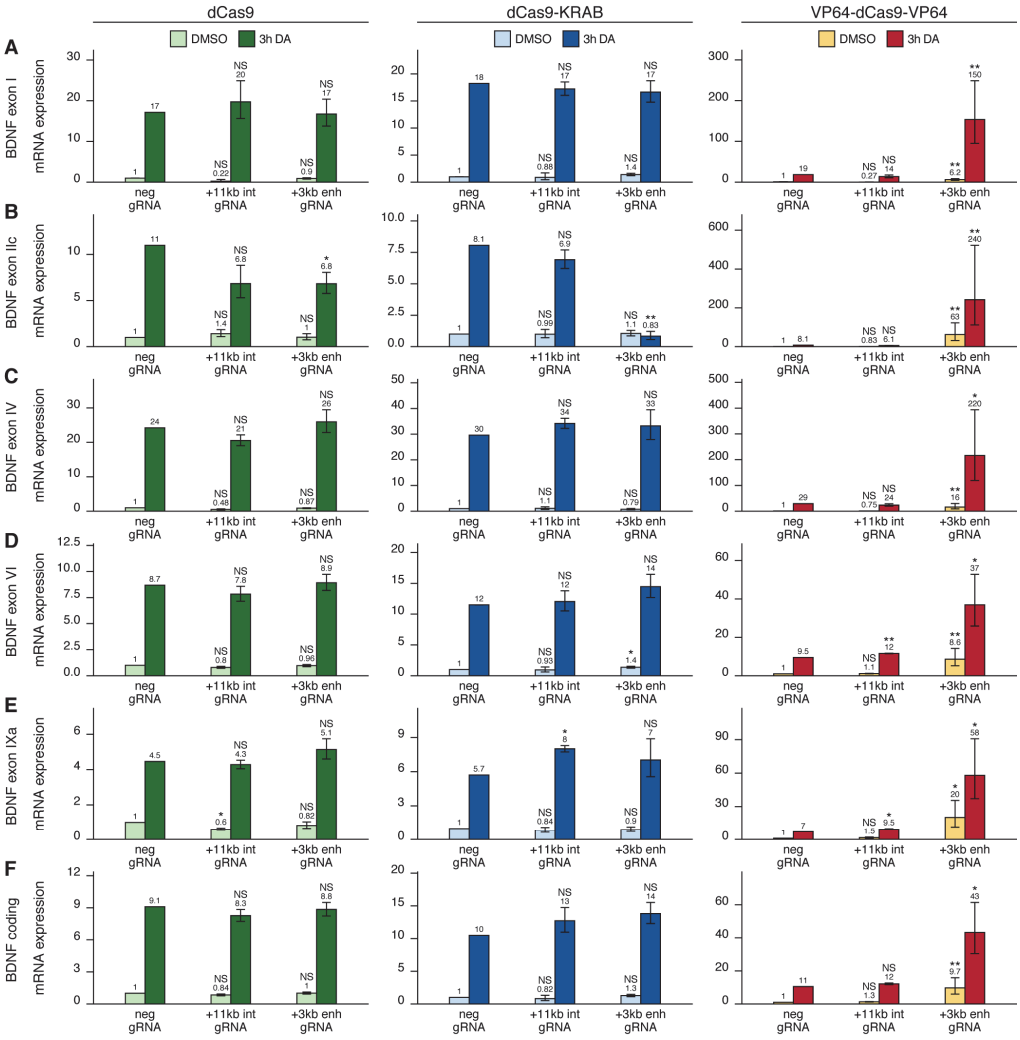

Figure 5. ***+3 kb enhancer region is mainly inactive in rat cortical astrocytes.*** *Rat cultured cortical astrocytes were transduced at 7 DIV with lentiviral particles encoding either catalytically inactive Cas9 (dCas9, left panel, green), dCas9 fused with Krüppel associated box domain (dCas9-KRAB, middle panel, blue) or 8 copies of VP16 domain (VP64-dCas9-VP64, right panel, orange) together with lentiviruses encoding either guide RNA that has no corresponding target sequence in the rat genome (neg gRNA), a mixture of four gRNAs directed to the putative +3 kb BDNF enhancer (+3 kb enh gRNA) or a mixture of four guide RNAs directed to + 11 kb intronic region (+11 kb int gRNA). Transduced astrocytes were treated with vehicle (CTRL) or with 150 µM dopamine (DA) for 3 hours at 15 DIV. Expression levels of different BDNF transcripts were measured with RT-qPCR. The levels of BDNF exon III-containing transcripts were too low to measure reliably. mRNA expression levels are depicted relative to the expression of the respective transcript in astrocytes treated with vehicle (CTRL) transduced with negative guide RNA within each set (dCas9, dCas9-KRAB, or VP64-dCas9-VP64). The average mRNA expression of independent experiments is depicted above the columns. Error bars represent SEM (n = 3 independent experiments). Statistical significance was calculated between respective mRNA expression levels in respectively treated astrocytes transduced with neg gRNA within each set (dCas9, Cas9-KRAB, or VP64-dCas9-VP64). NS – not significant, * p<0.05, ** p<0.01, *** p<0.001 (paired two-tailed t-test).*

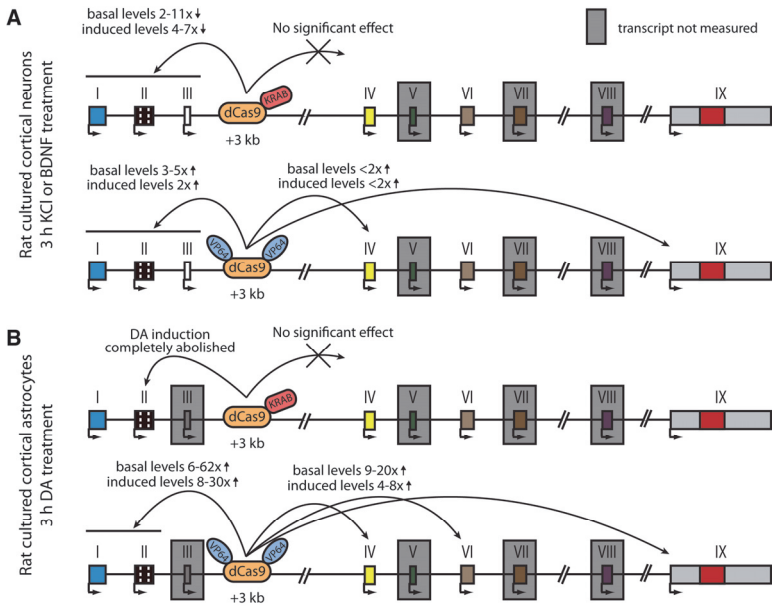

Figure 6. ***Summary of the CRISPRi and CRISPRa experiments with BDNF +3 kb enhancer region in rat cultured cortical neurons and astrocytes.*** *Graphical representation of the main results shown in Figure 4 (A) and Figure 5 (B). Different BDNF exons are shown with boxes, red box in exon IX indicates BDNF coding region. BDNF transcripts that were not measured or that had too low levels to measure reliably are indicated with a grey box around the respective 5' exon.*

5. Deletion of the +3 kb enhancer region in mouse embryonic stem cell-derived neurons decreases the expression of BDNF transcripts starting from the first cluster of exons

To test the regulatory function of the +3 kb enhancer directly and address biological significance of its interspecies conservation, we used CRISPR/Cas9 system to delete the conserved core sequence of the enhancer region in mouse embryonic stem cells engineered to express the pro-neural transcription factor Neurogenin2 from a doxycycline-inducible promoter. We selected single-cell clones containing the desired deletion, differentiated them into excitatory neurons (Ho et al., 2016; Thoma et al., 2012; Zhang et al., 2013) by the addition of doxycyclin, treated the stem cell-derived neurons with BDNF or KCl, and measured the expression of different BDNF transcripts using RT-qPCR.

The deletion of the +3 kb enhancer region strongly decreased both the basal and stimulus-dependent expression levels of BDNF exon I, IIc, and III-containing transcripts (Figure 7A-C). Notably, the effect was more prominent in clones containing homozygous deletion compared to heterozygous clones. We also noted a slight, albeit not statistically significant decrease in the expression of BDNF exon IV and VI-containing transcripts in cells where the +3 kb enhancer region was deleted (Figure 7D-E). This could be attributed to impaired BDNF autoregulatory loop caused by the deficiency of transcripts from the first cluster of exons. It is also possible that the +3 kb enhancer participates in the regulation of the transcripts from the second cluster of exons, but the effect is only very subtle, which would explain why it was not detected in our CRISPRi and CRISPRa experiments in cultured neurons. The levels of BDNF exon IXa were

too low to measure reliably (data not shown). The deletion of the +3 kb enhancer region respectively decreased the total levels of BDNF similarly to the first cluster of BDNF transcripts (Figure 7F). These results confirm the essential role of the +3 kb enhancer region in regulating the expression of BDNF exon I, IIc, and III-containing transcripts in rodent neurons.

