

**DOCTORAL THESIS**

# The Role of Enhancers in the Regulation of Brain- Derived Neurotrophic Factor Transcription

Annela Avarlaid

TALLINN UNIVERSITY OF TECHNOLOGY  
DOCTORAL THESIS  
/2024

# **The Role of Enhancers in the Regulation of Brain-Derived Neurotrophic Factor Transcription**

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The dissertation was accepted for the defence of the degree of Doctor of Philosophy in Gene Technology on 19/06/2024

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**Defence of the thesis:** 15/08/2024, Tallinn

**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.

Annela Avarlaid

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ISSN 2585-6898 (publication)

ISBN 978-9916-80-168-0 (publication)

ISSN 2585-6901 (PDF)

ISBN 978-9916-80-169-7 (PDF)

TALLINNA TEHNIKAÜLIKOOL  
DOKTORITÖÖ  
/2024

# Enhanseralade roll aju-päritolu neurotroofse teguri transkriptsiooni regulatsioonis

ANNELA AVARLAID





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## List of Publications

The list of author's publications, based on which the thesis has been prepared:

I Tuvikene J, Esvald EE\*, Rähni A\*, Uustalu K\*, Zhuravskaya A, **Avarlaid A**, Makeyev EV, Timmusk T

Intronic enhancer region governs transcript-specific Bdnf expression in rodent neurons

*Elife*. 2021;10:e65161. doi:10.7554/eLife.65161.

II **Avarlaid A**, Esvald EE, Koppel I, Parkman A, Zhuravskaja A, Makeyev EV, Tuvikene J#, Timmusk T#

An 840 kb distant upstream enhancer is a crucial regulator of catecholamine-dependent expression of the *Bdnf* gene in astrocytes

*Glia*. 2024;72(1):90-110. doi:10.1002/glia.24463

III **Avarlaid A**, Falkenberg K\*, Lehe K\*, Mudò G, Belluardo N†, Di Liberto V, Frinchi M, Tuvikene J#, Timmusk T#

An upstream enhancer and MEF2 transcription factors fine-tune the regulation of *Bdnf* gene in cortical and hippocampal neurons

*J Biol Chem*. 2024;300(6):107411. doi: 10.1016/j.jbc.2024.107411

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## **Author's Contribution to the Publications**

Contribution to the papers in this thesis are:

I The author contributed to the CRISPR/dCas9-related experiments, analysed results, and supervised part of the experiments conducted in astrocytes. The author edited the manuscript.

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

III The author conceptualised the idea, supervised the experiments, performed part of the experiments. The author wrote the first draft and final manuscript.

## Introduction

Approximately 2% of the total human genome is responsible for the synthesis of proteins, indicating that majority of our genome consists of non-coding DNA. Within this non-coding DNA, enhancers - key regulatory elements - play a pivotal role in controlling the activation of genes. These elements are sensitive to the specific cellular context, developmental stages, and environmental stimuli. The current doctoral thesis explores the enhancer regions that regulate brain-derived neurotrophic factor (*Bdnf*) gene.

BDNF, a member of the neurotrophin family, plays a crucial role in both developing and mature organism, contributing to neuronal survival, growth, and plasticity. Therefore, alterations in BDNF expression are linked to a wide range of neurodevelopmental, neuropsychiatric, and neurodegenerative disorders. Given its diverse roles in the nervous system, BDNF has great potential as a therapeutic agent. While the proximal regulatory regions of the *Bdnf* gene have been well studied, the location and function of enhancer regions have not been examined. This is a critical knowledge gap considering that enhancer regions refine the spatial, temporal, and stimulus-specific transcription and alterations in the enhancer regions are often linked to diseases.

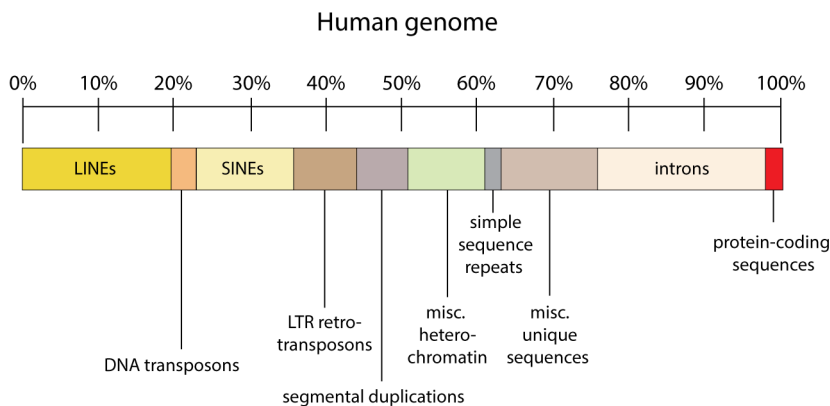
The studies underlying the current PhD thesis provide a detailed characterization of three novel enhancer regions that regulate the stimulus-specific transcription of *Bdnf* gene. These findings give new insights into the transcriptional regulation of *BDNF* and unveil potential molecular mechanisms that could be harnessed for therapeutic applications.

## Abbreviations

AP1	Activating protein 1
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
BET	Bromodomain and extra terminal domain
bHLH	basic helix–loop–helix
CREB	cAMP response element binding protein
CRE	cAMP-response element
CRISPR	Clustered regularly interspaced short palindromic repeats
ChIP	Chromatin immunoprecipitation
CTCF	CCCTC-binding factor
DMSO	Dimethyl sulfoxide
IEG	Immediate early gene
eRNA	Enhancer RNA
MEF2	Myocyte enhancer factor 2
p75NTR	P75 neurotrophin receptor
SNP	Single nucleotide polymorphism
TSS	Transcription start site
TrkB	Tropomyosin-related kinase B
UTR	Untranslated region

# 1 Review of literature

Protein coding sequences make up a remarkably small part of our total DNA. Roughly 98% of our genome consists of non-coding DNA, which does not contain information about protein production and was long considered as junk DNA (Dunham et al., 2012; Gregory, 2005). Nowadays the role of the non-coding DNA is increasingly recognized. In general, non-coding DNA includes intronic regions, long interspersed nuclear elements (LINEs), short interspersed nuclear elements (SINEs), long terminal repeat (LTR) retrotransposons, DNA transposons, segmental duplications, simple sequence repeats, miscellaneous heterochromatin and unique DNA sequences, **Figure 1**). Crucial components of the gene regulation are enhancers – non-coding DNA sequences that control the activation of genes according to cell type, developmental stage, and stimuli. It is estimated that while the human genome consists of ~25,000 protein-coding genes, there are hundreds of thousands enhancer regions (Alberts et al., 2015; Dunham et al., 2012; Kassouf et al., 2023; Mills et al., 2020; Shen et al., 2012). The target genes of most of the enhancers, and vice versa, are largely unknown. This also applies to one vital protein coding gene, brain-derived neurotrophic factor (*BDNF*). The upcoming chapters will provide a comprehensive overview of the regulatory and functional aspects of *BDNF*, enhancers and finally emphasize the current knowledge about the enhancers of *BDNF*.



*Figure 1. Percentage distribution of genomic elements in the human genome. Majority of the human genome consists of non-protein-coding DNA, which includes intronic regions, long interspersed nuclear elements (LINEs), short interspersed nuclear elements (SINEs), long terminal repeat (LTR) retrotransposons, DNA transposons, segmental duplications, simple sequence repeats, miscellaneous heterochromatin, and unique DNA sequences. Roughly 2% of the total human genome contains protein-coding sequences (modified from Alberts et al., 2015; Gregory, 2005)*

## 1.1 Neurotrophins

The family of neurotrophins consists of four homologous proteins that have an essential and multifunctional role in the mammalian brain (Lu et al., 2005). The first neurotrophin, nerve growth factor, was discovered and extracted from mouse sarcoma tumor in the early 1950s (Cohen et al., 1954). This discovery marked the beginning of neurotrophin research and resulted in Rita Levi-Montalcini and Stanley Cohen receiving the Nobel Prize in Physiology or Medicine in 1986 (Levi-Montalcini, 1987). Another neurotrophin, *BDNF*, was discovered in the 1978 when Barde and his colleagues demonstrated that medium conditioned by glioma cells promoted the survival of chicken embryonic sensory neurons

(Barde et al., 1978). In 1982, BDNF was first purified from a brain of a pig (Barde et al., 1982). Just before the 21<sup>st</sup> century, neurotrophin-3 and neurotrophin-4/5 were discovered based on structural homology and conservation with neurotrophins that had already been described (Hallböök et al., 1991; Maisonpierre et al., 1990). The non-mammalian neurotrophins, neurotrophin-6 and neurotrophin-7 have been described in different fish species (Gotz et al., 1994; Lai et al., 1998; Nilsson et al., 1998).

## 1.2 Brain-derived neurotrophic factor

### 1.2.1 Structure of the *BDNF* gene

In 1993, Timmusk et al. provided the very first description of the *Bdnf* gene structure (Timmusk et al., 1993). In 2007, the structure and nomenclature of the *Bdnf* gene was revisited (**Figure 2**) (Aid et al., 2007). Accordingly, rodent *Bdnf* gene is divided into eight 5' non-coding exons (exons I to VIII) and one 3' coding exon (exon IX). Each 5' exon of the *Bdnf* gene is regulated by its distinct promoter, leading to *Bdnf* transcripts that are composed of one 5' non-coding and the coding exon spliced together. Transcription can also start from the intron upstream of the coding exon, giving rise to a 5' extended variant of the coding exon (exon IXa). The *Bdnf* exon II consists of three alternative splice-donor sites (A, B, C) that can result in exon II-containing *Bdnf* transcripts with different 5' untranslated regions (UTR) (Aid et al., 2007). The coding exon has two possible polyadenylation sites that can generate *Bdnf* transcripts with either short (~1.6 kb) or long 3' UTR (~4.2 kb). Although the roles of different 5' and 3' UTRs of *Bdnf* remains to be elucidated, it gives several opportunities to regulate the fate of mRNA, subcellular localisation, and translation efficiency (Fukuchi & Tsuda, 2010; Lekk et al., 2023; Timmusk et al., 1993). For example, the 5' UTRs of *Bdnf* have shown repressive effects on translatability, while the 3' UTRs show non-repressive role in brain but repress translation in lung and heart (Lekk et al., 2023). The alternative polyadenylation signals in *Bdnf* exon IX are conserved between mammals, and both short and long *Bdnf* mRNAs are found in neural and non-neural tissues, while the ratio depends on specific tissue and stage of development (An et al., 2008; Esvald et al., 2023). Moreover, the long 3' UTR containing *Bdnf* transcripts are more stable and enriched in dendrites, while the *Bdnf* transcripts with short 3' UTR are found in the soma of neurons (An et al., 2008). The *Bdnf* exon I contains an alternative translation start codon, which leads to 8 amino acid longer preproBDNF compared to the canonical protein and is used more efficiently than the start codon in exon IX (Koppel et al., 2015; Timmusk et al., 1993).

The regulation of human *BDNF* is more complex because of additional 5' non-coding exons (Vh and VIIIh) and alternative splicing mechanisms (Pruunsild et al., 2007). Interestingly, Pruunsild et al., 2007 showed the presence of human specific non-coding antisense RNAs that are transcribed from human antisense-*BDNF* gene consisting of 10 exons and one promoter. The transcription start site of the antisense-*BDNF* is located 200 kb downstream of the *BDNF* gene. It has been shown that the overlapping region of human *BDNF* and antisense-*BDNF* can form double-stranded RNA duplexes (Pruunsild et al., 2007). Thereby, antisense-*BDNF* may serve a regulatory role in the BDNF expression (Lipovich et al., 2012; Modarresi et al., 2012; Pruunsild et al., 2007). It has been suggested that antisense-*BDNF* has evolved during the evolution of primates (Aid et al., 2007; Q. R. Liu et al., 2006).

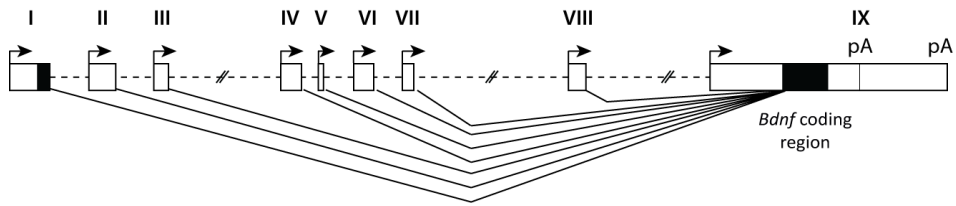


Figure 2. Structure of the rodent *Bdnf* gene. Introns are shown as dashed lines and exons as boxes, solid box stands for coding and white box for non-coding regions. Roman numerals above the boxes indicate the exons, arrows show transcription start sites. The transcripts of rodent *Bdnf* are generated by splicing one of the 5' non-coding (I-VIII) exon together with the common coding (IX) exon. The coding exon has two polyadenylation sites (pA) that give rise to *Bdnf* transcripts with either short or long 3' UTR.

### 1.2.2 Transcription factors regulating *BDNF* expression

The expression of BDNF is induced in response to various stimuli in both neuronal and non-neuronal cells. Different stimuli induce activation of distinct signalling cascades and binding of numerous transcription factors to the promoter regions of *BDNF*. In neurons the most studied and potent BDNF inducing stimuli is neuronal activity. This can be triggered, for example, due to physical activity, light stimulation, ischemia, and learning (West et al., 2014). In fact, in addition to immediate early genes (IEGs) like *ARC* and *FOS*, *BDNF* serves as a classical model of a neuronal activity-regulated gene (Yap & Greenberg, 2018). Neuronal activity induces the expression of all *BDNF* transcripts, with transcription from *BDNF* promoters I and IV showing the highest induction (Pruunsild et al., 2011), and thus these promoters are the most studied *BDNF* promoters (West et al., 2014). Briefly, neuronal activity induced transcription of *BDNF* exon I is promoted by transcription factors like cAMP response element binding protein (CREB), which binds to CRE-site (Pruunsild et al., 2011; Tabuchi et al., 2002), upstream stimulatory factor 1 (USF1) and upstream stimulatory factor 2 (USF2) binding to USF binding site that overlaps with CRE-site (Tabuchi et al., 2002), aryl hydrocarbon receptor nuclear translocator 2 (ARNT2) and neuronal PAS domain protein 4 (NPAS4), which binds to the bHLH-PAS transcription factor response element (PasRE) (Lin et al., 2008; Pruunsild et al., 2011). Neuronal activity causes binding of neuron-restrictive silencing factor (NRSF), also known as repressor element 1 (RE-1) silencing transcription factor (REST), to neuron-restrictive silencing element (NRSE) within *BDNF* exon II (Hara et al., 2009; Timmusk et al., 1999). In general, NRSE recruits co-repressors and restricts the neuron-specific expression of genes in neuronal progenitor cells and non-neural tissues (Chong et al., 1995). However, NRSE is also associated with silencing of *BDNF* expression from promoter I and II in neurons (Hara et al., 2009; Timmusk et al., 1999). Activity-dependent transcription from *BDNF* promoter IV is mediated by USF1/2 binding to USF-site (W. G. Chen et al., 2003; Pruunsild et al., 2011), CREB binding to CRE-site (Esvald et al., 2020; Hong et al., 2008; Pruunsild et al., 2011; Shieh et al., 1998; Tao et al., 1998), ARNT2 and NPAS4 dimer binding to PasRE region (Lin et al., 2008; Pruunsild et al., 2011). In addition, *BDNF* promoter IV has two calcium-response elements (CaREs), which bind transcription factors like calcium-response factor (CaRF) (Tao et al., 2002) and myocyte enhancer factor 2 (MEF2) (Hong et al., 2008; M. R. Lyons et al., 2012). CREB and NPAS4-ARNT4 binding to partially overlapping CRE and PasRE elements have been shown to regulate activity-dependent expression of human *BDNF* from promoter IX (Pruunsild et al., 2011).

In addition to neuronal activity, BDNF stimulates its own expression through a positive feedback loop. This occurs by activation of the tropomyosin-related kinase B (TrkB) signalling pathway, a mechanism evident both *in vitro* and *in vivo* (Cheng et al., 2011; Esvald et al., 2020; Nakajima et al., 2015; Tuvikene et al., 2016; Yasuda et al., 2007). Although less investigated than neuronal activity, BDNF-TrkB signalling induces the expression of all major *BDNF* transcripts and is mediated by AP1 and CREB family members (Esvald et al., 2020; Tuvikene et al., 2016). The AP1 family regulates *BDNF* exon I-, III- and VI-containing transcripts after TrkB-signalling, but direct effect of AP1 factors has only been observed through the two AP1 sites in *BDNF* promoter I (Tuvikene et al., 2016). Similarly, while all *BDNF* transcripts are induced in CREB-dependent manner, the CRE-site in *BDNF* promoter IV and in human, but not rat, *BDNF* promoter IX plays a role (Esvald et al., 2020). These results suggest that other *BDNF* transcripts induced by BDNF-TrkB signalling might be regulated via distal regulatory regions or indirectly, i.e., interplay of CREB and AP1 factors with transcription factors that regulate *BDNF* expression.

In non-neuronal cells, the molecular mechanism of *BDNF* transcriptional regulation has not been extensively investigated. With the exception of astrocytes, in which catecholamine signalling induces *Bdnf* expression, specifically *Bdnf* exon IV- and VI-containing transcripts, in CREB-dependent manner (Inoue et al., 1997; Jurič et al., 2006; Koppel et al., 2018; Zafra et al., 1992). Interestingly, the well-studied CRE-site in *BDNF* promoter IV is critical for stimulus-dependent expression of *BDNF* in neurons (Esvald et al., 2020; Hong et al., 2008; Pruunsild et al., 2011; Shieh et al., 1998; Tao et al., 1998), whereas it is not involved in catecholamine-dependent regulation of *Bdnf* in astrocytes (Koppel et al., 2018).

### 1.2.3 Translation and signalling of BDNF

Like all neurotrophins, BDNF is synthesized as a precursor protein (proBDNF) into the endoplasmic reticulum (ER). The pre region stands for signal peptide important for translocating the BDNF to ER lumen, where it is removed co-translationally to form ~32 kDa proBDNF (Lu et al., 2005). ProBDNF passes to trans-Golgi network (TGN), where it can be (1) secreted without further processing and signal via p75 neurotrophin receptor (p75<sup>NTR</sup>), (2) secreted following extracellular cleaving, or (3) intracellularly cleaved and secreted (Mowla et al., 1999, 2001). To form mature BDNF (~14 kDa), the pro-sequence is cleaved off intracellularly by furin and other protein convertases (Mowla et al., 1999), or extracellularly by plasmin and metalloproteinases (Lee et al., 2001). The proBDNF is secreted either in constitutive (spontaneous release) or in most cases, in regulated pathway (i.e., upon stimuli). The regulated secretion pathway depends on the interaction between BDNF prodomain and sortilin, as well as the interaction of carboxypeptidase E with a sorting motif found in the mature BDNF (Z. Y. Chen et al., 2005; Lou et al., 2005). Correct sorting of BDNF is essential, illustrated by a common and widely studied SNP located in the prodomain of BDNF (rs6265, Val66Met), which disrupts sortilin binding with the prodomain of BDNF and thereby decreases the neuronal activity-dependent sorting and secretion of BDNF (Egan et al., 2003). Both human and rodent models with Val66Met show crucial cognitive and structural abnormalities, e.g., impaired neurogenesis, synaptic plasticity and transmission, and tendency to neuropsychiatric disorders (S. J. Tsai, 2018).

ProBDNF, when signalling through its high affinity receptor p75<sup>NTR</sup>, triggers NF- $\kappa$ B dependent cell survival, Jun kinase mediated activation of proapoptotic genes or Rho GTPase-controlled suppression of neurite outgrowth (Bamji et al., 1998; Reichardt, 2006;

Teng et al., 2005). An opposite cascade of cellular events is triggered by mature BDNF binding to its high affinity TrkB receptor. TrkB, a member of the receptor tyrosine kinase superfamily, characteristically dimerizes after ligand binding, induces autophosphorylation and activation of the intracellular tyrosine kinase domain. As a result, three different signalling pathways can be triggered: phospholipase C $\gamma$ 1 (PLC $\gamma$ 1), phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways (Reichardt, 2006). Briefly, PLC $\gamma$ 1 cleaves the phosphatidylinositol-4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3). DAG activates protein kinase C (PKC) and IP3 increases the cytosolic concentration of calcium, thereby activating Ca<sup>2+</sup>-dependent cascades and promoting synaptic plasticity. The PI3K-Akt pathway promotes cell survival due to the activation of protein kinase B (PKB), which through phosphorylation inhibits the activation of proapoptotic genes. Finally, activation of MAPK pathway stimulates cell growth and differentiation by phosphorylating distinct kinases and transcription factors (Minichiello, 2009; Reichardt, 2006).