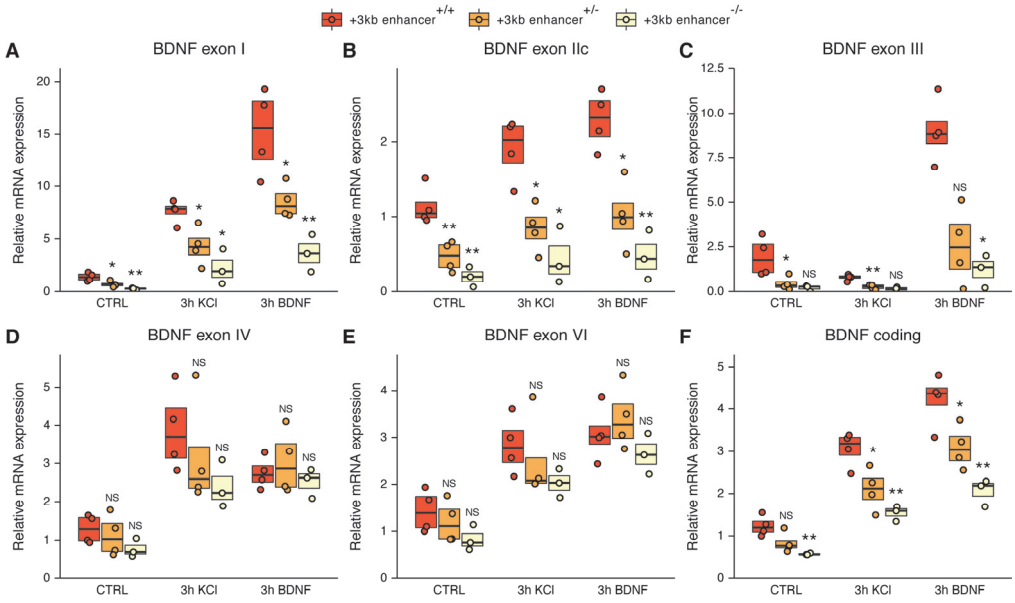

Figure 7. ***The deletion of the +3 kb enhancer region decreases the expression of BDNF exon I, IIc, and III-containing transcripts in mouse embryonic stem cell (mESC)-derived neurons.*** *CRISPR/Cas9 system was used to generate mESC cell lines with ~300-500 bp deletions of the conserved core region of the +3 kb BDNF enhancer. The obtained clonal cell lines containing intact +3 kb enhancer region (+/+), heterozygous deletion (+/-), or homozygous deletion (-/-) of the +3 kb enhancer region were differentiated into neurons using overexpression of Neurogenin2. After 12 days of differentiation, the cells were treated with vehicle (CTRL), 50 ng/ml BDNF or 25 mM KCl together with 25 μM D-APV for 3 hours. The expression levels of different BDNF transcripts were measured using RT-qPCR. The levels of respective BDNF transcripts measured in the parental cell line (also included as a data point in the +/+ group) was set as 1. All data points (obtained from independent cell clones and parental cell line) are depicted with circles. Box plot shows 25% and 75% quartiles and the horizontal line shows the median value. N = 3-4 independent cell clones for each group. Statistical significance was calculated compared to the expression level of the respective transcript in the +/+ genotype group at respective treatment. NS – not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (equal variance unpaired t-test).*

6. The activity of +3 kb enhancer region is regulated by CREB, AP-1 family and E-box-binding transcription factors

To investigate the molecular mechanisms that control the activity of the +3 kb enhancer region, we used *in vitro* DNA pulldown assay with 8 days old rat cortical nuclear lysates coupled with mass-spectrometric analysis to determine the transcription factors that bind to the +3 kb enhancer region (Figure 8A). Collectively, we determined 21 transcription factors that showed specific *in vitro* binding to the +3 kb enhancer region compared to the +11 kb intronic region in two independent experiments (Figure 8B). Of

note, we found numerous E-box-binding proteins, including USFs, TCF4 and the pro-neural transcription factors NeuroD2 and NeuroD6, possibly providing the neuron specific activity of the +3 kb enhancer region. We also detected binding of JunD, a member of the AP-1 transcription factor family.

Next, we used various ChIP-seq experiments in different human cell lines from the ENCODE project and determined numerous transcription factors that bind to the +3 kb enhancer region, including CREB, CEBPB, EGR1 and JunD (Supplementary figure 3). We further used publicly available ChIP-seq data (see Materials and methods section for references) to visualize the binding of different transcription factors to the +3 kb enhancer region in mouse neural cells and tissues (Figure 8C). This data shows neuronal activity-dependent binding of NPAS4, c-Fos, and coactivator CBP to the enhancer region. In agreement with our *in vitro* pulldown results, ChIP-seq analysis also revealed binding of EGR1, NeuroD2 and TCF4 to the +3 kb enhancer region in the endogenous chromatin context.

Considering the CREB binding in ENCODE data (Suplementary figure 2) and CBP binding in cultured cortical neurons (Figure 8C), we first decided to investigate whether CREB binds BDNF +3 kb enhancer region in our rat cortical neurons. We performed ChIP-qPCR and determined that in cultured cortical neurons CREB binds to the +3 kb enhancer region, whereas we found no significant CREB binding to the +21 kb negative control region, located directly downstream of BDNF exon VII (Figure 8D). Of note, the binding of CREB to the +3 kb enhancer region was ~2.6-times stronger than binding to BDNF promoter IV, which contains the well-described CRE element (Hong et al., 2008; Tao et al., 1998). Next, we focused on the various E-box-binding proteins, as many E-box-binding proteins are proneural and could therefore confer the neural specificity of the +3 kb enhancer region. As transcription factors from the NeuroD family need dimerization partner from the class I helix-loop-helix proteins, e.g. TCF4, to bind DNA (Massari and Murre, 2000; Ravanpay and Olson, 2008), we verified binding of TCF4 to the +3 kb region in our cultured neurons using TCF4 ChIP-qPCR (Figure 8D).

To determine functionally important transcription factors that regulate BDNF +3 kb enhancer region, we first screened a panel of dominant-negative transcription factors in luciferase reporter assay where the expression of luciferase was under control of the +3 kb enhancer region (Figure 8E). In agreement with the in *vitro* pulldown assay, ChIP-seq and ChIP-qPCR results, we found the strongest inhibitory effect using dominant negative versions of CREB (named A-CREB), ATF2 (named A-ATF2) and AP-1 family (named A-FOS). However, the effect of different dominant negative proteins was slightly lower when +3 kb enhancer region was in the reverse orientation. Our data suggests the role of CREB, AP-1 family proteins and ATF2 in regulating the neuronal activity-dependent activation of the +3 kb enhancer region, whereas we found no notable evidence of USF family transcription factors and CEBPB regulating the activity of +3 kb enhancer region.

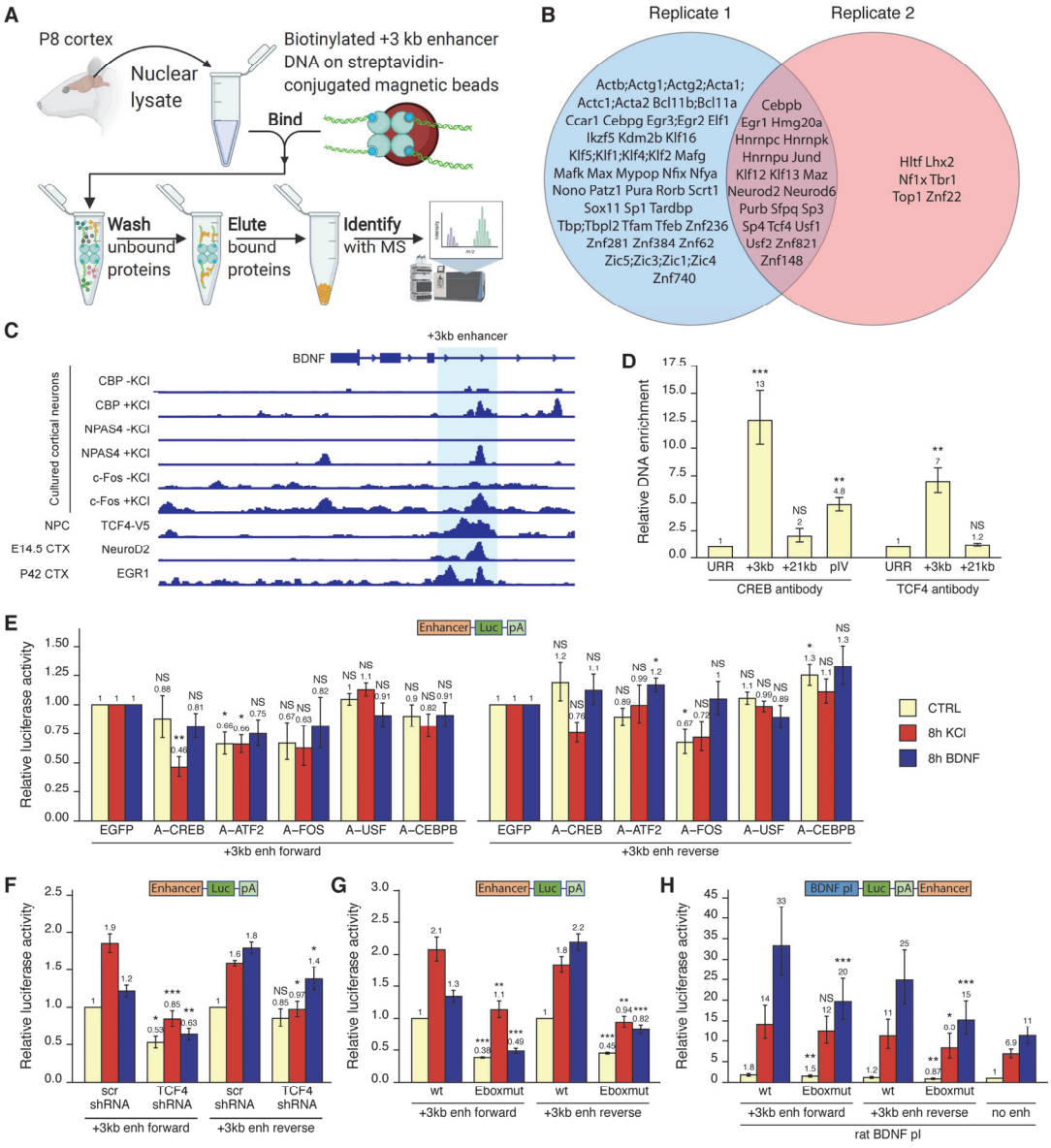

Figure 8. ***Various transcription factors, including CREB, AP-1 proteins and E-box-binding transcription factors regulate the activity of +3 kb enhancer region.*** *(A) Schematic overview of the in vitro DNA pulldown assay to determine transcription factors binding to the +3 kb enhancer region. The illustration was created with BioRender.com. (B) Transcription factors identified in the in vitro DNA pulldown assay in two biological replicates. Semicolon between protein names indicates uncertainty in the peptide to protein assignment between the proteins separated by the semicolons. (C) Previously published ChIP-seq experiments showing binding of different transcription factors to the +3 kb enhancer region. (D) ChIP-qPCR assay in cultured cortical neurons at 8 DIV with anti-CREB or anti-TCF4 antibody. Enrichment is shown relative to the enrichment of unrelated region (URR) with the respective antibody. +21 kb region (downstream of the BDNF exon VII) was used as a negative control. pIV indicates BDNF*

*promoter IV region. (E-H) Rat cortical neurons were transfected at 5 DIV (F) or 6 DIV (E-H) with reporter constructs where the +3 kb enhancer region was cloned in front of the luciferase coding sequence (E-G, see also Figure 2A) or with reporter constructs where the +3 kb enhancer region was cloned downstream of the BDNF promoter I-controlled firefly luciferase expression cassette (H, see Figure 3A). Schematic representations of the used reporter constructs are shown above the graphs, with Luc designating luciferase coding sequence and pA polyadenylation sequence. At 8 DIV, neurons were left untreated (CTRL) or treated with 25 mM KCl (with 5 μM D-APV) or 50 ng/ml BDNF for the indicated time, after which luciferase activity was measured. Luciferase activity is depicted relative to the luciferase activity in respectively treated cells transfected with EGFP and the respective +3 kb enhancer construct (E), relative to luciferase activity in untreated cells co-transfected with control shRNA (scr) and the respective +3 kb enhancer construct (F), relative to luciferase activity in untreated cells transfected with the respective wild-type (wt) +3 kb enhancer construct (G), or relative to luciferase activity in untreated cells transfected with rat BDNF promoter I construct containing no enhancer region (H). Eboxmut indicates mutation of a putative E-box element in the +3 kb enhancer region. Numbers above the columns indicate average, error bars represent SEM (n = 4 (D, TCF4 antibody), n = 3 (D, CREB antibody), n = 5-6 (E), n = 4-5 (F), n=4 (G), n = 7 (H) independent experiments). Statistical significance was calculated compared to the ChIP enrichment of DNA at the URR region using respective antibody (D), compared to the luciferase activity in respectively treated cells transfected with respective +3 kb enhancer construct and EGFP (E), scr shRNA (F), or respective wt +3 kb enhancer construct (G, H). NS – not significant, * p<0.05, ** p<0.01, *** p<0.001 (paired two-tailed t-test).*

Finally, we elucidated the role of E-box binding proteins in the regulation of +3 kb enhancer region. Using luciferase reporter assays, we found that silencing TCF4 expression with TCF4 shRNA-expressing plasmid decreased the activity of the +3 kb enhancer region in both unstimulated and KCl and BDNF-stimulated neurons. However, the effects were slightly smaller when the enhancer region was in the reverse orientation (Figure 8F). Based on the TCF4 and NeuroD2 ChIP-seq data (Figure 8C), we identified a putative E-box binding sequence in the +3 kb enhancer region (CAGATG). To determine the relevance of this E-box element, we generated +3 kb enhancer-containing reporter constructs where this E-box motif was mutated (CAGAAC). We determined that this motif participates in regulating both the basal activity and BDNF and KCl-induced activity of the enhancer region (Figure 8G). Importantly, mutating the E-box decreased the ability of the +3 kb enhancer region to potentiate transcription from BDNF promoter I in reporter assays (Figure 8H).

Collectively, we have identified numerous transcription factors that potentially regulate the activity of +3 kb enhancer region, and further discovered a functional E-box element in the enhancer, possibly conferring neuron-specific activity of the +3 kb enhancer region.

DISCUSSION

BDNF promoters I, II, and III are located within a relatively compact (~2 kb) region in the genome, making it possible that their activity is controlled by a common mechanism. A similar spatial clustering of BDNF exons seems to be conserved in vertebrates, with a similar genomic organization observed in frog (Kidane et al., 2009), chicken (Yu et al., 2009), zebrafish (Heinrich and Pagtakhan, 2004), rodents and human (Aid et al., 2007; Pruunsild et al., 2007). It has previously been suggested that BDNF promoters I and II could be co-regulated as one functional unit (Hara et al., 2009; Timmusk et al., 1999; West et al., 2014). Here, we show that the promoters of BDNF exons I, II, and III are co-regulated as a neuron-specific unit through a conserved enhancer region located downstream of exon III.

We have previously reported that exon I-containing BDNF transcripts contain in-frame alternative translation start codon that is used more efficiently for translation initiation than the canonical start codon in the exon IX (Koppel et al., 2015). As the BDNF exon I-containing transcripts are highly inducible in response to different stimuli, they could make a substantial contribution to the overall production of BDNF protein in neurons, despite the low basal expression levels of this transcript. Remarkably, the BDNF transcripts from the first cluster of exons have been shown to regulate important aspects of behavior. In

female mice, BDNF exon I-containing transcripts are important for proper sexual and maternal behavior (Maynard et al., 2018), whereas in male mice the BDNF exon I and exon II-containing transcripts regulate serotonin signaling and control aggressive behavior (Maynard et al., 2016). Furthermore, it has been shown that BDNF exon I-containing transcripts in the hypothalamus participate in energy metabolism and thermoregulation (You et al., 2020). The +3 kb enhancer identified in our work might therefore be an important regulator of BDNF gene expression in the formation of the neural circuits regulating both social behavior and energy metabolism. Further work will address this possibility experimentally.

The data from Nord et al. (2013) indicates that the highest H3K27ac modification, a hallmark of active regulatory region, at the +3 kb enhancer region in development occurs a week before and a week after birth in mice – coinciding with the period of late neurogenesis, neuronal migration, synaptogenesis, and maturation of neurons (Reemst et al., 2016). It appears that the +3 kb enhancer region is mostly active in early life and participates in the development of the central nervous system via regulating BDNF expression. However, it is also possible that the decline in H3K27ac mark in murine brain tissue during postnatal development is due to the increased amount of non-neuronal cells in the brain compared to neurons. Although the activity of the +3 kb enhancer seems to decrease with age, it is plausible that it remains active also in later postnatal life and upregulates BDNF expression, thereby regulating synaptic plasticity in the adult organism.

Based on the induction of eRNA expression from the +3 kb enhancer region upon depolarization and BDNF-TrkB signaling in both luciferase reporter assays and in the endogenous context, and binding of various activity-dependent transcription factors to the +3 kb region, our data indicates that in addition to conferring neuron-specificity, the +3 kb enhancer region also participates in BDNF-TrkB signaling and neuronal activity-induced expression of the first cluster of BDNF transcripts. Furthermore, repressing or activating the +3 kb enhancer region with CRISPRi or CRISPRa also affected the stimulus-induced levels of these transcripts. Notably, the part of the BDNF gene containing the +3 kb enhancer has previously been implicated in the Reelin-mediated induction of BDNF expression (Telese et al., 2015), indicating that the +3 kb enhancer could respond to other stimuli in addition to membrane depolarization and TrkB signaling.