Of note, mature BDNF can also signal via truncated TrkB receptor. The gene for TrkB, *NTRK2*, can encode either the full-length TrkB or, through alternative 3' exon usage, produce C-terminally truncated TrkB isoforms (TrkB.T1 and TrkB.Shc), both of which are missing the intracellular tyrosine kinase domain (Luberg et al., 2010). While TrkB.T1 and TrkB.Shc have been described as a dominant-negative for BDNF and full length TrkB signalling, several studies argue against this hypothesis (Tessarollo & Yanpallewar, 2022).

#### **1.2.4 Expression pattern of BDNF**

All the distinct transcripts of *BDNF* code for identical BDNF protein. Multiple promoters enable separate regulation for different *BDNF* mRNAs, which in turn allows complex spatiotemporal expression of BDNF protein (You & Lu, 2023). It is important to notice that the mRNA levels of *BDNF* do not always correlate with the levels of protein, an indication for BDNF transport between different brain regions and translational regulation via 5' and 3' UTRs. For example, while the mRNA of *BDNF* in striatum is almost undetectable (Esvald et al., 2023; Timmusk et al., 1994), BDNF protein is transported from cerebral cortex to striatum and is necessary for the normal morphology of striatal neurons (Baquet et al., 2004). In brain, the expression levels of BDNF are low during embryogenesis, but start to rise just before the birth and peak during the first postnatal weeks (Aid et al., 2007; Esvald et al., 2023; Timmusk et al., 1994). The highest levels of BDNF are found in hippocampal, hypothalamic, and cortical areas, while in non-neural tissues the highest BDNF levels are in lung, heart, stomach, bladder and in spleen. In non-neural tissues the levels of BDNF are generally lower and the developmental expression of BDNF is not so uniform compared to the BDNF expression in brain. For example, in skeletal muscle the levels of BDNF peak at P0 and decrease during the postnatal development, while the BDNF levels in heart stay similar during the whole postnatal development (Esvald et al., 2023).

#### **1.2.5 Functions of BDNF**

Since its discovery more than 40 years ago, BDNF has been mainly associated and studied in neuronal development, survival, and functioning. Specifically, the first studies of BDNF proved its importance as a survival factor for various neuron populations (Barde et al., 1980; Davies et al., 1986; Lindsay & Rohrer, 1985; Segal et al., 1992). Moreover, BDNF enhances the differentiation of new neurons from neural precursor cells (NPCs) (Ahmed et al., 1995; Ito et al., 2003), and promotes neurogenesis in the adult hippocampus (Benraiss et al., 2001; Y. Li



et al., 2008; Pencea et al., 2001; Scharfman et al., 2005; Taliatz et al., 2010). To illustrate, rodents who have been injected with BDNF to dentate gyrus or subventricular zone have elevated levels of adult-born neurons in these regions (Benraiss et al., 2001; Scharfman et al., 2005), and RNAi-mediated knockdown of BDNF in dentate gyrus inhibits neuronal differentiation (Taliatz et al., 2010). BDNF is also associated with neuroprotection, as BDNF administration prevents neuronal death and atrophy in rodent and primate Alzheimer's disease models (Nagahara et al., 2009).

It is evident that BDNF is important for the proper morphology of both dendrites and axons (Alonso et al., 2004; Horch & Katz, 2002; Jeanneteau et al., 2010; McAllister et al., 1995). Moreover, BDNF is well known to promote the formation of synapses (synaptogenesis) by increasing the growth and remodelling of neurites (Wang et al., 2022). In addition to synaptogenesis, BDNF also modulates synaptic plasticity, i.e., BDNF either increases or decreases the strength of synaptic transmission at already pre-existing synapses (Bramham & Messaoudi, 2005; Gibon & Barker, 2017; Korte et al., 1996). The role of BDNF in synaptic plasticity was illustrated already in the early BDNF knockout studies, where long term potentiation was reduced in hippocampus of BDNF knockout mice, an effect that was reversed with the administration of exogenous BDNF (Korte et al., 1996; Patterson et al., 1996).

In addition, BDNF in the hypothalamus has been strongly associated with energy homeostasis and behaviour. It has been shown that knockdown of BDNF in the hypothalamic regions causes obesity (An et al., 2015; Unger et al., 2007), and that BDNF is a downstream target of the melanocortin-4 receptor signalling pathway in hypothalamus (Nicholson et al., 2007; Xu et al., 2003). In fact, melanocortin pathway is one of the main systems regulating appetite and energy balance (Baldini & Phelan, 2019). Moreover, hypothalamus-specific BDNF is required for proper thermoregulation (An et al., 2015; You et al., 2020). Nonetheless, the exact molecular mechanisms mediating BDNF function in all these processes remain of great interest.

Although less studied, the importance of BDNF is becoming increasingly evident in non-neuronal cells. For example, BDNF derived from astrocytes, the most abundant glial cells in the brain, promotes the formation of oligodendrocytes and provides trophic support after demyelinating lesions (Fulmer et al., 2014; Miyamoto et al., 2015), modulates the morphology and survival of neurons (De Pins et al., 2019; Giralt et al., 2010, 2011), and is crucial for memory retention (Vignoli et al., 2016). Astrocyte-specific deletion of TrkB.T1 results in immature and morphologically abnormal astrocytes (Holt et al., 2019). Moreover, BDNF and its signalling in cardiomyocytes, probably via TrkB.T1, ensures the normal development and contraction of the heart (Feng et al., 2015; Fulgenzi et al., 2015; L. Li et al., 2022). In kidney, BDNF acts as a survival factor for podocytes (Endlich et al., 2018; M. Li et al., 2015). Muscle-specific ablation of BDNF results in structural and functional remodelling of neuromuscular junctions, which leads to slower locomotion (Delezie et al., 2019).

The expression levels of BDNF are changed within a variety of diseases. Decreased levels of BDNF are associated with drug addiction (Graham et al., 2007; Vargas-Perez et al., 2009) and neuropsychiatric disorders, such as depression and schizophrenia (Martinowich & Lu, 2008). Moreover, patients with different neurodevelopmental (e.g., Rett syndrome, autism) and neurodegenerative (e.g., Alzheimer's, Parkinson, Huntington) diseases show decreased levels of BDNF (Autry & Monteggia, 2012; Miranda et al., 2019), while increased levels of BDNF have been detected in the hippocampus and cortex of patients with temporal lobe epilepsy (Martínez-Levy et al., 2016, 2018). There is a lack of common understanding regarding whether the variable levels of BDNF are a cause or an effect of disease.

### 1.2.6 BDNF knockout models

To illustrate the importance and functions of BDNF more precisely, phenotypic and molecular studies of BDNF knockout animals have provided valuable insights. Homozygous BDNF knockout mice exhibit reduced number of sensory neurons, problems with locomotion, early postnatal lethality (die 2-4 week after birth) (Ernfors et al., 1994, 1995; Jones et al., 1994) and cardiovascular defects (Donovan et al., 2000). In contrast, heterozygous BDNF knockout mice have normal life span but show aggressive behaviour, obesity due to increased food intake (hyperphagia) and impairment of serotonergic system (Kernie et al., 2000; W. Lyons et al., 1999). Heterozygous BDNF animals also show reduced pain sensitivity (MacQueen et al., 2001; Sapio et al., 2019). Furthermore, knockout of BDNF in specific tissues and cell types illustrates the pleiotropic roles of BDNF. For instance, forebrain specific BDNF knockout animals show impaired neuronal morphology, learning and fertility (Gorski, Balogh, et al., 2003; Gorski, Zeiler, et al., 2003). Central nervous system specific BDNF knockout mice live up to 8 months, but suffer from obesity, hyperactivity and have peculiarities in brain morphology, especially in the striatum (Rauskolb et al., 2010). Post-mitotic neuron-specific ablation of BDNF causes obesity, aggressiveness and anxiety-related behaviour (Rios et al., 2001). Deletion of BDNF in astrocytes affects the myelination and maturation of oligodendrocytes (Fulmer et al., 2014; Miyamoto et al., 2015), neuronal maturation (De Pins et al., 2019), survival and excitability (Fernández-García et al., 2020). Cardiomyocyte-specific BDNF knockout mice exhibit crucial deficit in cardiac functions and have decreased survival rate (L. Li et al., 2022).

Although the current understanding of numerous individual promoters of *BDNF* is not fully understood, exon-specific knockout mice provide great insight into the complex regulation of BDNF. Deletion of *Bdnf* transcripts transcribed from promoter I causes decreased maternal care and sexual receptivity in female mice (Maynard et al., 2018), also severe obesity and impaired thermoregulation (You et al., 2020). Knockout of *Bdnf* produced from promoters I and II causes male-mice specific aggression (Maynard et al., 2016) and hyperphagia together with obesity (McAllan et al., 2018). Knockout of *Bdnf* exon IV-containing transcripts results in impaired synaptic transmission and plasticity (Sakata et al., 2009, 2013), GABAergic signalling (Martinowich et al., 2011; Maynard et al., 2016; Sakata et al., 2009), sleep (Hill et al., 2016; Martinowich et al., 2011) and depressive behaviour (Sakata et al., 2010). Finally, ablation of *Bdnf* promoter VI together with postnatal stress causes schizophrenia-like behaviour (Y. Chen et al., 2022). Taken together, the knowledge from different knockout models corroborates the diverse and vital roles of BDNF.

### 1.2.7 BDNF and its potential in therapeutics

The prevalence of BDNF in various diseases makes it a potential therapeutic target. However, currently there are no BDNF-based therapeutics, primarily due to its short half-life (<10 min), low penetration through the blood-brain barrier (BBB) and limited diffusion rate (Miranda-Lourenço et al., 2020; Wang et al., 2022; Wurzelmann et al., 2017). Numerous studies have attempted to overcome these challenges by using strategies such as drugs that enhance BDNF synthesis (Fukuchi et al., 2019), AAV-mediated gene-therapy (Kells et al., 2004; Queen et al., 2022), employing BDNF-producing cells (Rodrigues Hell et al., 2009) or providing the exogenous supply of BDNF through minipumps, injections, and intranasal delivery (Canals et al., 2004; Ma et al., 2016; Sansevero et al., 2019). Additionally, several small-molecule TrkB-agonists that

mimic BDNF have been explored. The most known among them, 7,8-dihydroxyflavon, has been found to mimic the functions of BDNF, has longer half-life and penetrates through BBB (Devi & Ohno, 2012; C. Liu et al., 2016; Z. Zhang et al., 2014). However, studies have revealed controversial results, thereby raising questions about its functionality (Boltaev et al., 2017). Despite efforts, none of the BDNF-specific therapeutics have proven their efficiency *in vivo*.

It is of great interest to a broad audience that regular exercise, social interactions and enriched environment are among the simplest methods for individuals to boost their BDNF levels (Hsiao et al., 2014; Miranda et al., 2019). To illustrate, Hsiao and colleagues showed that cohousing increases hippocampal levels of BDNF, leading to improved neurogenesis and memory recovery in animal models of Alzheimer's disease (Hsiao et al., 2014). Enriched environment (food hoppers, running wheels, tunnels, shelters, stairs, etc.) has similarly been associated with higher BDNF levels in the rodent hippocampus and cortex. This increase in BDNF has been associated with improved spatial memory and reduced depressive-like behaviour (Gutiérrez-Vera et al., 2022; Novkovic et al., 2015; Rossi et al., 2006; Shilpa et al., 2017). However, it has been proposed that the increased BDNF expression resulting from environmental enrichment might be due to the increased exercise (Kobilo et al., 2011). The link between heightened BDNF levels and various exercises has been well-established (Miranda et al., 2019). Voluntary free running-wheel exercise up to 30 days raises BDNF levels particularly in the hippocampus, enhancing adult neurogenesis and alleviating depression- and anxiety-like behaviours in rodents (Kobilo et al., 2011; Y. Li et al., 2008; Wrann et al., 2013). Specifically, running has been shown to boost the levels of *Bdnf* exon I- and II-containing transcripts in the mouse hippocampus, a process dependent on histone acetylases (Sleiman et al., 2016). In adult humans, a year of aerobic exercise has been demonstrated to increase hippocampal volume and enhance spatial memory, correlating with higher levels of serum BDNF (Erickson et al., 2011).

### 1.3 Enhancer regions

The expression of each gene represents a complex process, where enhancer regions have a crucial role (Hafner & Boettiger, 2023). Enhancers were discovered from viral genomes in the late 20<sup>th</sup> century, when Banerji and colleagues identified a small (72 bp) tandem repeat of simian virus 40 (SV40) DNA that enhanced the transcription of  $\beta$ -globin gene up to 200-fold (Banerji et al., 1981). Over the following decades, the understanding of enhancers has significantly evolved. Enhancers can be located upstream, downstream and inside of the target or unrelated genes, and are defined as non-coding DNA elements that orchestrate the spatiotemporal expression of target genes. Despite significant effort, the understanding of enhancers remains ambiguous (Pennacchio et al., 2013; Schoenfelder & Fraser, 2019).

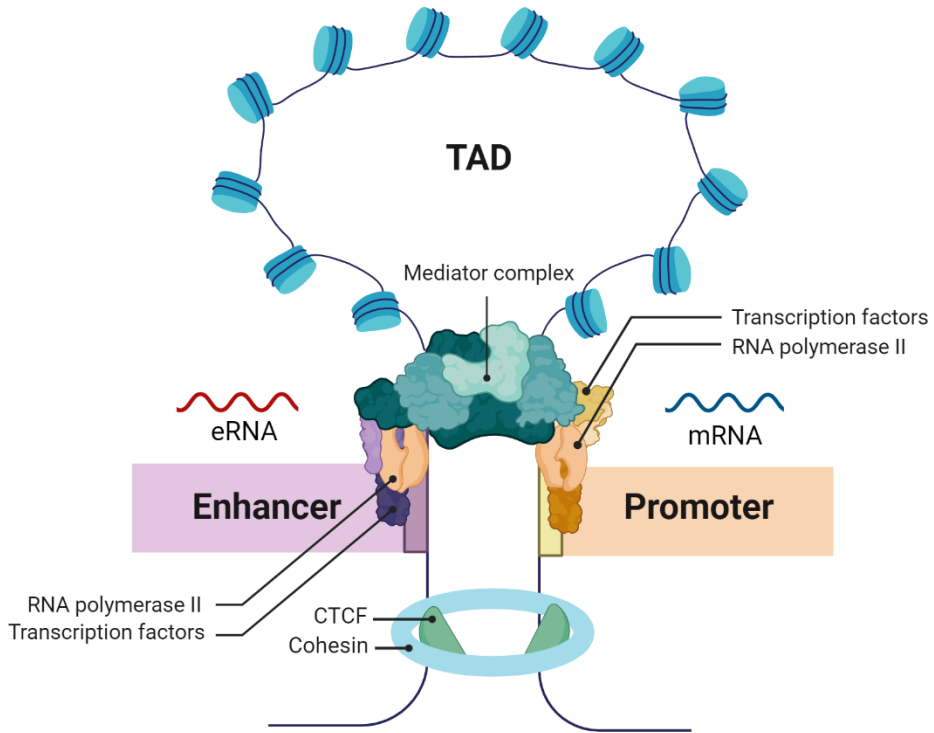
#### 1.3.1 Mechanism of enhancers

To regulate gene expression in cell-type, stimuli- and development-specific manner, enhancers switch their states. According to the functionality, enhancers can be divided into inactive, primed, poised or active state (Heinz et al., 2015). The inactive state enhancers are characterized with tightly condensed chromatin conformation and show no binding of transcription factors. The primed state is prior to active state, where enhancer regions contain repressive epigenetic marks (H3K4me1) but are bound by pioneer transcription factors that have triggered nucleosomal depletion. To become

active, primed enhancers need stimuli to bind the whole transcriptional machinery (Calo & Wysocka, 2013; Zentner et al., 2011). The poised state is similar to primed state (in addition marked with H3K27me3), but characterizes developmental enhancers that are found in ESCs, bound by polycomb protein complexes and activated in cell-specific manner upon differentiation (Crispatzu et al., 2021; Rada-Iglesias et al., 2011). In addition to nucleosome-free core region (DNase I hypersensitivity sites), active state enhancers are characterized by open chromatin mark (H3K27Ac) and bind numerous transcription factors together with RNA polymerase II to facilitate the transcription of enhancer RNAs (eRNAs) (T. K. Kim et al., 2010; Perenthaler et al., 2019).

To regulate genes, enhancer regions come to close physical proximity with their target promoter. The preferential interactions between a gene and its distal regulatory regions are restricted to topologically associated domain (TADs), up to mega base-sized compartments that separate regulatory hubs in the vertebrate genome (Dixon et al., 2012; Nora et al., 2012; Sexton et al., 2012). Most likely, the folding of genome and establishment of regulatory domains is caused by loop-extrusion model, i.e., an interplay between CCCTC-binding factor (CTCF) and cohesin. CTCF, a DNA binding protein, exhibits enriched binding across regulatory regions and TAD boundaries (Davidson et al., 2023). Cohesin is an ATP-dependent protein-complex, which is loaded onto chromatin to extrude DNA bidirectionally until meeting CTCF-bound sites, the anchors for chromatin loops (Davidson et al., 2019; Fudenberg et al., 2016; Y. Kim et al., 2019). It is suggested that cohesin can also occupy the enhancer and core promoter sites in conjunction with mediator (Kagey et al., 2010). Mediator, a multiprotein complex, is recruited by transcription factors within enhancers. Through the enhancer-promoter loop, the mediator is loaded onto the target gene promoters to complete the preinitiation complex and activate RNA polymerase II (Richter et al., 2022) (**Figure 3**).

If enhancer regions are found in large clusters (up to 50 kb), they are defined as super-enhancers. Super-enhancers are highly enriched with RNA polymerase II, various cofactors (e.g., CBP), chromatin regulators (e.g., cohesin, BRD4), and are especially important for genes that regulate cell identity (Hnisz et al., 2013; Whyte et al., 2013). Moreover, super-enhancers are frequently located near key oncogenes, as typical enhancers often transform into super-enhancers during the progression of tumor pathogenesis (Hnisz et al., 2013; Lovén et al., 2013).



*Figure 3. A model for enhancer-promoter interaction. CTCF and cohesin facilitate the folding of topologically associated domains (TADs), where enhancer and promoter interactions are favoured. Active enhancer regions bind transcription factors and RNA polymerase II to transcribe enhancer RNAs (eRNAs). The mediator complex, transcription factors and chromatin modifiers constitute a multiprotein complex that acts as a bridge between the enhancer and promoter regions, thereby enhancing transcription from target genes. Figure was created using BioRender.com.*

### 1.3.2 Enhancer RNA

The majority of active enhancers are known to facilitate bidirectional transcription of short (~500 bp), 5' capped, non-polyadenylated and unspliced non-coding eRNAs (Andersson et al., 2014; Han & Li, 2022). In contrast, super-enhancers generate longer (up to 5 kb), polyadenylated and overall higher levels of eRNAs than typical enhancers (Alvarez-Dominguez et al., 2017; Hnisz et al., 2013; Xiang et al., 2014). The transcription of eRNAs might be different between enhancers. For instance, some eRNAs are transcribed in unidirectional manner (Kouno et al., 2019; W. Li et al., 2013; P. F. Tsai et al., 2018), while others are transcribed bidirectionally, but functional eRNA is produced only from one strand (Hsieh et al., 2014). In general, the transcription of eRNA is correlated with the activity of enhancer and usually precedes the transcription of the target gene (Arner et al., 2015; De Santa et al., 2010; T. K. Kim et al., 2010). Interestingly, based on eRNA expression, brain and blood cells have more cell type-specific than ubiquitously transcribed enhancers (Andersson et al., 2014).

Research investigating the functional roles of eRNAs presents varying outcomes, but suggests that eRNAs are more than just transcriptional noise (Arnold et al., 2020). Moreover, instead of universally defined mechanism, the role of eRNAs seems to be specific for each enhancer-promoter connection. For example, eRNAs have been shown

to stabilize enhancer-promoter looping by (1) recruiting cohesin proteins to enhancers important for oestrogen-dependent transcriptional activation in human breast cancer cells (W. Li et al., 2013); or (2) forming complex with Mediator subunit in prostate cancer cells (Hsieh et al., 2014). In muscle cells the eRNAs transcribed from *MYOD1* enhancers direct the chromatin-remodelling cascade and RNA polymerase occupancy at the *MYOD1* promoter (Mousavi et al., 2013). For IEGs, the knockdown of eRNA does not affect the enhancer-promoter looping in neurons. Instead, eRNAs associate with the RNA recognition subunit of negative elongation factor (NELF), thereby promoting release of NELF from promoters, elongation of transcription and induction of target gene expression (Schaukowitch et al., 2014). Taken together, it is widely accepted that transcription from enhancers is in strong correlation with the target gene mRNA levels (Arner et al., 2015), but whether the eRNA is important for enhancer-promoter loops, chromatin modifications or transcriptional machinery, must be elucidated separately for each enhancer-promoter interaction.

### 1.3.3 Enhancer-associated diseases

It is speculated that variations in protein non-coding regions (hereafter non-coding) might play a significant role in many genetic diseases. A rough estimation based on Genome-Wide Association Studies (GWAS) suggests that approximately 90% disease-related single nucleotide polymorphisms (SNPs) are found in the non-coding genome (Maurano et al., 2012). Of course, it is important to note that enhancers make up only a small portion of the non-coding genome. Yet, changes in enhancers are linked to a range of diseases, many of which are still undiscovered. For example, it is estimated that approximately 60% of the SNPs responsible for autoimmune diseases are in enhancer-like elements specific for immune cells (Farh et al., 2015). The conditions where misregulation of enhancers leads to pathogenic outcomes - enhanceropathies - are particularly prevalent in various forms of cancer and Mendelian disorders. Different types of alterations in enhancers have been described, including insertions and deletions, chromosomal rearrangements, and single nucleotide changes. These alterations can result in either a change in enhancer activity or cause rewiring of enhancer-promoter interactions (Smith & Shilatifard, 2014; Zaugg et al., 2022). To illustrate, a SNP in  $\alpha$ -synuclein (*SNCA*) enhancer alters the binding of repressive transcription factors, increasing the risk of Parkinson's disease (Soldner et al., 2016). A TAD disruption between *MEF2C* and its distal enhancer causes 5q14.3 microdeletion syndrome (Redin et al., 2017). Mutations in an enhancer leads to the misregulation of *SHH* gene, resulting in abnormal digit development (preaxial polydactyly) (Lettice et al., 2002). Somatic duplications of super-enhancers have been implicated in the overexpression of *MYC* gene, a phenomenon prevalent in various types of carcinomas (X. Zhang et al., 2016). Finally, a hypoxia-inducible eRNA (HERNA1) promotes cardiac hypertrophy, a condition which was reversed *in vivo* by antisense oligonucleotides-mediated inactivation of the eRNAs (Mirtschink et al., 2019).

The increasing prevalence of enhanceropathies raises important questions about the potential of enhancer-related therapies. Currently, research has focused on two different approaches (Claringbould & Zaugg, 2021). First, epigenetic modulators such as bromodomain and extra terminal domain (BET) inhibitors, which are class of small molecule drugs, mostly studied and shown to work as anticancer treatments. The exact molecular mechanism is still under debate, but BET inhibitors preferably target cancer cells and interact with bromodomains of BET proteins in super-enhancers, thus initiating

transcriptional repression programs (Claringbould & Zaugg, 2021; Sermer et al., 2019). An example of BET inhibitors is JQ1, a drug that displaces BRD4 from the super-enhancers, leads to the downregulation of oncogenes and inhibits the proliferation of tumour cells (B. Liu et al., 2022; Lovén et al., 2013).

Second, recent achievements possess great potential to CRISPR-based therapies. Unlike epigenetic inhibitors, CRISPR-based tools could provide enhancer-specific treatment. For example, Matharu et al., 2019 showed that using CRISPR/dCas9 to activate the functional copy of a distant hypothalamic enhancer of *Sim1* gene could successfully reverse the severe obesity phenotype that arises from *Sim*<sup>+/-</sup> haploinsufficiency (Matharu et al., 2019). The world's first ever CRISPR/Cas-based gene therapy, Casgevy, was approved by FDA on December 8, 2023 (FDA, 2023). A breakthrough that, in fact, began with studying the enhancers of the *BCL11A* gene (Canver et al., 2015). Although it does not cure enhancer-related disease, Casgevy is used to treat sickle cell disease, a Mendelian-inherited blood disorder caused by mutations in both copies of the hemoglobin gene. In principle, the CRISPR/Cas9 system targets and disrupts the intronic enhancer of *BCL11A* gene. *BCL11A* encodes a transcriptional repressor that inhibits the expression of fetal hemoglobin in the adult organism. Consequently, this disruption leads to reinduction of fetal hemoglobin in an adult organism (Fрати & Miccio, 2021; Mullard, 2023).

#### 1.4 The enhancers of *BDNF* gene

Although the proximal regulatory regions of the *BDNF* gene have been well studied (Esvald et al., 2022; Pruunsild et al., 2011; Tuvikene et al., 2016; West et al., 2014), only a limited number of studies have focused on the distal regulatory regions governing the expression of *BDNF* gene. One of the first papers of a *Bdnf* enhancer was published in 2007, where a region 4.8 kb upstream of *Bdnf* promoter I was shown to bind myocyte enhancer factor-2 (MEF2) family transcription factor D (MEF2D) and enhance *Bdnf* promoter I activity in heterologous context in hippocampal neurons (Flavell et al., 2008). Few years later it was shown that the -4.8 kb region does not enhance *Bdnf* promoter I in cortical neurons in heterologous context (M. R. Lyons et al., 2012), indicating the -4.8 kb region to be a cell-specific enhancer of *Bdnf* gene. Recently an enhancer located +237 kb downstream of *Bdnf* exon I was described in detail (Brookes et al., 2023). The +237 kb enhancer is critical to ensure the expression of *Bdnf* during neuronal development by affecting dendritogenesis and cortical development (Brookes et al., 2023).

Recent genome-wide analyses have revealed several hints regarding the potential enhancers of *BDNF*:

- Schmitt et al. data indicates that the TAD of *BDNF* runs ~1.7 Mb upstream and ~0.2 Mb downstream of *BDNF* exon I TSS in multiple human neuronal and non-neuronal tissues (Schmitt et al., 2016).
- Brookes et al., have described a sub-TAD of *Bdnf* in NPCs and cortical neurons. The sub-TAD is located in the 3' end of the larger TAD, contains the *Bdnf* gene, downstream intergenic region and *Lin7c* gene, and the boundaries of this sub-TAD are CTCF and cohesin positive (Brookes et al., 2023).
- Genome folding maps around *Bdnf* gene show neuronal-activity dependent loops with ~840 kb (Beagan et al., 2020) and ~1.7 Mb regions in mouse cortical neurons (Beagan et al., 2020; Calderon et al., 2022).

- The -840 kb region is predicted as an activity-dependent enhancer of *Bdnf* in rat primary cortical neurons based on correlation analysis (Carullo et al., 2020).
- Protein crucial for connecting chromatin loops, CTCF, binds to the first and second cluster of *Bdnf* promoters in both cortical (Beagan et al., 2020; Bonev et al., 2017; Calderon et al., 2022; Chang et al., 2010) and hippocampal neurons (Sams et al., 2016). Moreover, loss of the CTCF-cohesin based chromatin loops decreases the expression of *Bdnf* (Calderon et al., 2022; Chang et al., 2010).
- A cell-specific PLAC-seq analysis in human post-mortem brain reveals *BDNF* interactions with ~-405 kb, ~-840 kb and ~-952 kb regions in neurons (Nott et al., 2019).

Moreover, several *BDNF*-related studies indicate the presence of yet unknown distal regulatory regions. In cortical astrocytes, neither *Bdnf* promoters IV nor VI are induced by catecholamines in luciferase reporter assays, however BAC-reporters containing the entire human or rat *BDNF* locus are induced by catecholamine treatments (Koppel et al., 2018). In cortical neurons there are indications for CREB-dependent enhancer(s) in the autoregulation of *Bdnf*. CREB has been shown to bind *Bdnf* promoter IV after *BDNF*-TrkB signalling in cortical neurons, while overexpression of dominant-negative protein for CBP/p300 (CCII), CREB family (A-CREB) or CREB-CBP/p300 interaction inhibitor (CCII) decreased the *BDNF*-TrkB-induced levels of all measured *Bdnf* transcripts (Esvald et al., 2020). In addition, the endogenous levels of all *Bdnf* transcripts are highly induced in response to TrkB signalling both *in vitro* and *in vivo* (Esvald et al., 2020; Tuvikene et al., 2016), while the *Bdnf* promoters and BAC-reporters with entire human or rat *BDNF* locus are induced less after *BDNF* stimulation in cortical neurons (Esvald et al., 2020, unpublished data from prof. Timmusk lab). Finally, alterations in the -840 kb region have been shown to decrease the levels of *BDNF* and cause phenotypic changes in both humans (Sha et al., 2007) and mice (Gray et al., 2006). Taking into account all the information above and considering that current understanding indicates that one gene can be regulated by numerous enhancers (Mills et al., 2020), it is evident that there are still several enhancer regions of *Bdnf* yet to be discovered. All currently known functional and potential enhancers of *BDNF* are listed in **Table 1**.



Table 1. List of potential and functional *BDNF* enhancer regions

Distance from rat <i>Bdnf</i> exon I TSS (rn6)	Methods	Study system	Stimulus	Mechanism	Reference
-840 kb	FISH, SB, ELISA, phenotyping	Human patient with chromosomal inversion	-	Disruption causes phenotype similar to <i>BDNF</i> KO mice	Gray et al., 2006
	Genotyping, NB, SB, WB, RT-PCR, phenotyping	Mice with transgene insertion			Sha et al., 2007
-4.8 kb	Luciferase reporter assay	Rat primary hippocampal	KCl	Binds MEF2D, regulates <i>Bdnf</i> pl	Flavell et al., 2008
-405 kb	PLAC-seq	Human postnatal neurons	-	Loops with <i>BDNF</i>	Nott et al., 2019
-840 kb					
-952 kb					
-840 kb	5C-seq	Mouse primary cortical neurons	Bicuculline		Beagan et al., 2020
-1.7 Mb					
-840 kb	ATAC-seq, total RNA-seq	Rat primary cortical neurons	KCl	eRNA levels correlate with <i>Bdnf</i> mRNA levels	Carullo et al., 2020
-1.7 Mb	5C-seq, ChIP-seq	Mouse primary cortical neurons	KCl	Looping with <i>Bdnf</i> reduced in cohesin-deficient neurons	Calderon et al., 2022
+237 kb	4C-seq, FISH, CRISPRi	Mouse NPCs and primary cortical cultures	Neuronal differentiation, KCl	Regulates <i>Bdnf</i> -dependent neuronal differentiation and cortical development	Brookes et al., 2023

*FISH* – Fluorescence in situ hybridization; *SB* – Southern Blot; *ELISA* – Enzyme-linked immunosorbent assay, *KO* – Knockout; *MEF2D* – Myocyte enhancer factor 2D; *NB* – Northern blot; *WB* – Western blot; *RT-PCR* – Quantitative real-time polymerase chain reaction; *PLAC-seq* – Proximity ligation-assisted chromatin immunoprecipitation sequencing; *5C-seq* – Chromosome-conformation-capture carbon-copy sequencing; *ATAC-seq* – Assay for transposase-accessible chromatin with sequencing; *ChIP-seq* – Chromatin immunoprecipitation sequencing; *4C-seq* – Chromosome conformation capture sequencing; *CRISPRi* – CRISPR interference; *NPCs* – Neural precursor cells

## 2 Aims of the study

The aim of this doctoral thesis was to identify and describe enhancer regions responsible for the stimulus-dependent expression of *Bdnf* in neuronal and non-neuronal cells. The more specific aims of the study were as follows:

- Determine the molecular mechanism of the +3 kb intronic enhancer in neurons and astrocytes
- Identify and characterize the enhancer(s) regulating catecholamine-dependent regulation of *Bdnf* in astrocytes
- Revisit the functionality of the MEF2-dependent -4.8 kb enhancer in cortical and hippocampal neurons

### 3 Materials and methods

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

- Growing of cell lines – Publications I, II, III
- Growing of primary neural cells (rat primary cortical and hippocampal neurons, rat primary cortical astrocytes) – Publications I, II, III
- Growing and differentiation of mouse embryonic stem cells – Publication I, II
- Molecular cloning – Publications I, II, III
- CRISPR/Cas9-mediated deletion of enhancer region in mouse embryonic stem cells – Publication I, II
- RNA extraction, cDNA synthesis, qPCR – Publications I, II, III
- Western blot analysis – Publications I
- Purification and use of lentivirus vectors – Publications I, II, III
- CRISPR activation and interference systems – Publication I, II
- RNA interference – Publication III
- Transfection, luciferase reporter assay – Publications I, II, III
- *In vitro* DNA pulldown coupled with mass-spectrometry – Publication I
- Chromatin immunoprecipitation – Publication I, II
- Immunocytochemistry – Publication II
- Confocal microscopy – Publication II
- Bioinformatic analysis – Publication I, II
- Site-directed mutagenesis – Publication II
- Chromosome conformation capture analysis – Publication II
- Intracerebroventricular injection of kainic acid – Publication III

## 4 Results

### 4.1 Results obtained in publication I

- The +3 kb region shows enhancer-associated characteristics in rodent and human neural tissues.
- The +3 kb region shows bidirectional transcription in heterologous context in rat cortical neurons.
- The +3 kb region potentiates the activity of *Bdnf* promoters I and IV in heterologous context in rat cortical neurons.
- The +3 kb region enhances the expression of *Bdnf* exon I-, IIc-, and III-containing transcripts in the endogenous context in rat cortical neurons.
- The +3 kb enhancer is inactive in the endogenous context in rat cortical astrocytes.
- Deletion of the +3 kb enhancer region in mESC-derived neurons decreases the expression of *Bdnf* exon I-, IIc-, and III-containing transcripts.
- The activity of the +3 kb enhancer region is regulated by numerous transcription factors, such as CREB, AP1 family and E-box-binding transcription factors, including Transcription Factor 4 (TCF4).

### 4.2 Results obtained in publication II

- Four candidate regions (-840 kb, -450 kb, -40 kb, +37 kb) were predicted as *Bdnf* enhancers in astrocytes using GeneHancer database in GeneCards.
- Out of the four candidate enhancers, only the -840 kb region shows properties of a catecholamine-dependent enhancer in astrocytes in heterologous context.
- The -840 kb region shows dopamine-dependent evolutionarily conserved bidirectional transcription in heterologous context in cultured rat cortical astrocytes.
- The -840 kb region potentiates dopamine-dependent transcription from *Bdnf* promoters in heterologous context in cultured rat cortical astrocytes.
- The -840 kb region is enriched with active transcription mark (H3K27Ac), and not with condensed chromatin mark (H3K27me3) in cultured rat cortical astrocytes.
- The -840 kb region is in close physical proximity to *Bdnf* promoter VI upon dopamine treatment in cultured rat cortical astrocytes as determined by chromosome conformation capture analysis.
- The *Bdnf* promoter VI interacts with -790 kb region in DMSO-treated astrocytes, with -990 kb and -2.65 Mb regions in dopamine-treated astrocytes.
- The *Bdnf* promoter VI interacts with -1.82 Mb region in both DMSO- and dopamine-treated astrocytes.
- The -840 kb region enhances catecholamine-dependent transcription of *Bdnf* in endogenous context in cultured rat cortical astrocytes.
- Deletion of the -840 kb region decreases catecholamine-dependent transcription of *Bdnf* in mESC-derived astrocytes.

- CREB and AP1 family transcription factors regulate the transcription of -840 kb enhancer RNA and *Bdnf* mRNA in cultured rat cortical astrocytes.
  - CREB and AP1 family transcription factors bind to the -840 kb enhancer region in cultured rat cortical astrocytes.
  - Mutations in CRE and AP1 sites in the -840 region decrease the transcriptional activity of the enhancer in heterologous context in cultured rat cortical astrocytes.

### 4.3 Results obtained in publication III

- The -4.8 kb enhancer potentiates activity from *Bdnf* promoter I in heterologous context in hippocampal and to a lesser extent in cortical neurons.
- The -4.8 kb enhancer exhibits enhancer-related characteristics in both rat and human hippocampus (e.g., open chromatin, kainic-acid induced transcription *in vivo*).
- The -4.8 kb region enhances KCl-dependent expression of the first cluster of *Bdnf* transcripts (exon I, IIc- and III-containing transcripts) in hippocampal neurons.
- MEF2D represses the activity of the -4.8 kb enhancer in cortical and hippocampal neurons, and MEF2C acts as a transcriptional activator of the -4.8 kb enhancer in KCl-treated hippocampal neurons.
- MEF2C and MEF2D are crucial regulators of the expression of all the major *Bdnf* transcripts in cortical and hippocampal neurons.

## 5 Discussion

### 5.1 Enhancers are crucial for stimulus-dependent expression of *BDNF*

While the proximal regulatory regions of *BDNF* have been well described (West et al., 2014), only a few studies have investigated the functional role of enhancers regulating *BDNF* expression (mentioned in 1.4, Table 1). This represents a critical knowledge gap, given that enhancers refine the spatiotemporal transcriptional regulation and alterations in enhancer regions are increasingly associated with diseases (mentioned in 1.3.3). The studies underlying current PhD thesis describe functionality of three new *Bdnf* enhancer regions, therefore providing valuable insight into the transcriptional regulation of this crucial neurotrophin.

The *BDNF* gene has two distinct cluster of 5' exons. The expression of the first cluster of *BDNF* exons (I, II and III) is commonly known as neuron specific, while the second cluster of *BDNF* exons (IV, V, VI and VII) is more universal and is expressed both in neuronal and non-neuronal cells (Aid et al., 2007; Esvald et al., 2023; Pruunsild et al., 2007; Timmusk et al., 1993). As the promoters situated in the first and second cluster of *BDNF* are in proximity (fit into ~2 kb region within cluster), it is tempting to speculate a coregulation of promoters in one cluster. The CTCF and cohesin have been shown to bind nearby of the first and second cluster of *Bdnf* 5' exons (Brookes et al., 2023; Chang et al., 2010; Sams et al., 2016), possibly pointing to the formation of local contact domain near each of the *Bdnf* cluster (Rinzema et al., 2022). The enhancer located downstream of the *Bdnf* cluster I (+3 kb enhancer, publication I) could partially mediate its effect on *Bdnf* gene expression by regulating the higher-order chromatin structure in the *Bdnf* gene locus by bringing together specific distal enhancer regions and *Bdnf* promoters from the first cluster of exons. Moreover, it is plausible that the -4.8 kb (Publication III) and +3 kb enhancer (Publication I) regions synergistically facilitate the neuronal activity dependent expression of the first cluster of *Bdnf* exons in the hippocampal neurons.