We also investigated the possibility that the +3 kb enhancer contributes to the catecholamine-induced expression of BDNF transcripts in rat cultured cortical astrocytes (Koppel et al., 2018) and noted that even though the activation of the +3 kb enhancer increased the basal and stimulus-induced expression of all BDNF transcripts, repression of the +3 kb enhancer had almost no effect on BDNF expression. Furthermore, the transcriptional activity of the +3 kb enhancer was not induced by dopamine-treatment in luciferase reporter assay, further indicating that the +3 kb region is not the enhancer responsible for catecholamine-dependent induction of BDNF expression. Interestingly, the dopamine-dependent induction of BDNF exon II-containing transcripts was abolished when the +3 kb enhancer was repressed using CRISPRi, suggesting that the +3 kb enhancer region might control the activity of stimulus-specific expression of BDNF promoter II in astrocytes. Since the activity of BDNF promoter II is regulated by neuron-restrictive silencer factor (NRSF) (Timmusk et al., 1999), it is possible that the drastic decrease in the dopamine-dependent induction of BDNF exon II-containing transcripts was due to the cooperative effect between NRSF and the +3 kb enhancer region. Further investigation is needed to determine whether this hypothesis is true and whether such cooperation between the +3 kb enhancer region and NRSF binding to BDNF exon II also happens in neurons. Although we have not tested this directly, our data does not support the notion that the +3 kb region is an active repressor in non-neuronal cells, e.g. astrocytes. Instead, it seems that the +3 kb enhancer is a positive regulator of BDNF gene operating

specifically in neurons. We conclude that the +3 kb enhancer region is largely inactive in rat cultured cortical astrocytes and it is distinct from the distal *cis*-regulatory region controlling the catecholamine-induced activities of BDNF promoters IV and VI.

Our results indicate that the +3 kb enhancer can receive regulatory inputs from various basic helix-loop-helix transcription factors, including TCF4 and its pro-neural heterodimerization partners NeuroD2 and NeuroD6. Single-cell RNA-seq analysis in the mouse cortex and hippocampus has indicated that NeuroD transcription factors are expressed mainly in excitatory neurons, similar to BDNF (Tasic et al., 2016). It has been reported that NeuroD2 preferentially binds to E-boxes CAGCTG or CAGATG (Fong et al., 2012), which is in agreement with the functional E-box CAGATG sequence found in the +3 kb enhancer. Furthermore, it has been previously shown that NeuroD2 knock-out animals exhibit decreased BDNF levels in the cerebellum (Olson et al., 2001). However, Olson et al found no change in BDNF levels in the cerebral cortex of these knock-out animals. It is possible that different NeuroD family transcription factors regulate BDNF expression in different brain areas and developmental stages, or that a compensatory mechanism between NeuroD2 and NeuroD6, both binding the +3 kb enhancer region in our *in vitro* DNA pulldown assay, exists in cortical neurons of NeuroD2 knock-out animals. It has been well-described that NeuroD transcription factors regulate neuronal differentiation (Massari and Murre, 2000), axonogenesis (Bormuth et al., 2013), neuronal migration (Guzelsoy et al., 2019), and proper synapse formation (Ince-Dunn et al., 2006; Wilke et al., 2012). As BDNF also has a role in the aforementioned processes (Park and Poo, 2013), it is plausible that at least some of the effects carried out by NeuroD family result from increasing BDNF expression. Further work is needed to clarify the exact role of TCF4 and NeuroD transcription factors in BDNF expression.

In conclusion, we have identified a novel intronic enhancer region governing the expression of neuron-specific BDNF transcripts starting from the first cluster of exons – exons I, II, and III – in mammals. Exciting questions for further work are whether the +3 kb enhancer region is active in all neurons or in specific neuronal subtypes, and whether the activity of this enhancer element underlies *in vivo* contributions of BDNF to brain development and function.

MATERIALS AND METHODS

1. Cultures of rat primary cortical neurons

All animal procedures were performed in compliance with the local ethics committee. Cultures of cortical neurons from embryonic day (E) 21 Sprague Dawley rat embryos of both sexes were prepared as described previously (Esvald et al., 2020). The cells were grown in Neurobasal A (NBA) medium (Gibco) containing 1× B27 supplement (Gibco), 1 mM L-glutamine (Gibco), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Gibco) or 100 µg/ml Primocin (Invivogen) instead of penicillin/streptomycin at 37 °C in 5% CO_2 environment. At 2 days *in vitro* (DIV), half of the medium was replaced with fresh supplemented NBA, or the whole medium was replaced for cells transduced with lentiviruses. To inhibit the proliferation of non-neuronal cells a mitotic inhibitor 5-fluoro-2'-deoxyuridine (FDU, final concentration 10 µM, Sigma-Aldrich) was added with the change of the medium.

2. Cultures of rat primary cortical astrocytes

Cultures of cortical astrocytes were prepared from E21 Sprague Dawley rat embryos of both sexes as described previously (Koppel et al., 2018). The cells were grown in 75 cm^2 tissue culture flasks in

Dulbecco's Modified Eagle Medium (DMEM with high glucose, PAN Biotech) supplemented with 10% fetal bovine serum (PAN Biotech) and 100 U/ml penicillin and 0.1 mg/ml streptomycin (Gibco) at 37 °C in 5% CO_2 environment. At 1 DIV, the medium was replaced with fresh growth medium to remove loose tissue clumps. At 6 DIV, the flasks were placed into a temperature-controlled shaker Certomat® BS-1 (Sartorius Group) for 17-20 hours and shaken at 180 rpm at 37 °C to detach non-astroglial cells from the flask. After overnight shaking, the medium was removed along with unattached non-astrocytic cells, and astrocytes were washed three times with 1× PBS. Astrocytes were detached from the flask with trypsin-EDTA solution (0.25% Trypsin-EDTA (1×), Gibco) diluted 4 times with 1× PBS at 37 °C for 3-5 minutes. Trypsinized astrocytes were resuspended in supplemented DMEM and centrifuged at 200 × g for 6 minutes. The supernatant was removed, astrocytes were resuspended in supplemented DMEM and seeded on cell culture plates previously coated with 0.2 mg/ml poly-L-lysine (Sigma-Aldrich) in Milli-Q. At 9 DIV, the whole medium was replaced with fresh supplemented DMEM.

3. Drug treatments

At 7 DIV, cultured neurons were pre-treated with 1 µM tetrodotoxin (Tocris) until the end of the experiment to inhibit spontaneous neuronal activity. At 8 DIV, neurons were treated with 50 ng/ml human recombinant BDNF (Peprotech) or with a mixture of 25 mM KCl and 5 µM NMDA receptor antagonist D-2-amino-5-phosphopentanoic acid (D-APV, Cayman Chemical Company) to study BDNF autoregulation or neuronal activity-dependent expression of the BDNF gene, respectively.

Cultured cortical astrocytes were treated at 15 DIV with 150 µM dopamine (Tocris) to study the regulation of the BDNF gene by catecholamines or 0.15% DMSO (Sigma) as a vehicle control in fresh serum-free and antibiotics-free DMEM (DMEM with high glucose, PAN Biotech).

4. Transfection of cultured cells and luciferase reporter assay

Rat +3 kb enhancer (chr3:100771267-100772697, rn6 genome assembly) or +11 kb intron (chr3:100778398-100779836, rn6 genome assembly) regions were amplified from rat BDNF BAC construct (Koppel et al., 2018) using Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific) and cloned into pGL4.15 vector (Promega) in front of the Firefly luciferase coding sequence. To generate reporter constructs containing both BDNF promoter and enhancer region, the hygromycin expression cassette downstream of Firefly luciferase expression cassette in pGL4.15 vector was replaced with a new multiple cloning site, into which the +3 kb enhancer or +11 kb intron regions were cloned in either forward or reverse orientation (respective to the rat BDNF gene). The BDNF promoter regions were obtained from rat BDNF promoter constructs (Esvald et al., 2020) and cloned in front of the Firefly luciferase coding sequence. Plasmids encoding control and TCF4 shRNA have been published previously (Sepp et al., 2017). Coding regions of different dominant negative transcription factors were subcloned from AAV plasmids (Esvald et al., 2020) into pRRL vector backbone under the control of human PGK promoter.

For transfection and luciferase reporter assays, rat cortical neurons or astrocytes were grown on 48-well cell culture plates. Transfections were carried out in duplicate wells.

Cultured cortical neurons were transfected as described previously (Jaanson et al., 2019) with minor modifications. Transfection was carried out in unsupplemented NBA using 500 ng of the luciferase reporter construct and 20 ng of a normalizer plasmid pGL4.83-mPGK-hRLuc at 5-6 DIV using Lipofectamine

2000 (Thermo Scientific) with DNA to Lipofectamine ratio of 1:2. Transfection was terminated by replacing the medium with conditioned medium, which was collected from the cells before transfection.