The transcriptional regulation of *BDNF* gene exhibits mysterious redundancy from various perspectives. For instance, different individual promoters drive the expression of identical *BDNF* protein, and several transcription factor binding sites are repeated within the proximal and distal regulatory regions (e.g., CREB, AP1 and MEF2 family transcription factors). For instance, functional CRE-sites can be found in *BDNF* promoters I, IV, IX (mentioned in 1.2.2), -840 kb enhancer (publication II) and potentially also in +3 kb *Bdnf* enhancer (cis-element not tested, binding of CREB has been shown, publication I). Given the numerous long-range chromatin interactions detected in our CAPTURE-3C-sequencing experiment in astrocytes (Publication II), it is plausible that the enhancers of *Bdnf* also display strong redundant effects, i.e., multiple enhancers ensure *Bdnf* expression simultaneously. According to our results, dopamine-dependent regulation of *Bdnf* promoter VI in astrocytes needs the proximity of -840 kb, -990 kb and -2.65 Mb regions, while interaction with only -790 kb region was observed in DMSO-treated cells, and -1.82 Mb region showed stimulus-independent interaction (interaction in both DMSO- and dopamine-treated astrocytes). It must be noted that the endogenous functionality of the -790, -990 kb, -1.82 Mb and -2.65 Mb regions needs to be confirmed in the future studies. Moreover, results from Koppel et al. (2018) suggest that that at least some enhancers regulating the catecholamine-induced levels of *Bdnf* in cortical astrocytes are located within or near the *Bdnf* gene (Koppel et al., 2018). It is important to note that our CAPTURE-3C-sequencing might

lack the resolution and statistical power to detect the possible short-range interactions within the *Bdnf* locus. The redundancy of enhancers has been shown to be a widespread feature of mammalian genomes, serving as a robust backup mechanism to ensure stable expression even if one of the regulatory regions is disrupted (Osterwalder et al., 2018). The overlapping effects observed in the regulation of the *Bdnf* gene, involving multiple promoters, enhancers and cis-elements, are yet to be fully understood. However, they likely contribute to the consistent expression of BDNF, which is essential for maintaining the normal functioning of the organism.

Two previous studies (Gray et al., 2006; Sha et al., 2007) have indicated the existence of a regulatory region ~840 kb upstream of the *BDNF* gene. In Gray et al., 2006, a *de novo* chromosomal inversion breakpoint situated 840 kb upstream of the *BDNF* gene was observed in an 8-year-old girl. This patient suffered from hyperphagia, severe obesity, impaired cognitive functions, and hyperactivity. Notably, despite no alteration in the *BDNF* gene itself, the patient's serum showed decreased levels of BDNF (Gray et al., 2006). In Sha et al., 2007, a mouse line with a transgene insertion near a conserved region approximately 840 kb upstream of the *Bdnf* gene displayed a similar phenotype and reduced lifespan. The disruption of the -840 kb region reduced BDNF levels in the brain during postnatal development starting from postnatal day 28, the first time point analysed, particularly in the hypothalamus and hippocampus. Additionally, the study observed decreased *Bdnf* mRNA levels in the heart and lower protein levels in the serum of adult mice (Sha et al., 2007). We have identified and characterized the -840 kb region as a catecholamine-dependent enhancer in cortical astrocytes (Publication II). Based on the phenotypic peculiarities described above, it is more than evident that the -840 kb region serves as an enhancer of *BDNF* in various other brain regions beyond cerebral cortex. Furthermore, there is high likelihood that the -840 kb region plays a role in the transcriptional regulation of *BDNF* in cell types other than astrocytes. For instance, analysis of two recent studies shows that *BDNF* looping with -840 kb region is evident also in human (Nott et al., 2019) and mouse neurons (Beagan et al., 2020). Moreover, the -840 kb region is predicted as an activity-dependent transcriptional enhancer of *Bdnf* in rat cortical neurons (Carullo et al., 2020). As the study conducted by Sha and colleagues observed *Bdnf* mRNA expression changes in heart, the -840 kb region might also regulate *Bdnf* expression in heart. The fascinating storyline regarding -840 kb enhancer (Table 1, publication II) points it to be a master regulator of *BDNF*. Further research is necessary to elucidate the widespread function of the -840 kb enhancer region across different cell and tissue types.

BDNF is known to be expressed in various regions of the hypothalamus, where it plays a critical role in controlling food intake and thereby regulates the overall energy balance of the organism (discussed in 1.2.5, 1.2.6) (Rios et al., 2001). It is plausible that BDNF expression in the hypothalamus is involved in the age-dependent regulation of the organism's overall energy-balance, and that the -840 kb enhancer might play a significant role in this process. This hypothesis is supported by following aspects: (1) the obesity phenotype of *BDNF*<sup>+/-</sup> mice becomes apparent from 2 to 4 months after birth (W. Lyons et al., 1999); (2) an 8-year-old patient with a chromosomal inversion began to rapidly gain weight around 4 months after birth (Gray et al., 2006); and (3) mice with the homozygous disruption in the -840 kb region exhibited a considerable decrease in BDNF, specifically in the hypothalamus, and showed a statistically significant weight increase from 7 to 8 weeks post-birth, along with insulin resistance and type 2 diabetes (Sha et al., 2007). These findings suggest

that the -840 kb region, in addition to possibly functioning as an enhancer of *BDNF* in various tissues and cell types (discussed in previous paragraph), may exhibit activity dependent on the developmental stage, e.g., regulating BDNF expression in the hypothalamus only during the postnatal period.

## 5.2 The mechanism of *Bdnf* enhancers

Stimulus-dependent gene regulation resembles a domino effect, where triggering of one event leads to a series of interconnected and precisely timed reactions, each influencing the subsequent one. For example, neuronal activity is known to trigger the activation of hundreds of genes. Among these, IEGs such as *Fos* respond rapidly, while secondary response genes like *Bdnf* require *de novo* protein synthesis and are typically activated in hours (Flavell et al., 2008; Tyssowski et al., 2018). In each of our enhancer studies (Publication I-III) we have consistently measured the eRNA levels concurrently with *Bdnf* mRNA, i.e., at the same treatment point as we used to measure *Bdnf* mRNA levels. We have seen that the eRNA levels of +3 kb, -840 kb and -4.8 kb enhancer peak and correlate with *Bdnf* mRNA levels regardless of cell type (astrocytes or neurons) and stimuli (KCl, BDNF or catecholamine treatment). This finding is particularly intriguing because it is commonly believed that eRNA transcription precedes mRNA transcription (mentioned in 1.3.2) (Arner et al., 2015). Our observations challenge the concept of universal enhancer-related kinetics and supports the idea that each gene has distinct enhancer dynamics. This implies that the relatively slower enhancer kinetics for *Bdnf* might be a contributing factor to its delayed expression. This is in good agreement with Beagan et al., 2020 showing that loops surrounding IEGs peak in signal strength ~20 min after the induction of neuronal activity, which is prior to the peak in mRNA levels. In contrast, the neuronal activity-dependent expression of eRNA and loops associated with *Bdnf* gain strength in parallel with *Bdnf* mRNA levels, indicating a significant delay in enhancer dynamics for *Bdnf* compared to IEGs. Additionally, loops connected with *Bdnf* gene are substantially longer and more complex than those associated with IEGs (Beagan et al., 2020), further emphasizing the unique nature of each enhancer-promoter interaction.

An open question concerns the role of eRNA in the transcriptional regulation of *Bdnf*. There is growing evidence, as highlighted in various studies (mentioned in 1.3.2), that eRNAs can differently (directly, indirectly or not having role) influence gene regulation. So far, all the described enhancers of *Bdnf* exhibit transcription of eRNAs, which modulation is in correlation with *Bdnf* levels (Brookes et al., 2023, publication I-III), possibly indicating the importance of eRNA in *Bdnf* regulation per se. Moreover, eRNAs transcribed from the +237 kb enhancer are important for BDNF-mediated neuronal migration and cortical development (Brookes et al., 2023). Previously it has been shown that in cortical neurons the CTCF and cohesin proteins are essential for the transcription of *Bdnf* – the absence of these proteins results in inactive chromatin at the *Bdnf* locus and consequently decreases the levels of *Bdnf* (Chang et al., 2010). Furthermore, in cohesin-deficient cortical neurons the activity-dependent expression of *Bdnf* is reduced. This is in contrast with other genes like *Fos* and *Arc*, whose enhancer-promoter loops are not dependent on cohesin (Calderon et al., 2022). Although the precise mechanism by which the eRNAs of *Bdnf* function remains unclear and is a subject for future research, we can hypothesize that based on our (Publication I-III) and other studies (Brookes et al., 2023; Calderon et al., 2022; Chang et al., 2010) the transcription of eRNAs from the enhancers of *Bdnf* is important and that these eRNAs might interact with cohesin to ensure the regulation of *Bdnf*. A similar mechanism has been observed for the regulation



of Myogenin gene, where the transcription of eRNAs is essential for directing the correct loading and maintenance of cohesin at the gene locus, ensuring the proper transcription of Myogenin (P. F. Tsai et al., 2018).

### 5.3 Transcription factors in the enhancer-dependent regulation of *Bdnf*

There are substantial differences in the regulation of *Bdnf* gene between different cell types and brain regions, even when the same transcription factor families are involved. For example, the best-described regulators of *Bdnf* - CREB family transcription factors - show more important role in the regulation of *Bdnf* in cortical than in hippocampal neurons (Esvald et al., 2022). Similarly, the well-known CRE element in *Bdnf* promoter IV does not participate in the catecholamine-dependent regulation of *Bdnf* in astrocytes (Koppel et al., 2018), but is essential for stimulus-dependent expression of *Bdnf* in neurons (mentioned in 1.2.2) (Esvald et al., 2020; Hong et al., 2008; Pruunsild et al., 2011). Our work (Publication I-III) provides additional insight into the transcription factors involved in the enhancer-dependent regulation of *Bdnf*.

- I. We show for the first time that various basic helix–loop–helix (bHLH) transcription factors, such as TCF4 and its heterodimerization partners (NeuroD2, NeuroD6), regulate stimulus-specific expression of *Bdnf* expression in neurons via +3 kb intronic enhancer. Additionally, known *Bdnf* regulators like CREB, AP1, USF, EGR family transcription factors bind the +3 kb enhancer region (Publication I).
- II. We expand the knowledge about transcriptional regulation of *Bdnf* in astrocytes (Publication II). Catecholamines have been shown to induce *Bdnf* gene expression in astrocytes via CREB-dependent signalling (Jurič et al., 2008; Koppel et al., 2018). We show for the first time that AP1 family transcription factors regulate the catecholamine-dependent expression of *Bdnf* in astrocytes by binding to the AP1 cis-element within the -840 kb enhancer (Publication II).
- III. The results from our research (Publication III) reveal that MEF2 transcription factors play a more significant role in regulating *Bdnf* expression than previously understood.

The silencing of MEF2 family members showed diverse effects to all major *Bdnf* transcripts in both hippocampal and cortical neurons (Publication III). For instance, silencing of *Mef2d* led to enormous increase in the mRNA levels of *Bdnf* in cortical neurons, while silencing of *Mef2c* reduced the levels of the -4.8 kb eRNA and overall expression of *Bdnf* especially in the hippocampal neurons. The diverse roles of MEF2 transcription factors to their target genes has been previously described (Shalizi & Bonni, 2005). However, the comprehensive role of MEF2 family transcription factors in the regulation of *Bdnf* remains mysterious, and the exact role is yet to be discovered. Several possibilities could explain the intriguing results from Publication III. For example, the binding of MEF2 transcription factors to unknown distal regulatory regions of *Bdnf*, and to the regulatory regions of transcription factors regulating *Bdnf*. Furthermore, previous genome-wide studies have shown that strong redundancy and compensation among MEF2 family members complicate the studies of these factors (Akhtar et al., 2012; Estrella et al., 2015; Flavell et al., 2008). This might suggest that silencing one of the MEF2 family factors can disrupt the balance of MEF2 family members in a cell and lead to artefactual results. Nevertheless, our findings (Publication III) confirm that MEF2 transcription factors are important regulators of *Bdnf* in both cortical and hippocampal neurons.

## 5.4 Future directions

Based on a very rough estimation, each enhancer is associated with an average of 2 genes, while each gene is linked to several enhancers (Mills et al., 2020). Moreover, GeneHancer database in Genecards (used and described in publication II) predicts the human *BDNF* to have in total of ~80 enhancer regions (accessed on 17<sup>th</sup> of January 2024). This number should be treated with significant caution, as GeneHancer predicts the genomic regulatory elements based on different human genome-wide analysis and some of the regions are categorized as *BDNF* enhancers based on a very low score. Currently, there are in total four enhancer regions which endogenous functionality is linked with *Bdnf*. Three of the enhancers (-840 kb, -4.8 kb, +3 kb enhancer) underlie current PhD thesis studies, and one enhancer (+237 kb) has been described in detail by Brookes et al., 2023. In addition to mapping the yet unknown enhancers of *Bdnf*, a crucial and intriguing next step would be to verify the *in vitro* confirmed enhancers *in vivo*. This could involve transgenic mice in which one or a combination of enhancers is deleted, followed by assessing the resultant phenotypes, conducting morphological studies, and employing high-throughput sequencing techniques. Altogether, there is still a lot to discover regarding the physiological roles of the enhancers of *Bdnf*.

The CRISPR-based strategies possess great potential in the field of gene therapy. To illustrate, Casgevy, the first ever CRISPR-therapy curing sickle-cell disease was recently approved by FDA (FDA, 2023). Moreover, an obese phenotype in mice caused by *SIM1* haploinsufficiency was rescued using CRISPR-activation system targeting the functional copy of the *SIM1* enhancer (mentioned in 1.3.3) (Matharu et al., 2019). Regarding the enhancer regions studied here, one of them has been associated with strong phenotypic peculiarities. As mentioned above, disruption of the -840 kb region causes downregulation of BDNF expression in humans and mice (Gray et al., 2006; Sha et al., 2007), resulting in phenotypic abnormalities similar to those of BDNF knockout animals (mentioned in 1.2.6). This refers to an enhanceropathy, a condition caused by a dysregulation of an enhancer in a functionally important gene regulation (mentioned in 1.3.3) (Claringbould & Zaugg, 2021; Smith & Shilatifard, 2014). As the transcripts of *BDNF* start independently and by distinct regulatory mechanism from promoters in front of each *BDNF* 5' exon (Aid et al., 2007; Pruunsild et al., 2011; Timmusk et al., 1993), promoter-targeted activation of *BDNF* upregulates only specific transcript. As a proof of concept, our results show that targeting the CRISPR/dCas9 activator system to enhancer regions significantly upregulates the mRNA levels of total *Bdnf* and major *Bdnf* transcripts (Publication I-III). Therefore, enhancer-targeted gene activation allows to increase the levels of all *Bdnf* transcripts or activate *Bdnf* promoters within one cluster, which raises new therapeutic potential to BDNF-dysregulated diseases. Whether the knowledge gained from the research underpinning this doctoral thesis is applicable for therapeutic uses remains a question for the future.

## 6 Conclusions

The studies underlying current PhD thesis have identified and characterized the endogenous function of three distinct *Bdnf* enhancer regions (**Figure 4**), specifically:

+3 kb enhancer – an evolutionarily conserved intronic enhancer region that regulates basal, neuronal activity and BDNF-TrkB-dependent expression of exon I-, II-, and III-containing *Bdnf* transcripts in both mouse and rat neurons. This regulatory mechanism is orchestrated by various transcription factors, including CREB, AP1, and bHLH family transcription factors.

-840 kb enhancer – the first described non-neuronal enhancer of *Bdnf* that regulates catecholamine-dependent expression of *Bdnf* in rodent astrocytes, a mechanism mediated by CREB and AP1 family transcription factors.

-4.8 kb enhancer – a MEF2-dependent enhancer that regulates the neuronal activity-dependent expression of the first cluster of *Bdnf* transcripts in hippocampal neurons. In addition, MEF2 family of transcription factors exerts multifaceted control over the expression of *Bdnf* via both proximal and distal regulatory regions, a regulation dependent on stimuli and brain region.

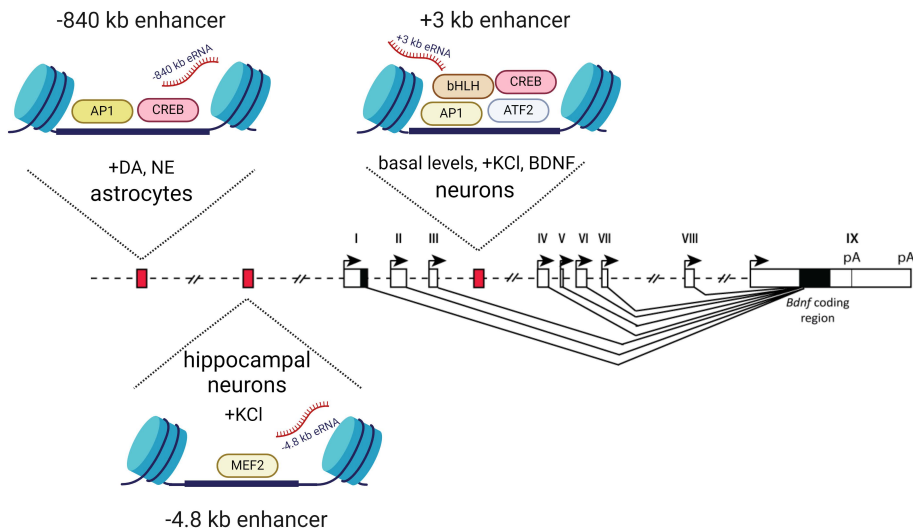


Figure 4. Illustration of enhancers discovered and characterized in studies underlying current doctoral thesis. All enhancer regions (red boxes) are named based on their location from rat *Bdnf* exon I TSS. Indicated transcription factors have shown to regulate the activity of respective enhancer. All studied enhancer regions facilitate the transcription of enhancer RNA (indicated as -840 kb, +3 kb, -4.8 kb eRNA). Figure was created using BioRender.com. DA – dopamine; NE – norepinephrine

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## Acknowledgements

I am grateful to my supervisor prof. Tõnis Timmusk who provided me with the opportunity to work in his molecular neurobiology laboratory. I will never forget the proud feeling when mentioning his name during conferences and discussions with esteemed neuroscientists. His contribution to the field of neuroscience is widely recognized. Working in prof. Timmusk lab has been a great privilege.

I would not have come this far without my co-supervisor dr. Jürgen Tuvikene. Jürgen's immense knowledge and commitment are admirable. Also, big thanks to dr. Eli-Eelika Esvald, whose high motivation and commitment made things work. My sincere gratitude to my students Kaisa and Karin, as well as to all members of the Neurolab and Protobios teams. Special thanks to Anastassia, dr. Tamberg, Carl Sander and dr. Sirp - colleagues who have become true friends. I am deeply grateful for the laughter you brought during both the toughest and most joyful moments.

Colleagues from the institute, I am extremely grateful to you: Indrek, Florencia, Helena and Olga, although connected from the end of my PhD journey, your help has been invaluable and I will follow your science with keen interest; Epp and Ants, your contribution to our research is invaluable; Pirjo and Cecilia, thank you for believing in me and really taking care; Vello, thank you for all these great opportunities; Urmas, thank you for the interesting scientific discussions not only with me, but also with my students; Tanja, thank you for all these cute rodents.

Although genetics largely defines what and how you are doing in life, there is still one more impact factor - the environment. Therefore, I would like to give my greatest gratitude to my family: mother, father, granny, Eric and Ilona; my best friend Kristin; friends from the Rakvere cheerleading team Eli, Karin, Kaisa, Marelle, Aili, Kristina, Ksenja, Berit; friends from Tallinn cheerleading team Brit, Anita, Ingrid; my chemistry and biotechnology girls dr. Kooli, dr. Välimets and dr. Tõlgo; Birgit, Kristjan and Säde; Angelis; Nadja. Thank you all for providing continuous motivation and support throughout my journey.

This work has been supported by Estonian Research Council (institutional research funding IUT19-18 and grant PRG805), European Union through the European Regional Development Fund (Project No. 2014–2020.4.01.15-0012) and H2020-MSCA-RISE-2016 (Grant EU734791). This work has also been partially supported by TUT Institutional Development Program for 2016–2022 Graduate School in Clinical medicine receiving funding from the European Regional Development Fund under program ASTRA 2014–2020.4.01.16-0032 in Estonia.

Kudos to ChatGPT4.0 for correcting grammar and finding synonyms.