Cultured cortical astrocytes were transfected as described previously (Koppel et al., 2018) using 190 ng of luciferase reporter construct and 10 ng of normalizer plasmid pGL4.83-SRα-hRLuc at 13 DIV using Lipofectamine 2000 (Thermo Scientific) with DNA to Lipofectamine ratio of 1:3.

The cells were lysed with Passive Lysis Buffer (Promega) and luciferase signals were measured with Dual-Glo® Luciferase assay kit (Promega) using GENios pro plate reader (Tecan). Background-corrected Firefly luciferase signals were normalized to background-corrected Renilla luciferase signals and the averages of duplicate wells were calculated. Data were log-transformed for statistical analysis, mean and standard error of the mean (SEM) were calculated, and data were back-transformed for graphical representation.

5. CRISPR interference and activator systems, RT-qPCR

pLV-hUbC-dCas9-KRAB-T2A-GFP plasmid used for CRISPR interference has been described previously (Esvald et al., 2020) and pLV-hUbC-VP64-dCas9-VP64-T2A-GFP plasmid used for CRISPR activation was obtained from Addgene (plasmid #59791). Lentiviral particles were produced as described previously (Koppel et al., 2018). Relative viral titers were estimated from provirus incorporation rate measured by qPCR and an equal amounts of functional viral particles were used for transduction in the following experiments.

Rat cortical neurons were transduced at 0 DIV, whereas cortical astrocytes were transduced after sub-culturing at 7 DIV. After treatments at 8 DIV for neurons or at 14 DIV for astrocytes, the cells were lysed and RNA was extracted with RNeasy Mini Kit (Qiagen) using on-column DNA removal with RNase-Free DNase Set (Qiagen). RNA concentration was measured with BioSpec-nano spectrophotometer (Shimadzu Biotech). cDNA was synthesized from equal amounts of RNA with Superscript® III or Superscript® IV reverse transcriptase (Invitrogen) using oligo$(dT)_{20}$ or a mixture of oligo$(dT)_{20}$ and random hexamer primer (ratio 1:1, Microsynth). To measure +3 kb enhancer eRNAs, cDNA was synthesized using a mixture of antisense-eRNA specific primer and HPRT1 primer (1:1 ratio). The primers used for cDNA synthesis are listed in Supplementary Table 1.

All qPCR reactions were performed in 10 µl volume in triplicates with 1× HOT FIREpol EvaGreen qPCR Mix Plus (Solis Biodyne) and primers listed in Supplementary Table 1 on LightCycler® 480 PCR instrument II (Roche). Gene expression levels were normalized to HPRT1 mRNA levels in neurons and Cyclophilin B mRNA levels in astrocytes. Data were log-transformed and autoscaled (as described in Vandesompele et al., 2002) for statistical analysis, mean and SEM were calculated, and data were back-transformed for graphical representation.

6. Mouse embryonic stem cells

A2Lox mouse embryonic stem cells (mESCs) containing doxycyclin-inducible Neurogenin2 transgene (Zhuravskaya and Makeyev, in preparation) were grown in 2i media as described in (Iacovino et al., 2011; Kainov and Makeyev, 2020). To delete the +3 kb enhancer region, 3 + 3 gRNAs targeting either side of the +3 kb enhancer core region (targeting sequences listed in Supplementary Table 2) were cloned into pX330 vector (Addgene plasmid #42230). mESCs were co-transfected with a mixture of all 6 CRISPR plasmids and a plasmid containing a blasticidin expression cassette for selection. One day post transfection, 8 µg/ml blasticidin (Sigma) was added to the media for 3 days after which selection was ended, and cells were

grown for an additional 11 days. Finally, single colonies were picked and passaged. The deletion of the +3 kb enhancer region was assessed from genomic DNA with PCR using primers flanking the desired deletion area. To rule out larger genomic deletions, qPCR-based copy number analysis was carried out with primers targeting the desired deletion area, and either side of the +3 kb region outside of the desired deletion area. All primers are listed in Supplementary Table 1. Cell clones containing no deletion, heterozygous or homozygous deletion of the core conserved enhancer region together with intact flanking regions were used for subsequent analysis.

Selected mESCs were differentiated into neurons as follows. Cells were plated on Matrigel (Gibco) coated 12-well plates at a density of ~25 000 cells/well in N2B27 media (1:1 DMEM F12-HAM and Neurobasal mixture, 1x N2, 1x B27 with retinoic acid, 1x penicillin-streptomycin, 1 µg/ml laminin, 20 µg/ml insulin, 50 µM L-glutamine) supplemented with 0.1M β-mercaptoethanol (Sigma) and 2 µg/ml doxycycline (Sigma). After 2 days the whole media was changed to N2B27 media containing 200 µM ascorbic acid (Sigma) and 1 µg/ml doxycycline. Next, half of the media was replaced every 2 days with new N2B27 media containing 200 µM ascorbic acid but no doxycycline. On the 12th day of differentiation, cells were treated with Milli-Q, BDNF (Peprotech), or KCl for 3 hours. All treatments were added together with 25 µM D-APV (Alfa Aesar). After treatment, the cells were lysed and RNA was extracted using EZ-10 DNAaway RNA Mini-Prep Kit (Bio Basic inc). cDNA was synthesized using Superscript IV (Thermo Fischer) and qPCR was performed with HOT FIREPol EvaGreen qPCR Mix Plus (Solis Biodyne) or qPCRBIO SyGreen Mix Lo-ROX (PCR Biosystems Ltd) on LightCycler 96 (Roche). The levels of CNOT4 mRNA expression were used for normalization. Used primers are listed in Supplementary Table 1.

7. In vitro DNA pulldown mass-spectrometry

855 bp region of the +3 kb enhancer and +11 kb intronic region were amplified with PCR using HotFirePol polymerase (Solis Biodyne) and primers listed in Supplementary Table 1, with the reverse primers having a 5' biotin modification (Microsynth). PCR products were purified using DNA Clean & Concentrator™-100 kit (Zymo Research) using a 1:5 ratio of PCR solution and DNA binding buffer. The concentration of the DNA was determined with Nanodrop 2000 spectrophotometer (Thermo Scientific).

The preparation of nuclear lysates was performed as follows. Cortices from 8 days old Sprague Dawley rat pups of both sexes were dissected and snap-frozen in liquid nitrogen. Nuclear lysates were prepared with high salt extraction as in (Wu, 2006) and (Lahiri and Ge, 2000) with minor modifications. Briefly, cortices were weighed and transferred to pre-cooled Dounce tissue grinder (Wheaton). 2 ml of ice-cold cytoplasmic lysis buffer (10 mM Hepes, pH 7.9 (adjusted with NaOH), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5% NP-40, 300 mM sucrose, 1x cOmplete™ Protease Inhibitor Cocktail (Roche), and phosphatase inhibitors as follows: 5 mM NaF (Fisher Chemical), 1 mM beta-glycerophosphate (Acros Organics), 1 mM Na_3VO_4 (ChemCruz, sc-3540A) and 1 mM $Na_4P_2O_7$ (Fisher Chemical)) was added and tissue was homogenized 10 times with tight pestle. Next, the lysate was transferred to a 15 ml tube and cytoplasmic lysis buffer was added to a total volume of 1 ml per 0.1 g of tissue. The lysate was incubated on ice for 10 minutes with occasional inverting. Next, the lysate was transferred to a 100 µm nylon cell strainer (VWR, ref nr 732-2759) to remove tissue debris and the flow-through was centrifuged at 2600 × g at 4 °C for 1 min to pellet nuclei. The supernatant (cytoplasmic fraction) was discarded and the nuclear pellet was resuspended in 1 ml per 1 g of tissue ice-cold nuclear lysis buffer (20 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 2.5% Glycerol, 1x cOmplete™ Protease Inhibitor Cocktail (Roche), and phosphatase inhibitors) and transferred to a new Eppendorf tube. To extract nuclear proteins, the pellet was rotated at 4 °C for

30 minutes and finally centrifuged at 11 000 × g at 4 °C for 10 min. The supernatant was collected as nuclear fraction and protein concentration was measured with BCA Protein Assay Kit (Pierce).

In vitro DNA pulldown was performed as follows. Two biological replicates were performed using nuclear lysates of cortices from pups of different litters. Pierce™ Streptavidin Magnetic Beads (50 µl per pulldown reaction) were washed 2 times with 1× binding buffer (BB, 5 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1 M NaCl, 0.05% Tween-20), resuspended in 2× BB, and an equal volume of 50 pmol biotinylated DNA (in 10 mM Tris-HCl, pH 8.5, 0.1 mM EDTA) was added and incubated at room temperature for 30 min with rotation. To remove the unbound probe, the beads were washed 3 times with 1× BB. Finally, 400 µg of nuclear proteins (adjusted to a concentration of 1.6 mg/ml with nuclear lysis buffer) and an equal volume of buffer D (20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 8% glycerol, 1x cOmplete™ Protease Inhibitor Cocktail (Roche), and phosphatase inhibitors) were added and incubated with rotation at 4 °C overnight. The next day, the beads were washed 3 times with 1× PBS, once with 100 mM NaCl and once with 200 mM NaCl. Bound DNA and proteins were eluted with 16 mM biotin (Sigma) in water (at pH 7.0) at 80 °C for 5 min, the eluate was transferred to a new tube and snap-frozen in liquid nitrogen.

Mass-spectrometric analysis of the eluates was performed with nano-LC-MS/MS using Q Exactive Plus (Thermo Scientific) at Proteomics core facility at the University of Tartu, Estonia, as described previously (Mutso et al., 2018) using label-free quantification instead of SILAC and Rattus norvegicus reference proteome for analysis. The full lists of proteins obtained from mass-spectrometric analysis are shown in Supplementary Table 3. Custom R-script was used to keep only transcription factors based on gene symbols of mammalian genes from gene ontology categories "RNA polymerase II cis-regulatory region sequence-specific DNA binding" and "DNA-binding transcription factor activity" obtained from www.geneontology.org (16.03.2020). At least 1.45-fold enrichment to the +3 kb enhancer probe compared to the +11 kb intronic probe was used as a cutoff for specific binding. The obtained lists were manually curated to generate Venn diagram illustration of the experiment.

8. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assay was performed as described previously (Esvald et al., 2020) using 10 min fixation with 1% formaldehyde. 5 µg of CREB antibody (catalog #06-863, lot 2446851, Merck Millipore) or TCF4 antibody (CeMines) was used per immunoprecipitation (IP). DNA enrichment was measured using qPCR. All qPCR reactions were performed in 10 µl volume in triplicates with 1× LightCycler 480 SYBR Green I Master kit (Roche) and primers listed in Supplementary Table 1 on LightCycler® 480 PCR instrument II (Roche). Primer efficiencies were determined by serial dilutions of input samples and were used for analysing the results. Percent of input enrichments were calculated for each region and IP, and data were log-transformed before statistical analysis.

ENCODE data of different ChIP-seq experiments was visualized using UCSC Genome Browser track "Transcription Factor ChIP-seq Peaks (340 factors in 129 cell types) from ENCODE 3 Data version: ENCODE 3 Nov 2018". Data of previously published ChIP-seq experiments were obtained from Gene Expression Omnibus with accession numbers GSM530173, GSM530174, GSM530182, GSM530183 (Kim et al., 2010), GSM1467429, GSM1467434 (Malik et al., 2014), GSM1820990 (Moen et al., 2017), GSM1647867 (Sun et al., 2019), GSM1649148 (Bayam et al., 2015), and visualized using Integrative Genomics Viewer version 2.8.0 (Robinson et al., 2011).

9. Statistical analysis

All statistical tests and tested hypotheses were decided before performing the experiments. As ANOVA's requirement of homoscedasticity was not met, two-tailed paired or unpaired equal variance t-test, as reported at each figure, was used for statistical analysis using Excel 365 (Microsoft). To preserve statistical power, p-values were not corrected for multiple comparisons as recommended by (Feise, 2002; Rothman, 1990; Streiner and Norman, 2011).

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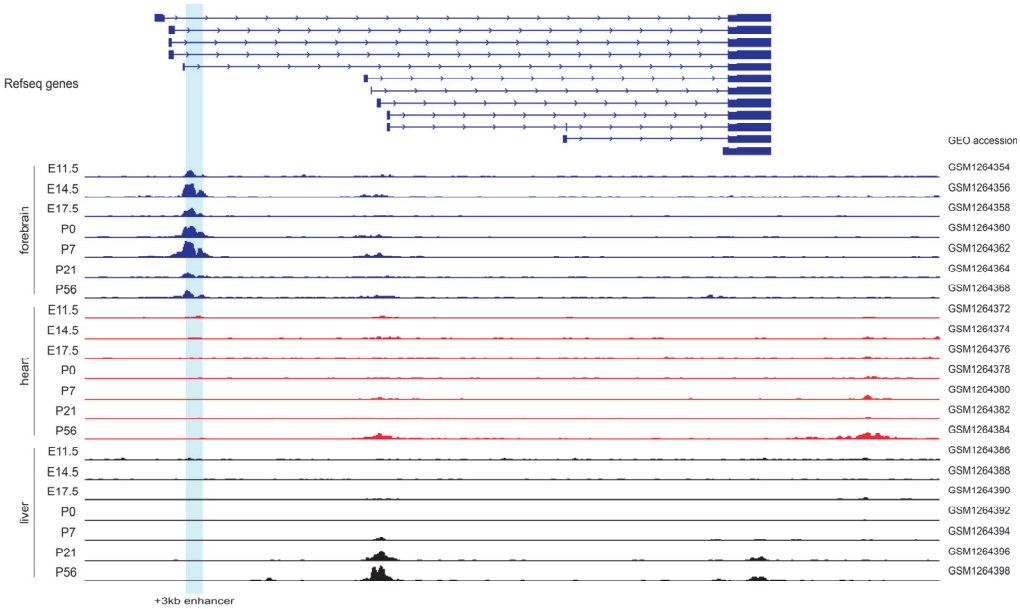
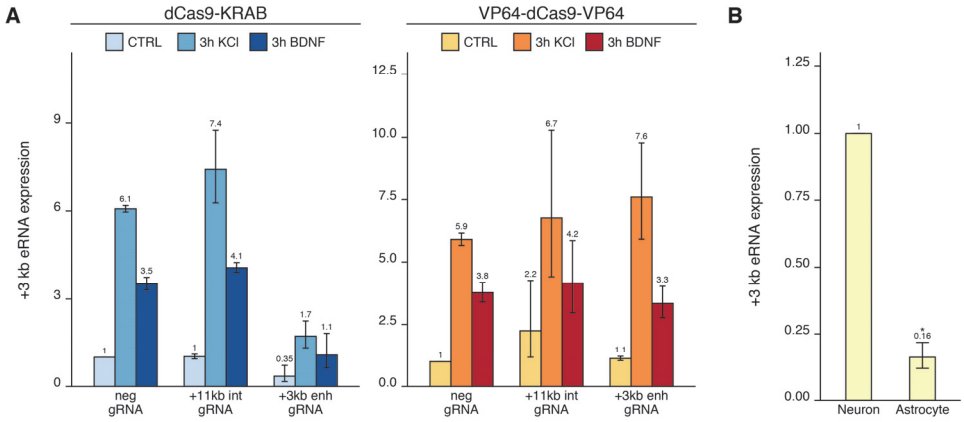
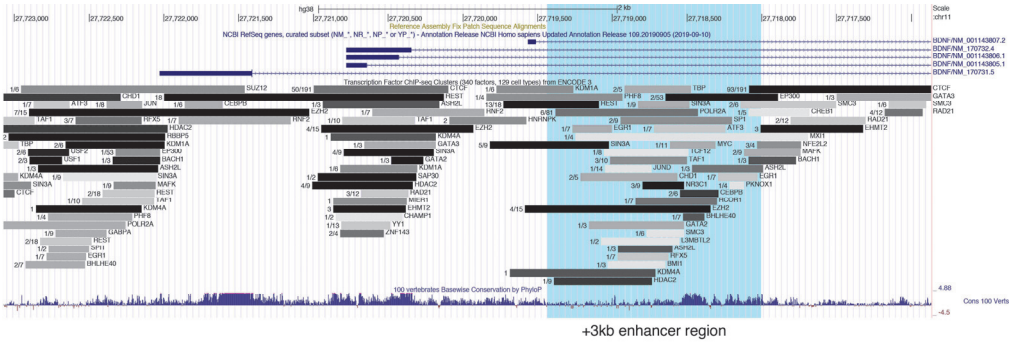
SUPPLEMENTARY FIGURES



Supplementary figure 1. ***+3 kb enhancer region shows brain-specific H3K27ac histone modification.*** *Integrative Genomics Viewer was used to visualize H3K27ac ChIP-seq data of different mouse tissues throughout the development (Nord et al., 2013). Different BDNF transcripts are shown in the upper part of the figure, developmental stage and tissue are shown on the left. E indicates embryonic day, P postnatal day. +3 kb enhancer region is marked with light blue. GEO accession numbers of the data are shown on the right.*