I acknowledge the wise words of Stanley B. Prusiner:

*Neuroscience is by far the most exciting branch of science because the brain is the most fascinating object in the universe. Every human brain is different - the brain makes each human unique and defines who he or she is.*

## Abstract

### The Role of Enhancers in the Regulation of Brain-Derived Neurotrophic Factor Transcription

Genes make up a remarkably small part of our total DNA - about 2% of our genome is responsible for protein production. Rough estimates based on GWAS studies suggest that approximately 90% of disease-related SNPs are found in the non-coding genome. Enhancer regions, elements within the non-coding DNA, control gene activation according to the cell type, developmental stage and stimuli. It is estimated that there are hundreds of thousands of enhancers in the genome, but so far, the target genes of most enhancers, and vice versa, remain unknown. This also applies to brain-derived neurotrophic factor (BDNF), a neurotrophin that plays a fundamental role in the developing and adult nervous system, contributing to neuronal survival, differentiation, and synaptic plasticity.

The expression of *Bdnf* gene is a result of a complex transcriptional regulation: each of the 5' exon has a distinct promoter, regulation of which is dependent on various stimuli and transcription factors. While the proximal regulatory regions of the *Bdnf* gene have been well described, only few studies have investigated the functional role of enhancers regulating *Bdnf* expression. This is a critical knowledge gap considering that alterations in enhancer regions are increasingly associated with diseases. Additionally, studies from our and other laboratories suggest that the stimulus-dependent expression of the *Bdnf* gene is regulated by several yet undescribed enhancers. Accordingly, the aim of the current thesis was to discover and characterize distal regulatory regions that participate in the stimulus-dependent expression of *Bdnf* in both neuronal and non-neuronal cells. As a result, the studies underlying current thesis have described the endogenous function of three distinct enhancer regions of *Bdnf* gene. Specifically:

- I. We studied an evolutionarily conserved intronic enhancer region located 3 kb downstream of *Bdnf* that regulates the basal, neuronal activity and BDNF-TrkB-dependent expression of exon I-, II-, and III-containing *Bdnf* transcripts in rodent neurons. The +3 kb enhancer binds numerous transcription factors, and the activity of the enhancer is regulated by CREB, AP1, and bHLH family transcription factors.
- II. We described the first non-neuronal enhancer of *Bdnf* gene. A region 840 kb upstream of *Bdnf* regulates the catecholamine-dependent expression of *Bdnf* in rodent astrocytes. This regulatory mechanism is orchestrated by CREB and AP1 family transcription factors. Importantly, disruption of the -840 kb region has been previously described to cause downregulation of BDNF expression in humans and mice, resulting in phenotypic abnormalities similar to those of BDNF knockout animals.
- III. We revisited the previously described MEF2-binding enhancer of *Bdnf*. We show for the first time that the enhancer 4.8 kb upstream of *Bdnf* is important for the endogenous expression of *Bdnf* in hippocampal neurons. The -4.8 kb enhancer regulates the first cluster of *Bdnf* transcripts after neuronal activity in hippocampal neurons. Additionally, we demonstrate that MEF2 transcription factors exert multifaceted control over the expression of *Bdnf* via both proximal and distal regulatory regions – a regulation dependent on stimuli and brain region.

Collectively, our results elucidate the regulation of the *Bdnf* gene and could provide valuable insights into the development of BDNF-related therapeutic applications.

## Lühikokkuvõte

### Enhanseralade roll aju-päritolu neurotroofse teguri transkriptsiooni regulatsioonis

Geenid moodustavad märkimisväärselt väikese osa kogu meie DNA-st – umbes 2% meie genoomist vastutab valkude tootmise eest. Ülegenoomilised uuringud näitavad, et ligikaudu 90% haigus-seoselistest ühe nukleotiidi muutustest leidub just mittekodeerivas genoomis. Enhanseralad, mittekodeeriva DNA elemendid, kontrollivad geenide aktiveerimist vastavalt rakutüübile, arengufaasile ja stiimulile. Hinnanguliselt on genoomis sadu tuhandeid enhanseralasid, kuid seni on enamiku enhanserite sihtmärkgeenid, ja ka vastupidiselt, teadmata. See kehtib ka käesoleva töö keskmes oleva geeni, aju-päritolu neurotroofse teguri (*Bdnf*), kohta. BDNF on neurotrofiinide perekonda kuuluv valk, mis mängib olulist rolli arenevas ja täiskasvanud närvisüsteemis, aidates kaasa neuronite ellujäämisele, diferentseerumisele ja sünaptilisele plastilisusele.

BDNF-i ekspressioon on keerulise transkriptsioonilise regulatsiooni tulemus: igal 5' eksonil on eraldiseisev promootor, mille aktiveerimine sõltub erinevatest stiimulitest ja transkriptsiooniteguritest. Kuigi *Bdnf* geeni promootoreid ja nende regulatsiooni on palju uuritud, on vähesed uuringud keskendunud *Bdnf* geeni enhanseralade kirjeldamisele. Seda on aga äärmiselt oluline uurida, sest enhanserid vastutavad geenide ajalis-ruumilise ekspressiooni eest ning on üha enam seostatud haigustega. Lisaks näitavad meie ja teiste laborite uuringud, et *Bdnf* geeni stiimul-sõltuvat ekspressiooni reguleerivad potentsiaalselt mitmed veel kirjeldamata enhanseralad. Seeläbi oli käesoleva doktoritöö eesmärk leida ja kirjeldada enhanseralad, mis osalevad *Bdnf* geeni stiimul-sõltuvas ekspressioonis nii neuronaalsetes kui ka mitte-neuronaalsetes rakkudes. Tulemusena kirjeldab käesolev doktoritöö kolme uue *Bdnf* geeni enhanserala asukoha ja endogeense funktsiooni:

- Kirjeldasime *Bdnf* geenist 3 kb allavoolu asuva evolutsiooniliselt konserveerunud introonse enhanserala, mis reguleerib *Bdnf* eksonite I, II ja III sisaldavate transkriptide baas-, neuronaalset aktiivsust ja BDNF-TrkB-sõltuvat ekspressiooni näriliste neuronites. Näitasime, et +3 kb enhanserala seob mitmeid erinevaid transkriptsioonitegureid ning ala aktiivsust reguleerivad CREB, AP1 ja bHLH perekonna transkriptsioonitegurid.
- Kirjeldasime esimese *Bdnf* geeni enhanserala mitteneuronaalses rakkudes. *Bdnf* geenist 840 kb ülavoolu asuv piirkond reguleerib katehoolamiinidest sõltuvat *Bdnf* geeni ekspressiooni näriliste astrotsüütides. Seda mehhanismi vahendavad CREB ja AP1 perekonna transkriptsioonitegurid. Varasemalt on kirjeldatud, et -840 kb enhanserala muutused põhjustavad nii inimestel kui ka hiirtel BDNF tasemete langust, mille tulemuseks on BDNF *knockout* loomadele sarnased fenotüübilised iseärasused.
- Uuendasime *Bdnf* geenist 4.8 kb ülavoolu asuva ja MEF2 transkriptsioonitegureid siduva enhanserala kirjeldust. Näitasime, et -4.8 kb enhanser reguleerib neuronaalsest aktiivsusest sõltuvat *Bdnf* geeni ekspressiooni hipokampaalsetes neuronites. Lisaks kirjeldasime, et MEF2 transkriptsioonitegurid kontrollivad *Bdnf* geeni ekspressiooni nii promootorite kui ka enhanserite kaudu - regulatsioon, mis sõltub nii stiimulitest kui ka ajupiirkonnast.

Kokkuvõtvalt, meie tulemused selgitavad nii *Bdnf* geeni reguleerimist kui ka pakuvad väärtuslikke teadmisi BDNF-ga seotud terapeutiliste rakenduste arendamiseks.

# Appendix 1

## Publication I

Tuvikene J, Esvald EE\*, Rähni A\*, Uustalu K\*, Zhuravskaya A, **Avarlaid A**, Makeyev EV, Timmusk T

Intronic enhancer region governs transcript-specific *Bdnf* expression in rodent neurons  
Elife. 2021;10:e65161. doi:10.7554/eLife.65161.





# Intronic enhancer region governs transcript-specific *Bdnf* expression in rodent neurons

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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* 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 mouse and rat 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.

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**Competing interest:** See page 21

**Funding:** See page 22

**Received:** 25 November 2020

**Accepted:** 08 February 2021

**Published:** 09 February 2021

**Reviewing editor:** Anne E. West, Duke University School of Medicine, United States

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## 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*).

Rodent *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 humans (*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., 2016; Maynard et al., 2018; 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 12,000 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 is 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.

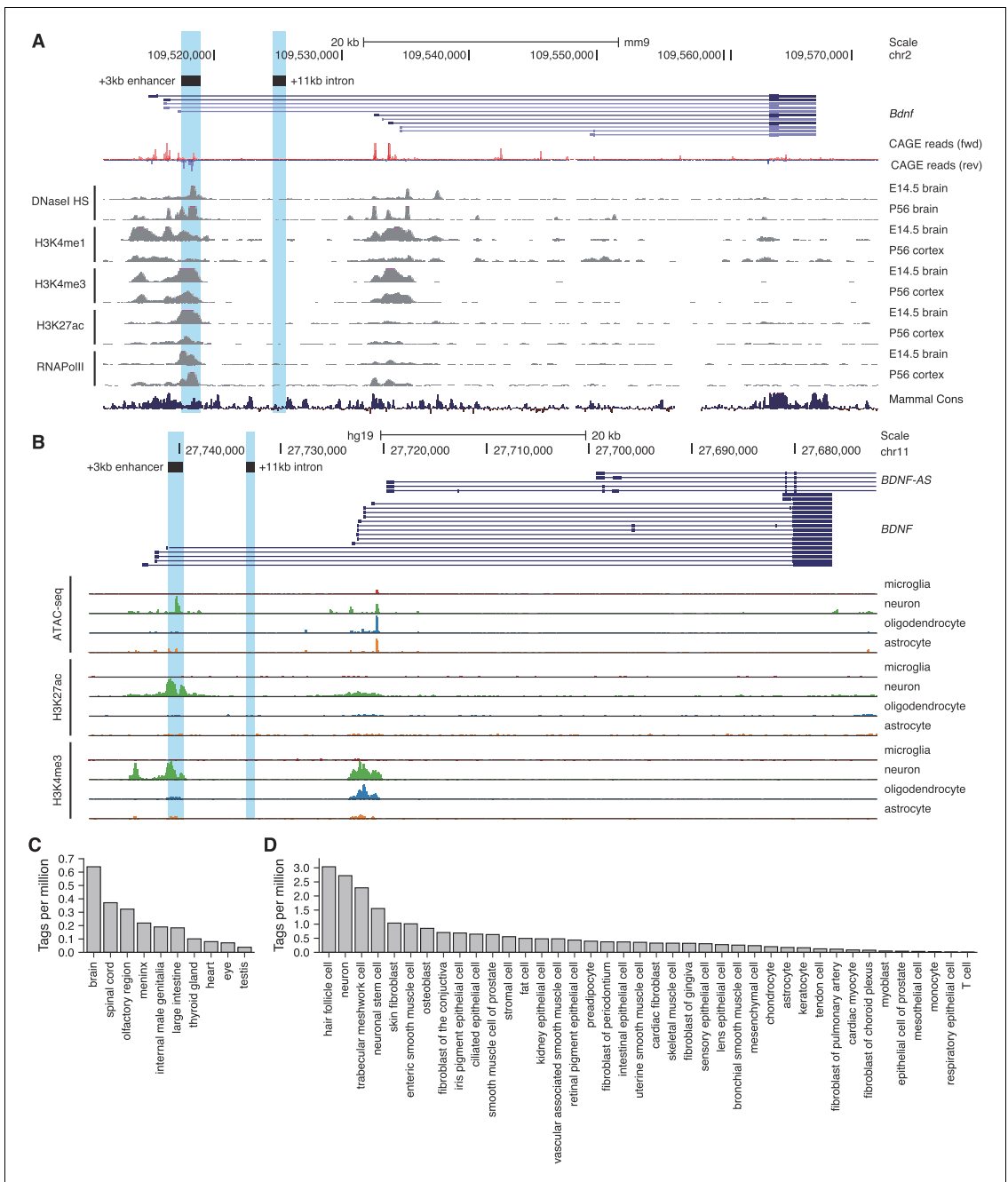
## Results

### The *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 chromatin immunoprecipitation sequencing (ChIP-seq) data from the ENCODE project and transcription start site (TSS) data from the FANTOM5 project, 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., 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 (Figure 1—figure supplement 1). This suggests that the +3 kb enhancer region might be active mainly in neural tissues in late prenatal and early postnatal life.

To investigate whether the +3 kb region might also function as an enhancer in rat, we analyzed published ATAC-seq and RNA-seq data for rat cortical and hippocampal neurons (Carullo et al., 2020). The ATAC-seq analysis was consistent with open chromatin structure of the +3 kb region, and the RNA-seq revealed possible eRNA transcription from the antisense strand (Figure 1—figure supplement 2). Unfortunately, the expression of the sense-strand eRNA could not be assessed since the *Bdnf* pre-mRNA is transcribed in the same direction, masking the sense-strand eRNA signal.



**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 project (all tissues and cell types), and (B) open chromatin (ATAC-seq) and ChIP-seq in different human brain cell types  
*Figure 1 continued on next page*

Figure 1 continued

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) enhancer RNA (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 cell types (D). Only tissue and cell types with non-zero eRNA expression are shown.

The online version of this article includes the following figure supplement(s) for figure 1:

**Figure supplement 1.** The +3 kb enhancer region shows brain-specific H3K27ac histone modification.

**Figure supplement 2.** The +3 kb enhancer region shows open chromatin structure and enhancer RNA (eRNA) expression in rat cultured neurons.

Together, open chromatin and possible eRNA expression suggest that the +3 kb region could be an enhancer in rat neurons.

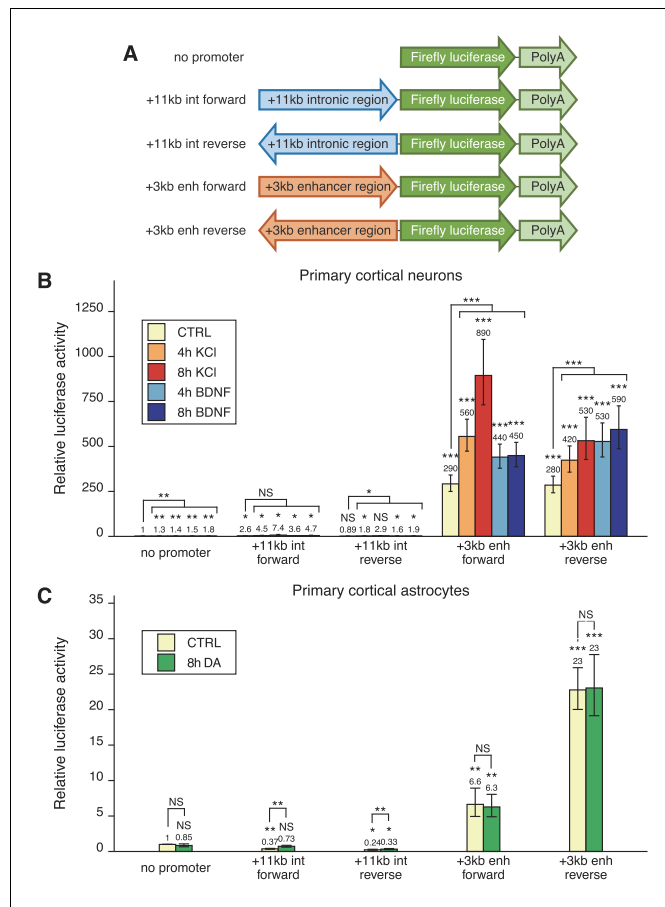
To further investigate which cell types the +3 kb region could be active in human in vivo, we used data from a recently published human brain cell-type-specific ATAC-seq and ChIP-seq experiments (**Nott et al., 2019**) (**Figure 1B**). We found that the +3 kb region shows remarkable neuron specificity, 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 TSSs from FANTOM5 project and summarizes eRNA transcription levels based on various tissue and cell types. We found that in humans 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.

### The +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 is well known that enhancer regions can initiate RNA polymerase II-dependent bidirectional transcription of eRNAs (**Kim and Shiekhattar, 2015; Nord and West, 2020; Sartorelli and Lauberth, 2020**) and that the expression of these eRNAs 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 an ~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.



**Figure 2.** The +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 (relative 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. Two days post-transfection, neurons were left untreated (CTRL) or treated with 25 mM KCl (with 5  $\mu$ M D-APV) or 50 ng/ml brain-derived neurotrophic factor (BDNF) for the indicated time (B); astrocytes were treated with 150  $\mu$ M 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).

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.

### 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 (relative to the rat *Bdnf* gene) downstream of *Bdnf* promoter-driven luciferase expression cassette (Figure 3A).

First, we transfected rat cultured cortical neurons with constructs containing *Bdnf* promoter I or 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 approximately threefold 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 approximately threefold and KCl- and BDNF-induced activity levels approximately twofold. The +11 kb intronic region failed to potentiate the activity of *Bdnf* promoters I and IV.

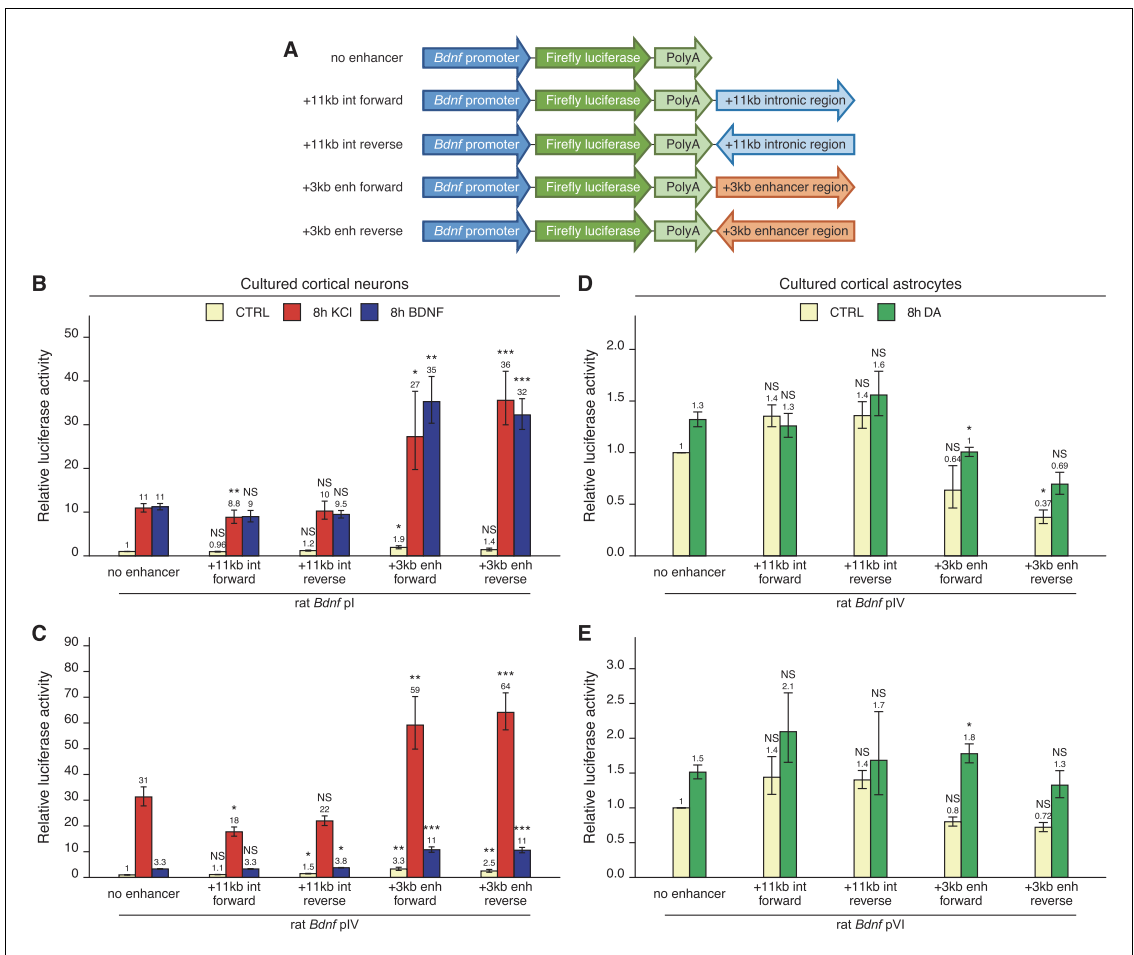
Cortical astrocytes preferentially express *Bdnf* transcripts containing 5' exons IV and VI (Koppel et al., 2018). Notably, the +3 kb enhancer failed to increase the activity of *Bdnf* promoters IV and VI in unstimulated astrocytes and had little effect on the response of these promoters to dopamine (Figure 3D, E).

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.

### The +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 catalytically inactive Cas9 (dCas9) fused with Krüppel-associated box domain (dCas9-KRAB, CRISPRi), or 8 copies of VP16 activator 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 notably 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, although these effects were not statistically significant (Figure 4A–C, middle panel). In contrast, no notable effect was seen for basal levels of *Bdnf* exon IV-, VI-, and IXa-containing transcripts (Figure 4D–F, 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

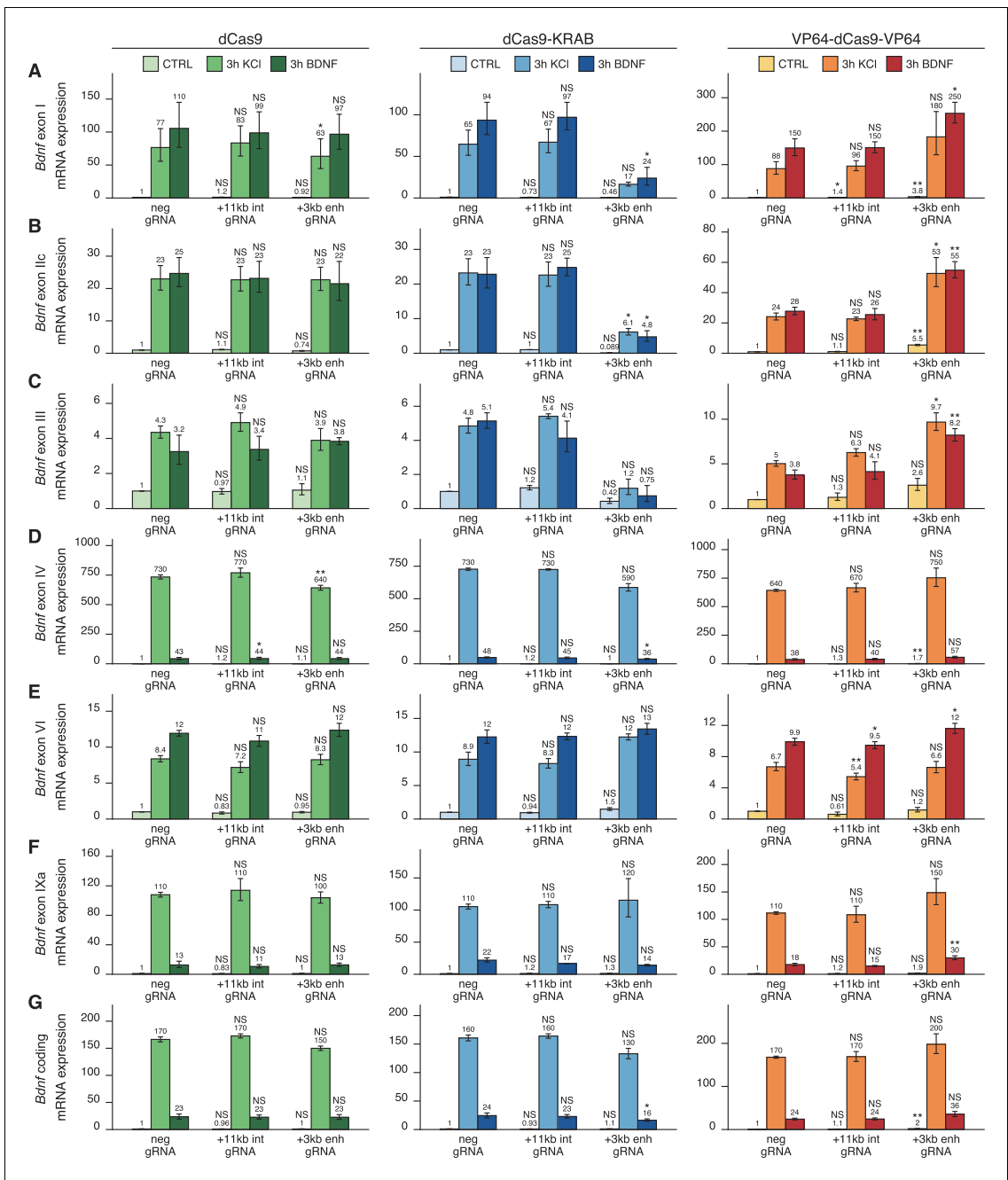


**Figure 3.** The +3 kb enhancer region potentiates the activity of *Bdnf* promoters in luciferase reporter assay in rat cortical neurons but not in astrocytes. (A) A diagram of the luciferase reporter constructs used in this experiment, with a *Bdnf* promoter in front of the firefly luciferase coding sequence and the +3 kb enhancer or +11 kb intronic region in either forward or reverse orientation (relative 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  $\mu$ M D-APV) or 50 ng/ml brain-derived neurotrophic factor (BDNF) for 8 hr (B, C); astrocytes were treated with 150  $\mu$ M dopamine (DA) or respective volume of vehicle (CTRL) for 8 hr (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).

*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 notable effect on any of the *Bdnf* transcripts (Figure 4A–F, middle panel).

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





**Figure 4.** The +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 activator domain (VP64-dCas9-VP64, right panel, orange), Figure 4 continued on next page

Figure 4 continued

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 gRNAs directed to +11 kb intronic region (+11 kb int gRNA). Transduced neurons were left untreated (CTRL) or treated with 50 ng/ml brain-derived neurotrophic factor (BDNF) or 25 mM KCl (with 5  $\mu$ M D-APV) for 3 hr at 8 DIV. Expression levels of different *Bdnf* transcripts (A-F) or total *Bdnf* (G) 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).

The online version of this article includes the following figure supplement(s) for figure 4:

**Figure supplement 1.** The +3 kb enhancer shows stimulus-dependent enhancer RNA (eRNA) transcription in neurons.

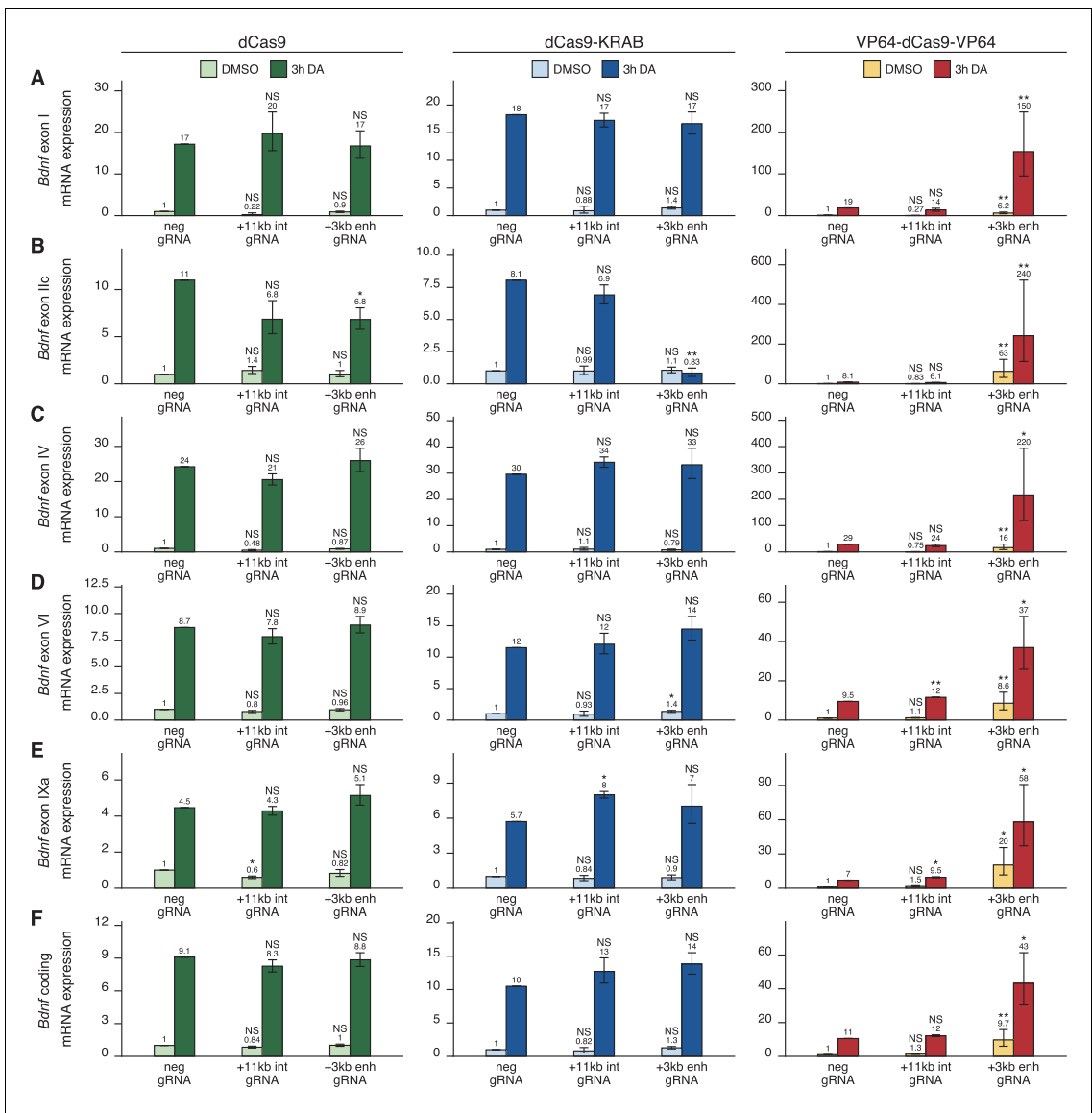
**Figure supplement 2.** The +3 kb enhancer shows membrane depolarization-induced enhancer RNA (eRNA) transcription in rat cultured cortical and hippocampal neurons.

was also seen for *Bdnf* exon IV- and IXa-containing transcripts (**Figure 4D, F**, 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 dramatic effect on the expression of any of the *Bdnf* transcripts, despite some minor changes reaching statistical significance (**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 most *Bdnf* transcripts (**Figure 5A and C–F**, left panel), although a slight decrease could be seen for exon IIc-containing transcripts in cells treated with dopamine (**Figure 5B**). Targeting CRISPRi to the +3 kb enhancer region in astrocytes did not decrease 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 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 cultured 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 (**Figure 4—figure supplement 1**). 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. We also analyzed RNA-seq data from **Carullo et al., 2020** and confirmed the membrane depolarization-dependent induction of +3 kb antisense eRNA expression in rat cultured cortical and hippocampal neurons (**Figure 1—figure supplement 2**, **Figure 4—figure supplement 2**). 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 (**Figure 5—figure supplement 1**), 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 enhancer of the *Bdnf* gene 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.** The +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 activator 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 the +11 kb intronic region (+11 kb int gRNA). Transduced astrocytes were treated with vehicle (CTRL) or with 150  $\mu$ M dopamine (DA) for 3 hr at 15 DIV. Expression levels of different *Bdnf* transcripts (A-E) or total *Bdnf* (F) 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  
 Figure 5 continued on next page

Figure 5 continued

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).

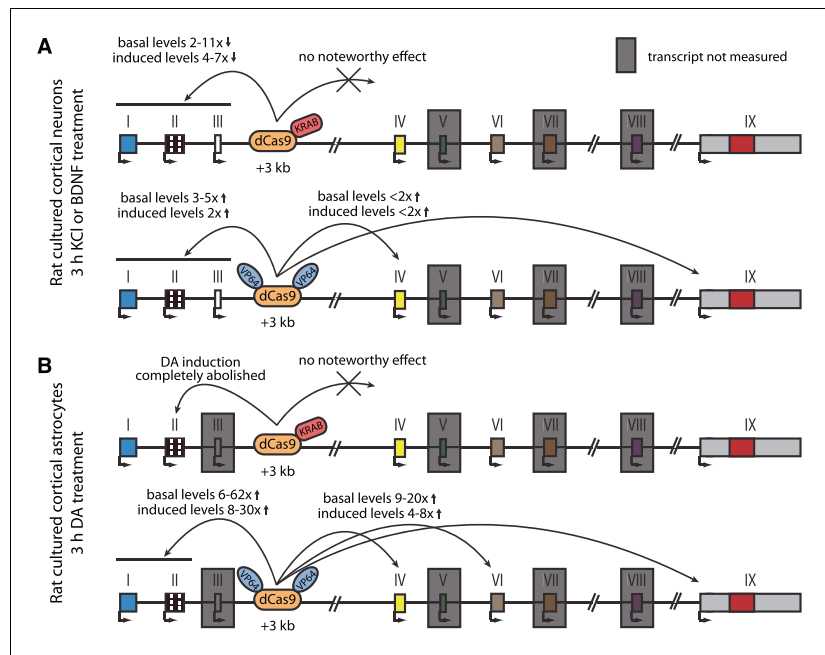
The online version of this article includes the following figure supplement(s) for figure 5:

**Figure supplement 1.** The +3 kb enhancer shows lower enhancer RNA (eRNA) expression in astrocytes than in neurons.

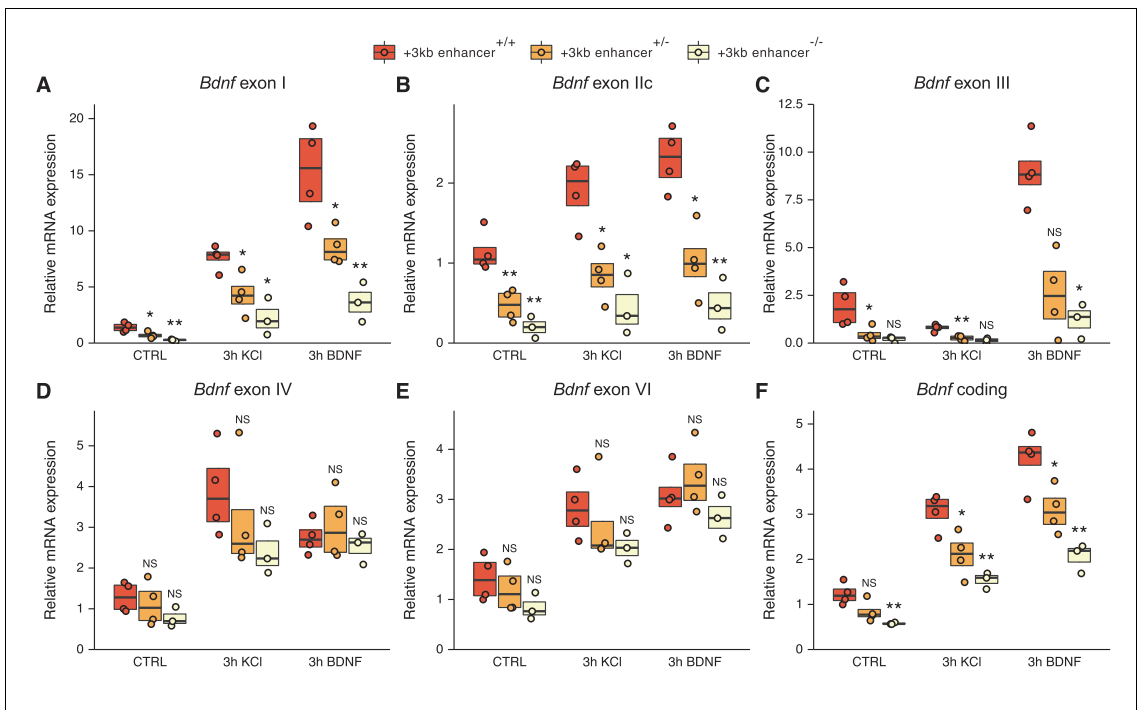
### Deletion of the +3 kb enhancer region in mouse embryonic stem cell-derived neurons decreases the expression of *Bdnf* transcripts starting from the first cluster of exons

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 (mESC) engineered to express the pro-neural transcription factor Neurogenin2 from a doxycycline-inducible promoter. We selected single-cell clones containing the desired deletion (Figure 7—figure supplement 1), differentiated them into neurons (Figure 7—figure supplement 2, Ho et al., 2016; Thoma et al., 2012; Zhang et al., 2013) by the addition of doxycycline, treated the stem cell-derived neurons with BDNF or KCl, and measured the expression of different *Bdnf* transcripts using RT-qPCR (Figure 7).

The deletion of the +3 kb enhancer region strongly decreased both the basal and stimulus-dependent expression levels of *Bdnf* exon I-, II-, 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



**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 Figures 4 (A) and 5 (B). Different *Bdnf* exons are shown with boxes, and 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 gray box around the respective 5' exon.



**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 brain-derived neurotrophic factor (BDNF) or 25 mM KCl together with 25  $\mu$ M D-APV for 3 hr. The expression levels of different *Bdnf* transcripts (A-E) or total *Bdnf* (F) 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) were 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).

The online version of this article includes the following figure supplement(s) for figure 7:

**Figure supplement 1.** Verification of the deletion of the +3 kb enhancer region in mouse embryonic stem cell (mESC) clones.

**Figure supplement 2.** Characterization of mouse embryonic stem cell (mESC) differentiation into neurons.

*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 could explain why it was not detected in our CRISPRi and CRISPRa experiments in cultured neurons. The levels of *Bdnf* exon IXa-containing transcripts were too low to measure reliably (data not shown). The deletion of the +3 kb enhancer region 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.

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

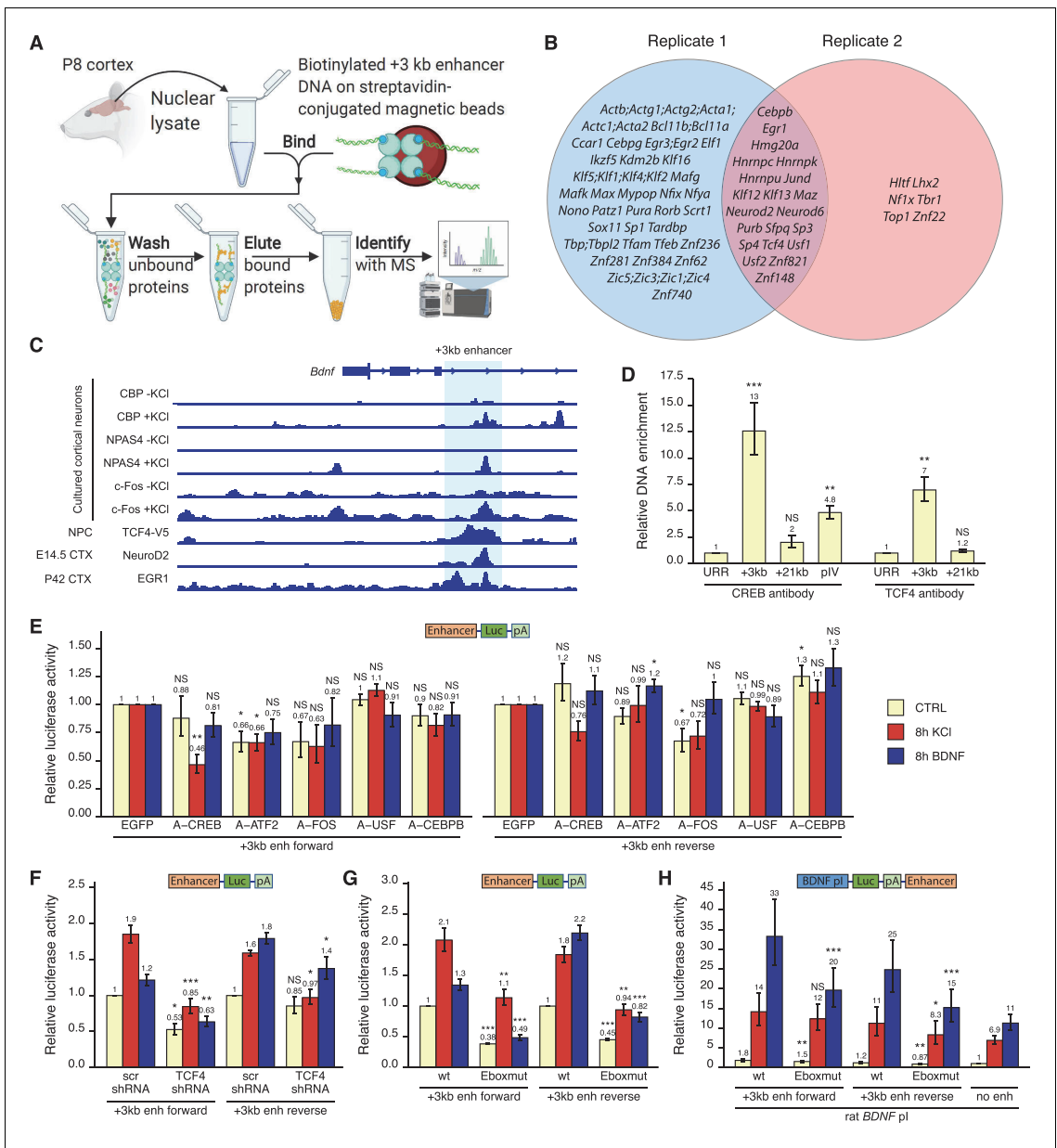
To investigate the molecular mechanisms that control the activity of the +3 kb enhancer region, we used in vitro DNA pulldown assay with 8-day-old rat cortical nuclear lysates coupled with mass spectrometric analysis to determine the transcription factors that bind to the +3 kb enhancer region (Figure 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 (Figure 8—figure supplement 1). 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 (Figure 8—figure supplement 1) and CBP binding in cultured cortical neurons (Figure 8C), we first investigated 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 pro-neural 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, for example, TCF4, to bind DNA (Massari and Murre, 2000; Ravanpay and Olson, 2008), we verified the 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). The effect of different dominant negative proteins was slightly lower when the +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 the +3 kb enhancer region.