Supplementary figure 2. ***+3 kb enhancer shows stimulus-dependent eRNA transcription in neurons.*** *(A) Measurement of +3 kb eRNA in CRISPRi and CRISPRa experiments in cultured neurons. Rat cultured cortical neurons were transduced at 0 DIV with lentiviral particles encoding either dCas9 fused with Krüppel associated box domain (dCas9-KRAB, blue) or 8 copies of VP16 domain (VP64-dCas9-VP64, orange) together with lentiviruses encoding either guide RNA that has no corresponding target sequence in the rat genome (neg gRNA), a mixture of four gRNAs directed to the putative +3 kb BDNF enhancer (+3 kb enh gRNA) or a mixture of four guide RNAs directed to +11 kb intronic region (+11 kb int gRNA). Transduced neurons were left untreated (CTRL) or treated with 50 ng/ml BDNF or 25 mM KCl (with 5 μM D-APV) for 3 hours at 8 DIV. Expression levels of +3 kb eRNA were measured with RT-qPCR and normalized to HPRT1 expression levels. eRNA expression levels are depicted relative to the eRNA expression in untreated (CTRL) neurons transduced with negative guide RNA within each set (dCas9-KRAB or VP64-dCas9-VP64). The average eRNA expression of independent experiments is depicted above the columns. Error bars represent SEM (n = 2 independent experiments). No statistical analysis was performed. (B) Comparison of +3 kb enhancer eRNA expression levels in cultured neurons and astrocytes. eRNA expression was measured using RT-qPCR, unnormalized average Ct values were used for the analysis and transformed to linear scale for graphical representation. The +3 kb eRNA expression level in neurons was set as 1. The average eRNA expression of independent experiments is depicted above the columns. Error bards represent SEM (n = 3 independent experiments). * p<0.05 (paired two-tailed t-test).*



Supplementary figure 3. ***+3 kb enhancer region binds various transcription factors in human cell lines.*** *UCSC Genome Browser was used to visualize ENCODE ChIP-seq data track "Transcription Factor ChIP-seq Peaks (340 factors in 129 cell types) from ENCODE 3 Data version: ENCODE 3 Nov 2018" at the +3 kb enhancer region. Numbers indicate cell lines with binding of the indicated transcription factor/number cell lines assayed for the binding of indicated transcription factor. The +3 kb enhancer region is shown in light blue.*

Curriculum vitae

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Language competence

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Professional employment

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11.2018–2.2019	King's College London, UK, Visiting scientist
2017–...	Tallinn University of Technology, School of Science, Department of Chemistry and Biotechnology, Engineer (0,50)
2013–2016	Tallinn University of Technology, Faculty of Science, Department of Gene Technology, Engineer (1,00)
2011–2013	Competence Center for Cancer Research, Engineer (0,20)

R&D related managerial and administrative work

2017–...	Tallinn University of Technology, council of School of Science, member, representative of PhD students
2017–2019	Tallinn University of Technology, council of Department of Chemistry and Biotechnology, member, representative of PhD students

Scholarships & awards

2018	Dora Pluss T1.1 mobility scholarship to attend „NGF meeting 2018" conference
2017	The best scientific publication of Tallinn University of Technology in the field of natural, exact and medical sciences in 2016
2016	Dora Pluss T1.1 mobility scholarship to attend „Neuroscience 2016" conference
2016	EEA/Norway grants mobility scholarship
2011	State Contest of Student Research 2011, Diploma

Courses and conferences

November 2019 Participation in 21st EstSHG annual conference, Pärnu, Estonia

August 2019 Participation in course „Baltic summer school on behavioural characterization of rodent models of major brain disorders“, Pühajärve, Estonia

June 2019 Participation in conference „Nordic Neuroscience 3“, Helsinki, Finland; poster presentation "Identification of an intragenic enhancer region regulating BDNF gene expression"

November 2018 Participation in 20th EstSHG annual conference, Viljandi, Estonia

November 2018 Participation in conference „Neuroscience 2018“, San Diego, USA; poster presentation „Identification of enhancer regions regulating BDNF gene expression“

September 2018 Participation in course „Developmental Biology Minisymposium“, Tallinn, Estonia

September 2018 Participation in workshop „From Nanotechnology to Nanomedicine 2018“, Tallinn, Estonia

June 2018 Participation in conference „NGF meeting 2018“, Salamanca, Spain; poster presentation „Identification of enhancer regions regulating BDNF gene expression“

March 2018 Participation in SZTest mini-symposium „Gene expression in health and disease“, Tallinn, Estonia; oral presentation „Enhancer regions of the BDNF gene“

January 2018 Participation in conference „Teaching for Learning – the University Perspective“, Tartu, Estonia

November 2017 Participation in 19th EstSHG annual conference, Rakvere, Estonia

November 2016 Participation in conference „Neuroscience 2016“, San Diego, USA; poster presentation „Identification of novel regulatory mechanisms responsible for TrkB signaling-dependent transcription of BDNF in cortical neurons“

October 2016 Participation in 18th EstSHG annual conference, Pärnu, Estonia

August 2016 Participation in course „Behavioural phenotyping of rodent disease models – potentials and pitfalls“, Pühajärve, Estonia

Supervised dissertations

Kaie Uustalu, Master's Degree, 2019, (sup) Jürgen Tuvikene; Tõnis Timmusk, Characterisation of enhancer regions regulating brain-derived neurotrophic factor transcription in rat primary neurons and astrocytes, Tallinn University of Technology School of Science, Department of Chemistry and Biotechnology.

Annika Rähni, Master's Degree, 2019, (sup) Jürgen Tuvikene; Tõnis Timmusk, The role of putative enhancer regions in the regulation of the brain-derived neurotrophic factor gene, Tallinn University of Technology School of Science, Department of Chemistry and Biotechnology.

Annela Avarlaid, Master's Degree, 2018, (sup) Jürgen Tuvikene; Tõnis Timmusk, Stimulus-dependent enhancer regions of the BDNF gene in primary cortical neurons, Tallinn University of Technology School of Science, Department of Chemistry and Biotechnology.

Eli-Eelika Esvald, Master's Degree, 2017, (sup) Jürgen Tuvikene; Tõnis Timmusk, The role of CREB family transcription factors in BDNF transcriptional

autoregulation in cortical neurons, Tallinn University of Technology School of Science, Department of Chemistry and Biotechnology.

Annika Rähni, Bachelor's Degree, 2017, (sup) Jürgen Tuvikene; Tõnis Timmusk, Local translation of brain-derived neurotrophic factor in synaptoneurosomes, Tallinn University of Technology School of Science, Department of Chemistry and Biotechnology.

Kaie Uustalu, Bachelor's Degree, 2017, (sup) Jürgen Tuvikene; Tõnis Timmusk, Determining the role of an intragenic enhancer region in the transcription of brain-derived neurotrophic factor, Tallinn University of Technology School of Science, Department of Chemistry and Biotechnology.

Alex Sirp, Master's Degree, 2016, (sup) Jürgen Tuvikene; Tõnis Timmusk, Screening of Transcription Factors Regulating BDNF Positive Feedback Loop in Cortical Neurons, Tallinn University of Technology Faculty of Science, Department of Gene Technology, Chair of Molecular Biology.

Marko Susi, Bachelor's Degree, 2014, (sup) Jürgen Tuvikene; Tõnis Timmusk, Regulation of BDNF gene expression by EGR family transcription factors, Tallinn University of Technology Faculty of Science, Department of Gene Technology, Chair of Molecular Biology.

Publications

Sirp, A; Leite, K; Tuvikene, J; Nurm, K; Sepp, M; Timmusk, T. (2020). The Fuchs corneal dystrophy-associated CTG repeat expansion in the TCF4 gene affects transcription from its alternative promoters. Scientific Reports 10(1):18424.

Tamberg, L; Jaago, M; Säälik, K; Sirp, A; Tuvikene, J; Shubina, A: Kiir, CS; Nurm, K; Sepp, M; Timmusk, T; Palgi, M. (2020). Daughterless, the Drosophila orthologue of TCF4, is required for associative learning and maintenance of the synaptic proteome. Disease Models & Mechanisms 13(7):dmm042747.

Esvald, E-E*; Tuvikene, J*; Sirp, A; Patil, S; Bramham, CR; Timmusk, T. (2020). CREB Family Transcription Factors Are Major Mediators of BDNF Transcriptional Autoregulation in Cortical Neurons. J Neurosci 40:1405–1426.

Taal, K; Tuvikene, J; Rullinkov, G; Piirsoo, M; Sepp, M; Neuman, T; Tamme, R; Timmusk, T. (2019). Neuralized family member NEURL1 is a ubiquitin ligase for the cGMP-specific phosphodiesterase 9A. Scientific Reports 9:1–12.

Koppel, I; Jaanson, K*; Klasche, A*; Tuvikene, J*; Tiirik, T; Pärn, A; Timmusk, T. (2018). Dopamine cross-reacts with adrenoreceptors in cortical astrocytes to induce BDNF expression, CREB signaling and morphological transformation. Glia, 66, 206−216.

Tuvikene, J; Pruunsild, P; Orav, E; Esvald, EE; Timmusk, T. (2016). AP-1 transcription factors mediate BDNF-positive feedback loop in cortical neurons. Journal of Neuroscience, 36, 1290−1305.

Jaagura, M; Taal, K; Koppel, I; Tuvikene, J; Timmusk, T; Tamme, R. (2016). Rat NEURL1 3'UTR is alternatively spliced and targets mRNA to dendrites. Neuroscience Letters, 635, 71−76.

Koppel, I; Tuvikene, J; Lekk, I; Timmusk, T. (2015). Efficient use of a translation start codon in BDNF exon I. Journal of Neurochemistry, 134 (6), 1015−1025.