Finally, we elucidated the role of E-box-binding proteins in the regulation of the +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).



**Figure 8.** Various transcription factors, including CREB, AP-1 proteins, and E-box-binding transcription factors, regulate the activity of the +3 kb enhancer region. (A) Schematic overview of the in vitro DNA pull-down assay to determine transcription factors binding to the +3 kb enhancer region. The illustration was created with BioRender.com. (B) Gene names of the transcription factors identified in the in vitro DNA pull-down assay in two biological replicates of postnatal day 8 (P8) rat cortices. Semicolon between gene names indicates uncertainty in the peptide to protein assignment between the genes 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. The +21 kb region (downstream of the *Bdnf* exon VII) was used as a Figure 8 continued on next page

Figure 8 continued

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  $\mu$ M D-APV) or 50 ng/ml brain-derived neurotrophic factor (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 enhanced green fluorescent protein (EGFP)-encoding plasmid and the respective +3 kb enhancer construct (E), relative to the luciferase activity in untreated cells co-transfected with control shRNA (scr) and the respective +3 kb enhancer construct (F), relative to the luciferase activity in untreated cells transfected with the respective wild-type (wt) +3 kb enhancer construct (G), or relative to the 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, CREB antibody], n = 3 [D, TCF4 antibody], n = 5–6 [E], n = 4–5 [F], n = 4 [G], and 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 the respective +3 kb enhancer construct and EGFP (E), scr shRNA (F), or the 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).

The online version of this article includes the following figure supplement(s) for figure 8:

**Figure supplement 1.** The +3 kb enhancer region binds various transcription factors in human cell lines.

Collectively, we have identified numerous transcription factors that potentially regulate the activity of the +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. Spatial clustering of *Bdnf* exons seems to be conserved in vertebrates (Keifer, 2021), with a similar genomic organization observed in frog (Kidane et al., 2009), chicken (Yu et al., 2009), zebrafish (Heinrich and Pagtakhan, 2004), rodents (Aid et al., 2007), and human (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 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 rodent 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, respectively, 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.

Recently, it has been shown that other distal enhancer regions form chromatin loops with the *Bdnf* first cluster of exons and the +3 kb enhancer region (Beagan et al., 2020). Similarly, long-distance interactions with the +3 kb enhancer region have been reported in neurons isolated from the human brain (Nott et al., 2019). Furthermore, CTCF and RAD21, both important regulators of the chromatin 3D structure (Rowley and Corces, 2018), seem to bind near the +3 kb enhancer region (Figure 8—figure supplement 1). Therefore, it is possible that the +3 kb enhancer could partially mediate its effect on *Bdnf* gene expression by regulating the higher-order chromatin structure in the *Bdnf* gene locus by bringing together distal enhancer regions and *Bdnf* promoters from the first cluster of exons. Further work is necessary to determine the role of the +3 kb enhancer region in the regulation of higher order chromatin structure.

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 IIc-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 IIc-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 it directly, our data does not support the notion that the +3 kb region is an active repressor in non-neuronal cells, for example, 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 mRNAs of 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* mRNA and protein 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

### 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 (Esvold et al., 2020). The cells were grown in Neurobasal A (NBA) medium (Gibco, Waltham, MA) containing 1× B27 supplement (Gibco, Waltham, MA), 1 mM L-glutamine (Gibco, Waltham, MA), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Gibco, Waltham, MA) or 100 µg/ml Primocin (Invivogen, San Diego, CA) instead of penicillin/streptomycin at 37°C in 5% CO<sub>2</sub> 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 (final concentration 10 µM, Sigma-Aldrich, Saint Louis, MO) was added with the change of the medium.

### 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<sup>2</sup> tissue culture flasks in Dulbecco's Modified Eagle Medium (DMEM with high glucose, PAN Biotech, Aidenbach, Germany) supplemented with 10% fetal bovine serum (PAN Biotech, Aidenbach, Germany) and 100 U/ml penicillin and 0.1 mg/ml streptomycin (Gibco, Waltham, MA) at 37°C in 5% CO<sub>2</sub> 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, Goettingen, Germany) for 17–20 hr 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× phosphate-buffered saline (PBS). Astrocytes were detached from the flask with trypsin-EDTA solution (0.25% Trypsin-EDTA [1×], Gibco, Waltham, MA) diluted four times with 1× PBS at 37°C for 3–5 min. Trypsinized astrocytes were collected in supplemented DMEM and centrifuged at 200 × g for 6 min. 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, Saint Louis, MO) in Milli-Q. At 9 DIV, the whole medium was replaced with fresh supplemented DMEM.

### Drug treatments

At 7 DIV, cultured neurons were pre-treated with 1 µM tetrodotoxin (Tocris, Bristol, UK) 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, London, UK) or with a mixture of 25 mM KCl and 5 µM N-Methyl-D-aspartate receptor antagonist D-2-amino-5-phosphopentanoic acid (D-APV, Cayman Chemical Company, Ann Arbor, MI) 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, Bristol, UK) to study the regulation of the *Bdnf* gene by catecholamines or 0.15% dimethyl sulfoxide (Sigma-

Aldrich, Saint Louis, MO) as a vehicle control in fresh serum-free and antibiotics-free DMEM (DMEM with high glucose, PAN Biotech, Aidenbach, Germany).

### 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, Waltham, MA) and cloned into pGL4.15 vector (Promega, Madison, WI) 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 (relative 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-SR $\alpha$ -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, Madison, WI) and luciferase signals were measured with Dual-Glo Luciferase assay kit (Promega, Madison, WI) 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.

### 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 equal amounts of functional viral particles were used for transduction in the following experiments. The efficiency of viral transduction was at least 90–95% based on EGFP expression in transduced cells.

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, Hilden, Germany) using on-column DNA removal with RNase-Free DNase Set (Qiagen, Hilden, Germany). RNA concentration was measured with BioSpec-nano spectrophotometer (Shimadzu Biotech, Kyoto, Japan). cDNA was synthesized from equal amounts of RNA with Superscript III or Superscript IV reverse transcriptase (Invitrogen, Waltham, MA, USA) using oligo(dT)<sub>20</sub> or a mixture of oligo(dT)<sub>20</sub> and random hexamer primer (ratio 1:1, Microsynth, Balgach, Switzerland). 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 file 1**.

All qPCR reactions were performed in 10  $\mu$ l volume in triplicates with 1  $\times$  HOT FIREpol EvaGreen qPCR Mix Plus (Solis Biodyne, Tartu, Estonia) and primers listed in **Supplementary file 1** on

LightCycler 480 PCR instrument II (Roche, Basel, Switzerland). 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.

### Bioinformatic analysis

RNA-seq and ATAC-seq data from [Carullo et al., 2020](#) was obtained from Sequence Read Archive (accession numbers [SRP261474](#) and [SRP261642](#)) and processed as described in [Sirp et al., 2020](#) with minor modifications. Trimming was performed with BBDuk using the following parameters: `ktrim=r k=23 mink=11 hdist=1 tbo qtrim=lr trimq=10 minlen=50`.

For RNA-seq data, STAR aligner (version 2.7.3a, [Dobin et al., 2013](#)) was used to map the reads to the Rn6 genome. Featurecounts (version 2.0.0, [Liao et al., 2014](#)) was used to assign reads to genes (annotation from Ensembl, release 101), and a custom-made SAF file was used to count reads mapping to +3 kb antisense eRNA (coordinates chr3:100771209–100772077). Counts were normalized using DESeq2 R package (version 1.28.1, [Love et al., 2014](#)) and visualized using ggplot2 R package (version 3.3.2, [Wickham, 2016](#)).

For ATAC-seq data, reads were mapped to the Rn6 genome with Bowtie2 (version 2.3.5.1, [Langmead and Salzberg, 2012](#)) using the following parameters: `--local --very-sensitive-local -X 3000`. The resultant SAM files were converted to BAM, sorted and indexed using Samtools (version 1.9, [Li et al., 2009](#)).

For both RNA-seq and ATAC-seq, aligned reads of biological replicates were merged using Samtools, converted to bigWig format using bamCoverage (version 3.3.0, [Ramírez et al., 2016](#)) with CPM normalization, and visualized using Integrative Genomics Viewer ([Robinson et al., 2011](#)).

### Mouse embryonic stem cells

A2Lox mESCs were a kind gift from Michael Kyba. The cells were authenticated based on their ability to form stable G418-resistant colonies after transfecting doxycycline-treated cultures with the p2Lox plasmid (Addgene plasmid #34635, [Iacovino et al., 2011](#)). The cells were tested negative for mycoplasma using PCR Mycoplasma Test Kit I/C (PromoCell, Heidelberg, Germany). A2Lox mESC line containing doxycycline-inducible Neurogenin2 transgene (Zhuravskaya et al., in preparation) was generated using recombination-mediated cassette exchange procedure ([Iacovino et al., 2011](#)). mESCs were grown in 2i media as described in [Iacovino et al., 2011](#) and [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 file 2](#)) were cloned into pX330 vector (Addgene plasmid #42230). A2Lox-Neurogenin2 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-Aldrich, Saint Louis, MO) 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. qPCR results were normalized to the levels of the *Sox2* genomic locus, and the copy number of each region in wild-type cells was set as 2. Normalized copy numbers in recombinant clones were rounded to the nearest integer. All primers used for genotyping are listed in [Supplementary file 1](#). Cell clones containing no deletion, heterozygous or homozygous deletion of the core conserved enhancer region, together with intact distal flanking regions, were used for subsequent analysis.

Selected mESC clones were differentiated into neurons as follows. Cells were plated on 12-well plates coated with Matrigel (Gibco, Waltham, MA) at a density of ~25,000 cells per well in N2B27 media (1:1 DMEM F12-HAM and Neurobasal mixture, 1× N2, 1× B27 with retinoic acid, 1× penicillin-streptomycin, 1 µg/ml laminin, 20 µg/ml insulin, 50 µM L-glutamine) supplemented with 0.1 M β-mercaptoethanol (Sigma-Aldrich, Saint Louis, MO) and 2 µg/ml doxycycline (Sigma-Aldrich, Saint Louis, MO). After 2 days, the whole media was changed to N2B27 media containing 200 µM ascorbic acid (Sigma-Aldrich, Saint Louis, MO) 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, London, UK), or KCl for 3 hr. All treatments were added together with 25  $\mu$ M D-APV (Alfa Aesar, Kandel, Germany). After treatment, the cells were lysed and RNA was extracted using EZ-10 DNAaway RNA Mini-Prep Kit (Bio Basic inc, Markham, Canada). cDNA was synthesized using Superscript IV (Thermo Fischer, Waltham, MA), and qPCR was performed with HOT FIREPol EvaGreen qPCR Mix Plus (Solis Biodyne, Tartu, Estonia) or qPCRBIO SyGreen Mix Lo-ROX (PCR Biosystems Ltd, London, UK) on LightCycler 96 (Roche, Basel, Switzerland). The levels of *Cnot4* mRNA expression were used for normalization. RT-qPCR was also used to characterize differentiation of A2Lox-Neurogenin2 parental cells into neurons (**Figure 7—figure supplement 2**). All primers used in the RT-qPCR are listed in **Supplementary file 1**.

### In vitro DNA pulldown mass spectrometry

The 855 bp region of the +3 kb enhancer and +11 kb intronic region were amplified with PCR using HotFirePol polymerase (Solis Biodyne, Tartu, Estonia) and primers listed in **Supplementary file 1**, with the reverse primers having a 5' biotin modification (Microsynth, Balgach, Switzerland). PCR products were purified using DNA Clean and Concentrator–100 kit (Zymo Research, Irvine, CA) 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-day-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). Also, 2 ml of ice-cold cytoplasmic lysis buffer (10 mM HEPES, pH 7.9 [adjusted with NaOH], 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.5% NP-40, 300 mM sucrose, 1x cOmplete Protease Inhibitor Cocktail [Roche, Basel, Switzerland], and phosphatase inhibitors as follows: 5 mM NaF [Fisher Chemical, Pittsburgh, PA], 1 mM beta-glycerophosphate [Acros Organics, Pittsburgh, PA], 1 mM Na<sub>3</sub>VO<sub>4</sub> [ChemCruz, Dallas, TX], and 1 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub> [Fisher Chemical, Pittsburgh, PA]) 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 min with occasional inverting. Next, the lysate was transferred to a 100  $\mu$ m nylon cell strainer (VWR) to remove tissue debris and the flow-through was centrifuged at 2600  $\times$  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<sub>2</sub>, 0.1 mM EDTA, 2.5% glycerol, 1x cOmplete Protease Inhibitor Cocktail [Roche, Basel, Switzerland], and phosphatase inhibitors) and transferred to a new Eppendorf tube. To extract nuclear proteins, the pellet was rotated at 4°C for 30 min and finally centrifuged at 11,000  $\times$  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  $\mu$ l per pulldown reaction) were washed 2 times with 1 $\times$  binding buffer (BB, 5 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1 M NaCl, 0.05% Tween-20), resuspended in 2 $\times$  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 three times with 1 $\times$  BB. Finally, 400  $\mu$ g 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, Basel, Switzerland], and phosphatase inhibitors) were added and incubated with rotation at 4°C overnight. The next day the beads were washed three times with 1 $\times$  PBS, once with 100 mM NaCl and once with 200 mM NaCl. Bound DNA and proteins were eluted with 16 mM biotin (Sigma-Aldrich, Saint Louis, MO) in water (at pH 7.0) at 80°C for 5 min, and 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 file 3**. Custom R script (available at [Tuvikene, 2021](#)) 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' from <http://geneontology.org/> (obtained March 16, 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.

### 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, Burlington, MA) or TCF4 antibody (CeMines, Golden, CO) 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, Basel, Switzerland) and primers listed in **Supplementary file 1** on LightCycler 480 PCR instrument II (Roche, Basel, Switzerland). Primer efficiencies were determined by serial dilutions of input samples and were used for analyzing the results. Percentage of input enrichments was calculated for each region and IP, and data were log-transformed before statistical analysis.

ENCODE data of different ChIP-seq experiments were 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](#)).

### Statistical analysis

Sample size estimation was not performed, and randomization and blinding were not used. Statistical analysis was performed on data obtained from independent biological replicates – cultured primary cells obtained from pups of different litters, or different clones of mESCs. 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](#), and [Streiner and Norman, 2011](#).

### Acknowledgements

This work was supported by Estonian Research Council (institutional research funding IUT19-18 and grant PRG805), Norwegian Financial Mechanism (Grant EMP128), European Union through the European Regional Development Fund (Project No. 2014–2020.4.01.15-0012), H2020-MSCA-RISE-2016 (Grant EU734791), and the Biotechnology and Biological Sciences Research Council (BB/M001199/1, BB/M007103/1, and BB/R001049/1). This work has also been partially supported by 'TUT Institutional Development Program for 2016–2022' Graduate School in Clinical medicine receiving funding from the European Regional Development Fund under program ASTRA 2014–2020.4.01.16-0032 in Estonia. We thank Epp Väli and Andra Moistu for technical assistance, and Indrek Koppel and Priit Pruunsild for critical reading of the manuscript.

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### Additional information

#### Competing interests

Jürgen Tuvikene, Eli-Eelika Esvald, Annika Rähni, Tõnis Timmusk: is an employee of Protobios LLC. The other authors declare that no competing interests exist.

**Funding**

<b>Funder</b>	<b>Grant reference number</b>	<b>Author</b>
Estonian Research Council	IUT19-18	Jürgen Tuvikene Eli-Eelika Esvald Annika Rähni Kaie Uustalu Annela Avarlaid Tõnis Timmusk
Estonian Research Council	PRG805	Jürgen Tuvikene Eli-Eelika Esvald Annela Avarlaid Tõnis Timmusk
Norwegian Financial Mechanism	EMP128	Jürgen Tuvikene Eli-Eelika Esvald Annika Rähni Kaie Uustalu Tõnis Timmusk
European Regional Development Fund	2014-2020.4.01.15-0012	Jürgen Tuvikene Eli-Eelika Esvald Annika Rähni Kaie Uustalu Annela Avarlaid Tõnis Timmusk
H2020 Marie Skłodowska-Curie Actions	EU734791	Jürgen Tuvikene Eli-Eelika Esvald Anna Zhuravskaya Annela Avarlaid Eugene V Makeyev Tõnis Timmusk
Biotechnology and Biological Sciences Research Council	BB/M001199/1	Anna Zhuravskaya Eugene V Makeyev
Biotechnology and Biological Sciences Research Council	BB/M007103/1	Anna Zhuravskaya Eugene V Makeyev
Biotechnology and Biological Sciences Research Council	BB/R001049/1	Anna Zhuravskaya Eugene V Makeyev
European Regional Development Fund	ASTRA 2014-2020.4.01.16-0032	Jürgen Tuvikene Eli-Eelika Esvald Annela Avarlaid Tõnis Timmusk

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

**Author contributions**

Jürgen Tuvikene, Conceptualization, Data curation, Formal analysis, Supervision, Investigation, Visualization, Methodology, Writing - original draft, Writing - review and editing; Eli-Eelika Esvald, Conceptualization, Formal analysis, Investigation, Methodology, Writing - review and editing; Annika Rähni, Formal analysis, Investigation, Methodology, Writing - review and editing; Kaie Uustalu, Formal analysis, Investigation, Methodology; Anna Zhuravskaya, Supervision, Investigation, Methodology, Writing - review and editing; Annela Avarlaid, Formal analysis, Supervision, Investigation, Methodology, Writing - review and editing; Eugene V Makeyev, Conceptualization, Supervision, Funding acquisition, Methodology, Writing - review and editing; Tõnis Timmusk, Conceptualization, Supervision, Funding acquisition, Project administration, Writing - review and editing

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- Supplementary file 1. List of used primers.
- Supplementary file 2. List of gRNA targeting sequences.
- Supplementary file 3. Mass-spectrometry results of the in vitro DNA pulldown experiment. MaxQuant ProteinGroups tables of two biological replicates are shown in separate Excel sheets. Refer to the MaxQuant documentation (<http://coxdocs.org/doku.php?id=maxquant:table:proteingrouptable>) for detailed column descriptions.
- Transparent reporting form

**Data availability**

Mass-spectrometry results of the in vitro DNA pulldown experiment are provided in Supplementary file 3.