* equal contribution

Elulookirjeldus

Isikuandmed

Nimi	Jürgen Tuvikene
Sünniaeg	08.12.1988
Sünnikoht	Tallinn, Eesti
Kodakondsus	Eesti

Kontaktandmed

E-post	jurgen.tuvikene@taltech.ee

Hariduskäik

2013–2021	Tallinna Tehnikaülikool, PhD
2011–2013	Tallinna Tehnikaülikool, MSc (geenitehnoloogia), diplom cum laude
2008–2011	Tallinna Tehnikaülikool, BSc (geenitehnoloogia), diplom cum laude
1996–2008	Tallinna Kuristiku Gümnaasium

Keelteoskus

Eesti keel	emakeel
Inglise keel	kõrgtase
Vene keel	algtase

Teenistuskäik

2018–...	Protobios OÜ, teadur-spetsialist (0,08)
11.2018–2.2019	King's College London, külalisteadlane
2017–...	Tallinna Tehnikaülikool, Loodusteaduskond, Keemia ja biotehnoloogia instituut, insener (0,50)
2013–2016	Tallinna Tehnikaülikool, Matemaatika-loodusteaduskond, Geenitehnoloogia instituut, insener (1,00)
2011–2013	Vähiuuringute Tehnoloogia Arenduskeskus AS, insener (0,20)

Teadusorganisatsiooniline ja -administratiivne tegevus

2017–...	Tallinna Tehnikaülikool, Loodusteaduskonna nõukogu liige, doktorantide esindaja
2017–2019	Tallinna Tehnikaülikool, Keemia ja biotehnoloogia instituudi nõukogu liige, doktorantide esindaja

Stipendiumid ja tunnustused

2018	Dora Pluss T1.1 mobiilsusstipendium „NGF meeting 2018" konverentsil osalemiseks
2017	Tallinna Tehnikaülikooli 2016. aasta parim teadusartikkel loodus-, täppis- ja terviseteaduste valdkonnas
2016	Dora Pluss T1.1 mobiilsusstipendium „Neuroscience 2016" konverentsil osalemiseks
2016	EEA/Norra välismobiilsusstipendium
2011	Eesti üliõpilaste teadustööde riiklik konkurss 2011, tänukiri

Kursused ja konverentsid

November 2019	Osalemine konverentsil „Eesti Inimesegeneetika ühingu 21. aastakonverents", Pärnu, Eesti
August 2019	Osalemine kursusel „Baltic summer school on behavioural characterization of rodent models of major brain disorders", Pühajärve, Eesti

Juuni 2019 Osalemine konverentsil Nordic Neuroscience 3, Helsingi, Soome; posterettekanne „Identification of an intragenic enhancer region regulating BDNF gene expression“

November 2018 Osalemine konverentsil „Eesti Inimesegeneetika ühingu 20. aastakonverents“, Viljandi, Eesti

November 2018 Osalemine konverentsil Neuroscience 2018, San Diego, USA; posterettekanne „Identification of enhancer regions regulating BDNF gene expression“

September 2018 Osalemine kursusel „Developmental Biology Minisymposium“, Tallinn, Eesti

September 2018 Osalemine kursusel „From Nanotechnology to Nanomedicine 2018“, Tallinn, Eesti

Juuni 2018 Osalemine konverentsil NGF meeting 2018, Salamanca, Hispaania; posterettekanne „Identification of enhancer regions regulating BDNF gene expression“

Märts 2018 Osalemine SZTest minisümpoosiumil „Gene expression in health and disease“, Tallinn, Eesti; suuline ettekanne „Enhancer regions of the BDNF gene“

Jaanuar 2018 Osalemine konverentsil „Teaching for Learning – the University Perspective“, Tartu, Eesti

November 2017 Osalemine konverentsil „Eesti Inimesegeneetika ühingu 19. aastakonverents“, Rakvere, Eesti

November 2016 Osalemine konverentsil Neuroscience 2016, San Diego, USA; posterettekanne „Identification of novel regulatory mechanisms responsible for TrkB signaling-dependent transcription of BDNF in cortical neurons“

Oktoober 2016 Osalemine konverentsil „Eesti Inimesegeneetika ühingu 18. aastakonverents“, Pärnu, Eesti

August 2016 Osalemine kursusel „Behavioural phenotyping of rodent disease models – potentials and pitfalls“, Pühajärve, Eesti

Juhendatud väitekirjad

Kaie Uustalu, magistrikraad, 2019, (juh) Jürgen Tuvikene; Tõnis Timmusk, Characterisation of enhancer regions regulating brain-derived neurotrophic factor transcription in rat primary neurons and astrocytes (Aju-päritolu neurotroofse teguri transkriptsiooni reguleerivate enhanseralade iseloomustamine roti primaarsetes neuronites ja astrotsüütides), Tallinna Tehnikaülikool, Loodusteaduskond, Keemia ja biotehnoloogia instituut.

Annika Rähni, magistrikraad, 2019, (juh) Jürgen Tuvikene; Tõnis Timmusk, The role of putative enhancer regions in the regulation of the brain-derived neurotrophic factor gene (Potentsiaalsete enhanseralade roll aju-päritolu neurotroofse teguri geeni regulatsioonis), Tallinna Tehnikaülikool, Loodusteaduskond, Keemia ja biotehnoloogia instituut.

Annela Avarlaid, magistrikraad, 2018, (juh) Jürgen Tuvikene; Tõnis Timmusk, Stimulus-dependent enhancer regions of the BDNF gene in primary cortical neurons (BDNF geeni stiimul-sõltuvad enhanseralad primaarsetes kortikaalsetes neuronites), Tallinna Tehnikaülikool, Loodusteaduskond, Keemia ja biotehnoloogia instituut.

Eli-Eelika Esvald, magistrikraad, 2017, (juh) Jürgen Tuvikene; Tõnis Timmusk, The role of CREB family transcription factors in BDNF transcriptional autoregulation in cortical neurons (CREB perekonna transkriptsioonifaktorite roll BDNF geeni transkriptsioonilises autoregulatsioonis ajukoore neuronites), Tallinna Tehnikaülikool, Loodusteaduskond, Keemia ja biotehnoloogia instituut.

Annika Rähni, bakalaureusekraad, 2017, (juh) Jürgen Tuvikene; Tõnis Timmusk, Aju-päritolu neurotroofse teguri lokaalne translatsioon sünaptoneurosoomides, Tallinna Tehnikaülikool, Loodusteaduskond, Keemia ja biotehnoloogia instituut.

Kaie Uustalu, bakalaureusekraad, 2017, (juh) Jürgen Tuvikene; Tõnis Timmusk, Geenisisese enhanserala mõju aju-päritolu neurotroofse teguri transkriptsioonile, Tallinna Tehnikaülikool, Loodusteaduskond, Keemia ja biotehnoloogia instituut.

Alex Sirp, magistrikraad, 2016, (juh) Jürgen Tuvikene; Tõnis Timmusk, Screening of Transcription Factors Regulating BDNF Positive Feedback Loop in Cortical Neurons (BDNF geeni positiivse tagasiside regulatsioonis osalevate transkriptsioonifaktorite tuvastamine ajukoore neuronites), Tallinna Tehnikaülikool, Matemaatika-loodusteaduskond, Geenitehnoloogia instituut, Molekulaarbioloogia õppetool.

Marko Susi, bakalaureusekraad, 2014, (juh) Jürgen Tuvikene; Tõnis Timmusk, BDNF geeni ekspressiooni regulatsioon EGR perekonna transkriptsioonifaktorite poolt, Tallinna Tehnikaülikool, Matemaatika-loodusteaduskond, Geenitehnoloogia instituut, Molekulaarbioloogia õppetool.

Publikatsioonid

Sirp, A; Leite, K; Tuvikene, J; Nurm, K; Sepp, M; Timmusk, T. (2020). The Fuchs corneal dystrophy-associated CTG repeat expansion in the TCF4 gene affects transcription from its alternative promoters. Scientific Reports 10(1):18424.

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Taal, K; Tuvikene, J; Rullinkov, G; Piirsoo, M; Sepp, M; Neuman, T; Tamme, R; Timmusk, T. (2019). Neuralized family member NEURL1 is a ubiquitin ligase for the cGMP-specific phosphodiesterase 9A. Scientific Reports 9:1–12.

Koppel, I; Jaanson, K*; Klasche, A*; Tuvikene, J*; Tiirik, T; Pärn, A; Timmusk, T. (2018). Dopamine cross-reacts with adrenoreceptors in cortical astrocytes to induce BDNF expression, CREB signaling and morphological transformation. Glia, 66, 206−216.

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Jaagura, M; Taal, K; Koppel, I; Tuvikene, J; Timmusk, T; Tamme, R. (2016). Rat NEURL1 3'UTR is alternatively spliced and targets mRNA to dendrites. Neuroscience Letters, 635, 71−76.

Koppel, I; Tuvikene, J; Lekk, I; Timmusk, T. (2015). Efficient use of a translation start codon in BDNF exon I. Journal of Neurochemistry, 134 (6), 1015−1025.

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