The following previously published datasets were used:

Author(s)	Year	Dataset title	Dataset URL	Database and Identifier
Kim T, Hemberg M, Gray JM, Kreiman G, Greenberg ME	2010	Widespread transcription at neuronal activity-regulated enhancers	<a href="https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE21161">https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE21161</a>	NCBI Gene Expression Omnibus, GSE21161
Nord AS, Visel A, Pennacchio LA	2013	Rapid and Pervasive Changes in Genome-Wide Enhancer Usage During Mammalian Development	<a href="https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE52386">https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE52386</a>	NCBI Gene Expression Omnibus, GSE52386
Malik AN	2014	Genome-wide identification and characterization of functional neuronal activity-dependent enhancers	<a href="https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE60192">https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE60192</a>	NCBI Gene Expression Omnibus, GSE60192
Dunn GI	2015	NeuroD2 ChIP-SEQ from embryonic cortex	<a href="https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE67539">https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE67539</a>	NCBI Gene Expression Omnibus, GSE67539
Brandsma JH, Moen MJ, Poot RA	2016	A protein interaction network of mental disorder factors in neural stem cells	<a href="https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE70872">https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE70872</a>	NCBI Gene Expression Omnibus, GSE70872
Sun Z, Sun M, He J, Xie H	2019	Genome-wide maps of EGR1 binding in mouse frontal cortex	<a href="https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE67482">https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE67482</a>	NCBI Gene Expression Omnibus, GSE67482
Carullo NVN, Phillips RA, Ianov L, Day JJ	2020	ATAC-seq datasets for chromatin accessibility quantification in "Enhancer RNAs predict enhancer-gene regulatory links and are critical for enhancer function in neuronal systems"	<a href="https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE150589">https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE150589</a>	NCBI Gene Expression Omnibus, GSE150589
Carullo NVN, Phillips RA, Ianov L, Day JJ	2020	RNA-seq datasets for enhancer RNA quantification in "Enhancer RNAs predict enhancer-gene regulatory links and are critical for enhancer function in neuronal systems"	<a href="https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE150499">https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE150499</a>	NCBI Gene Expression Omnibus, GSE150499



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## Appendix 2

### Publication II

**Avarlaid A**, Esvald EE, Koppel I, Parkman A, Zhuravskaja A, Makeyev EV, Tuvikene J<sup>#</sup>, Timmusk T<sup>#</sup>

An 840 kb distant upstream enhancer is a crucial regulator of catecholamine-dependent expression of the *Bdnf* gene in astrocytes

*Glia*. 2024;72(1):90-110. doi:10.1002/glia.24463



## RESEARCH ARTICLE

# An 840 kb distant upstream enhancer is a crucial regulator of catecholamine-dependent expression of the *Bdnf* gene in astrocytes

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## Funding information

Biotechnology and Biological Sciences Research Council, Grant/Award Numbers: BB/R001049/1, BB/V006258/1; Estonian Research Council, Grant/Award Numbers: IUT19-18, PRG805; European Regional Development Fund, Grant/Award Numbers: 2014-2020.4.01.15-0012, ASTRA 2014-2020.4.01.16-0032; HORIZON EUROPE Marie Skłodowska-Curie Actions, Grant/Award Number: EU734791

## Abstract

Brain-derived neurotrophic factor (BDNF) plays a fundamental role in the developing and adult nervous system, contributing to neuronal survival, differentiation, and synaptic plasticity. Dysregulation of BDNF synthesis, secretion or signaling has been associated with many neurodevelopmental, neuropsychiatric, and neurodegenerative disorders. Although the transcriptional regulation of the *Bdnf* gene has been extensively studied in neurons, less is known about the regulation and function of BDNF in non-neuronal cells. The most abundant type of non-neuronal cells in the brain, astrocytes, express BDNF in response to catecholamines. However, genetic elements responsible for this regulation have not been identified. Here, we investigated four potential *Bdnf* enhancer regions and based on reporter gene assays, CRISPR/Cas9 engineering and CAPTURE-3C-sequencing we conclude that a region 840 kb upstream of the *Bdnf* gene regulates catecholamine-dependent expression of *Bdnf* in rodent astrocytes. We also provide evidence that this regulation is mediated by CREB and AP1 family transcription factors. This is the first report of an enhancer coordinating the transcription of *Bdnf* gene in non-neuronal cells.

## KEYWORDS

AP1 transcription factors, astrocyte, BDNF, catecholamines, CREB, enhancer, neurotrophins

## 1 | INTRODUCTION

In recent decades, the study of astrocytes has garnered considerable attention. In addition to their supportive and protective roles, astrocytes contribute to the development and homeostasis of the nervous system (Mederos et al., 2018; Verkhratsky et al., 2016) and modulate synaptic plasticity in the tripartite synapse (Allen, 2014;

Farhy-Tselnicker & Allen, 2018). Similar to neurons, astrocytes respond to a variety of stimuli (Araque & Durkee, 2019), including catecholamine-dependent signaling in different brain regions in vitro and in vivo (Galloway et al., 2018; Jennings et al., 2017; Pittolo et al., 2022; Vaarmann et al., 2010; Wahis & Holt, 2021). For instance, catecholamine-evoked signaling in astrocytes modulates synaptic transmission and plasticity (Gordon et al., 2005) to regulate sleep and arousal states (Reitman et al., 2023; Wang et al., 2023), and reward system (Corkrum et al., 2020).

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Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family with important functions in the developing and adult nervous system. The expression of the *Bdnf* gene is a result of complex transcriptional regulation: rodent *Bdnf* gene has eight 5' non-coding exons (exons I–VIII) and one 3' coding exon (exon IX), each of which is controlled by a separate promoter. To produce *Bdnf* transcripts, one of the 5' non-coding exons is spliced together with the common 3' coding exon (Aid et al., 2007; Timmusk et al., 1993). Multiple promoters allow stimulus- and tissue-specific expression of *Bdnf*. Although the regulation of *Bdnf* expression has been studied predominantly in neurons (West et al., 2014), *Bdnf* is also expressed in glial cells (Dai et al., 2003; Elkabes et al., 1996; Koppel et al., 2018; Zafra et al., 1992; Zhang et al., 2014). Astrocytes express *Bdnf* in response to catecholamine signaling (Inoue et al., 1997; Jurič et al., 2006; Koppel et al., 2018; Zafra et al., 1992), indicating that neuron-astrocyte crosstalk is one of the mechanisms modulating BDNF signaling in the brain. BDNF derived from astrocytes has been shown to promote oligodendrogenesis and provide trophic support after demyelinating lesions (Fulmer et al., 2014; Miyamoto et al., 2015). Moreover, astrocytic BDNF has been shown to modulate the morphology and survival of neurons (De Pins et al., 2019; Giralte et al., 2010, 2011) and is crucial for memory retention (Vignoli et al., 2016).

While the proximal regulatory regions of the *Bdnf* gene have been well described (West et al., 2014), only few studies have investigated the role of different enhancer regions regulating *Bdnf* gene expression in neuronal cells (Beagan et al., 2020; Brookes et al., 2023; Calderon et al., 2022; Flavell et al., 2008; Lyons et al., 2012; Tuvikene et al., 2021). This is a critical knowledge gap considering that enhancer regions refine the spatial, temporal, stimulus and cell type-specific transcriptional regulation (Nord & West, 2020; Yap & Greenberg, 2018) and alterations in enhancer regions are increasingly associated with diseases (Maurano et al., 2012). Enhancers can be intra- or extragenic, located upstream or downstream of a gene, and can be located close or far away from the transcription start sites (TSS) of a gene (Perenthaler et al., 2019). To date, three enhancer regions for *Bdnf* gene have been reported: (1) a MEF2-binding region located 4.8 kb upstream of *Bdnf* exon I that enhances neuronal activity-dependent transcription from *Bdnf* promoter I in hippocampal neurons (Flavell et al., 2008) but not in cortical neurons (Lyons et al., 2012); (2) an intronic enhancer region that regulates basal and stimulus-dependent expression of first cluster of *Bdnf* transcripts in neurons (Tuvikene et al., 2021); and (3) an enhancer located ~237 kb downstream of *Bdnf* exon I that is involved in *Bdnf* expression during neuronal differentiation (Brookes et al., 2023).

We have previously shown that *Bdnf* proximal promoters IV and VI need distal regulatory element(s) for catecholamine-dependent induction in astrocytes (Koppel et al., 2018). Here, we aimed to identify the regulatory region(s) involved in catecholamine-dependent *Bdnf* gene transcription in astrocytes. To this end, we first screened several *in silico* predicted putative *Bdnf* enhancer regions in cortical astrocytes using reporter assays. We characterized a region ~840 kb upstream of *Bdnf* that showed strong dopamine-dependent bidirectional transcription and potentiated the activity of *Bdnf* promoters in rat cortical astrocytes. We demonstrated that the –840 kb enhancer

forms long-range chromatin interactions with *Bdnf* gene, and further validated its activity in the endogenous context using CRISPR interference and activator systems as well as CRISPR-mediated deletion of the region. Finally, we show that the activity of the enhancer is regulated by CREB and AP1 family transcription factors. Together, our results provide a novel mechanism for the regulation of *Bdnf* gene in astrocytes.

## 2 | MATERIALS AND METHODS

### 2.1 | Cultures of rat cortical astrocytes

All animal procedures were performed in accordance with European Directive 2010/63/EU. Animals were maintained under 12 h light/dark cycle in a humidity (50 ± 10%) and temperature (22 ± 1°C) controlled room. Rats were group-housed (2–4 animals per cage) in conventional polycarbonate or H-TEMP polysulfone cages with ad libitum access to water and food. Rat cortical astrocyte culture was generated from embryonic (E20/21) Sprague Dawley rats as described previously (Tuvikene et al., 2021).

Briefly, rat cortical astrocytes were grown in 75 cm<sup>2</sup> cell culture flasks in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Pan Biotech) and 100 U/mL penicillin and 0.1 mg/mL streptomycin (Gibco) at 37°C and 5% CO<sub>2</sub>. At 1 DIV, the whole medium was replaced with fresh supplemented DMEM. At 6 DIV, cell flasks were shaken in a 37°C shaker Certomat® BS-1 (Sartorius Group) for 18–20 h at 180 rpm. For splitting, medium was removed along with unattached non-astrocytic cells. Astrocyte monolayer was washed with phosphate-buffered saline (PBS) and detached from the flask with trypsin-EDTA solution (0.0625% trypsin, 0.25 mM EDTA, Gibco) in 1× PBS at 37°C for 5 min. Astrocytes were collected in supplemented DMEM and centrifuged at 200 g for 6 min. Supernatant was aspirated, astrocytes were resuspended in supplemented DMEM, and plated on cell culture plates coated with 0.2 mg/mL poly-L-lysine (Sigma-Aldrich) in Milli-Q. At 9 DIV, the whole medium was changed. At 15 DIV > 95% of the cells were GFAP-positive by immunocytochemical analysis and quantification (Figure S1).

### 2.2 | Deletion of the –840 kb enhancer region in mouse embryonic stem cells

A2Lox mouse embryonic stem cells (mESCs) were a kind gift from Michael Kyba. Doxycycline-inducible Neurogenin2 was inserted to A2Lox mESC genome using recombination-mediated cassette exchange procedure (Iacovino et al., 2011). mESCs were grown and passaged as described in Kainov and Makeyev (2020). The enhancer region located –840 kb from *Bdnf* gene was deleted in the mESCs as described in Tuvikene et al. (2021). Briefly, CRISPR/Cas9 system was used to delete ~500 bp of –840 kb enhancer core region. For that, a total of 6 gRNAs (listed in Supplementary file 1) targeting either upstream or downstream of the mouse –840 kb enhancer core region



were designed using the Benchling CRISPR tool, and chosen so that the gRNAs had no predicted off-targets (maximum of 2 mismatches, allowing both 5'NGG and 5'NAG PAM-sites) in the protein-coding regions. The gRNAs were cloned into the pX330 vector (Addgene plasmid #42230). A2Lox-Neurogenin-2 mESCs were cotransfected with a mixture of gRNA-encoding plasmids together with a plasmid containing a blasticidin S deaminase expression cassette. To create control cell lines without any deletion, the mESCs were transfected with pX330 vector containing no gRNA targeting sequence and blasticidin S deaminase-encoding plasmid. One day after transfection, 8 µg/mL blasticidin S (Sigma-Aldrich) was added to the media for 3 days to select for transfected cells. The cells were grown for ~2 weeks in 2i media containing LIF and 1% FBS (HyClone™ Fetal Bovine Sera) before selecting single colonies, passaging and genotyping. Deletion of the -840 kb region was determined by PCR and verified using qPCR-based copy number analysis as described in Tuvikene et al. (2021). PCR amplicons were confirmed with sequencing. All used primers are listed in Supplementary file 1.

### 2.3 | Astrocytic differentiation of mouse embryonic stem cells

To produce astrocytes from mESCs, the cells were first differentiated into neuronal progenitor cells (NPCs) based on previously published protocols (Conti et al., 2005; Kleiderman et al., 2016; Pollard et al., 2006; Tiwari et al., 2018) with some modifications. First, 100,000 cells/cm<sup>2</sup> wildtype mESCs and mESCs with deletion in the -840 kb region were plated on cell culture plates coated with 0.1% gelatin (Sigma-Aldrich, #ES-006-B) and grown for 3 days in N2B27 neuronal differentiation media (DMEM F12-HAM [Sigma-Aldrich] and Neurobasal [Invitrogen] 1:1 mixture, 1× in-house N2, 1× B27 with retinoic acid [Gibco, #17504044], 1× penicillin-streptomycin [Gibco, #15140122], 1 µg/mL laminin [Sigma-Aldrich, #L2020], 20 µg/mL insulin [Sigma-Aldrich, #I0516], 500 µM L-glutamine [Invitrogen]) and media was changed every day. Starting from 4th day, the cells were grown in N2B27/NPC media (DMEM F12-HAM [Sigma-Aldrich] and Neurobasal [Invitrogen] 1:1 mixture, 0.5× in-house N2, 0.5× B27 with retinoic acid [Gibco], 1× penicillin-streptomycin [Gibco, #15140122], 2 mM L-glutamine [Invitrogen]), and added BSA and insulin as the higher concentrations of BSA (final 37.5 µg/mL) and insulin (final 12.5 µg/mL, Sigma-Aldrich, #I9278) improve neural cell attachment and survival (Pollard et al., 2006). The used in-house 100× N2 was prepared as follows: 5 mg/mL BSA (Invitrogen), 2 µg/mL progesterone (Sigma-Aldrich), 1.6 mg/mL putrescine dihydrochloride (Sigma-Aldrich), 3 µM sodium selenite (Sigma-Aldrich), 10 mg/mL apo-transferrin (Sigma-Aldrich), 1 mg/mL insulin (Sigma-Aldrich, #I0516) in DMEM F12-HAM (Sigma-Aldrich).

At 7th day, the cells were passaged to produce suspension cultures. For that, the cells were trypsinized with 0.05% trypsin-EDTA (Gibco, #15400054), washed with Neurobasal media, and centrifuged at 300 g for 5 min. Finally, the cell pellets were resuspended in NSA media (EuroMed-N [EuroClone, #ECM0883L], 15 µg/mL insulin

[Sigma-Aldrich, #I9278], and 2 mM L-glutamine [Invitrogen]) with freshly added 10 ng/mL EGF (Peprotech, #AF-100-15) and 10 ng/mL FGF2 (Peprotech, #100-18B) and plated onto suspension-culture 6-well plates (Greiner bio-one, #657185). Aliquots of growth factors were stored at 4°C and used within 1 week. Fresh media was added to the cells every day and on 3rd day the formed neurospheres were replated to new suspension-culture 6-well plates.

On 7th day of suspension culture, the cells were plated to produce adherent monolayer of NPCs. For that, the neurospheres were washed from the well and collected in a tube, centrifuged at 72 g for 30 s, resuspended in NSA media with growth factors and plated onto 0.1% gelatin-coated 6-well plates. In the next days, the neurospheres sedimented and produced adherent monolayer cultures of NPCs.

Routinely, the cell media was changed every other day and NPCs were passaged when >90% confluency was reached (3rd or 4th day depending on the clonal cell line). For passaging, cell media was collected to a tube, and cells were trypsinized with 0.025% trypsin-EDTA (Gibco, #15400054) at room temperature for 1 min, diluted in 10 times volume of prewarmed DMEM, and collected to the same tube. The cells were centrifuged at 300 g for 5 min and resuspended in NSA media with freshly added growth factors. Routinely, 50,000 NPCs/cm<sup>2</sup> were plated onto 0.1% gelatin-coated cell culture multi-well plates.

The identity of the NPCs was verified using RT-qPCR for *Nes* mRNA (primers are listed in Supplementary file 1) and NESTIN immunocytochemistry using anti-NES antibody (Developmental Studies Hybridoma Bank, #rat-401) at 0.2 µg/mL. 96% of the cells were NES-positive (Figure S6B). To determine *Bdnf* mRNA levels in the NPCs, the clonal cell lines were independently plated at different times and lysed for RNA 2 days after plating.

NPCs were differentiated to astrocytes on PLL-coated wells (0.2 mg/mL PLL in Milli-Q) in DMEM with high D-glucose (Corning) and 1× penicillin-streptomycin (Gibco, #15140122) supplemented with (1) Tet-system approved 10% FBS only (Gibco, #A4736101); (2) Tet-system approved 10% FBS (Gibco, #A4736101) with 1× B27 with retinoic acid (Gibco); or (3) Tet-system approved 10% FBS (Gibco, #A4736101) with 1× B27 with retinoic acid (Gibco) and 1000 U/mL LIF (Sigma-Aldrich, #ESG1107) for 21 days. The cell media was changed every second day. The identity of the cells was described at different days (1, 5, and 21 days of induction) with RT-qPCR using primers listed in Supplementary file 1 and by immunocytochemical analysis at 21 days of differentiation. The *Bdnf* mRNA levels were measured with RT-qPCR using primers listed in Supplementary file 1.

### 2.4 | DNA constructs

The selected putative enhancer regions were amplified from genomic DNA and cloned in both forward and reverse orientation in front of the Firefly luciferase reporter gene into pGL4.15 luciferase reporter vector (Promega) without an additional promoter as described previously (Tuvikene et al., 2021). Highly conserved ~500 bp regions from

rat, mouse and human –840 kb region were cloned in forward or reverse orientation in front of Firefly luciferase coding sequence in pGL4.15 vector. The coordinates of the cloned regions are listed in Supplementary file 1.

The pRRL-hPGK-A-CREB plasmid has been published previously (Koppel et al., 2018). The coding region of dominant-negative for AP1 family (A-FOS) (Ahn et al., 1998), CEBP $\alpha$  (A-CEBP $\alpha$ ), CEBP $\beta$  (A-CEBP $\beta$ ), USF (A-USF), and ATF2 (A-ATF2) (described previously in Esvald et al., 2020) were subcloned to pRRL lentiviral vector under the control of human PGK promoter. The plasmids used to overexpress constitutively active CREB (pQM-VP16-CREB) and FOS (pQM-VP16-FOS) have been published previously (Pruunsild et al., 2011; Tuvikene et al., 2016). The gRNA targeting sequences (Supplementary file 1) of enhancers were designed using Benchling CRISPR tool (<http://www.benchling.com>) and cloned into the pRRL-U6-gRNA-hPGK-EGFP plasmid. We selected gRNAs with high off-target score (>60) (Hsu et al., 2013) and screened in silico the possible CRISPR gRNA off-targets based on PAM-sites (both 5'NGG and 5'NAG) and mismatches (maximum of 2 mismatches), and found no off-targets for the CRISPR/dCas9 gRNA sequences near the TSS-s of annotated genes. The lentiviral plasmid pLV-hUbc-dCas9-KRAB-T2A-GFP used for CRISPR interference, and pLV-hUbc-VP64-dCas9-VP64-T2A-GFP plasmid used for CRISPR activation have been described previously (Tuvikene et al., 2021). Site-directed mutagenesis was performed as described previously (Tuvikene et al., 2016) using mutations containing complementary primers (Supplementary file 1).

For CAPTURE-3C-sequencing, the FLAG-tagged biotin acceptor site-containing nuclease-deficient Cas9 (FB-dCas9) and biotin ligase BirA plasmids were obtained from Addgene (plasmid #10054 and #100548) and Liu et al. (2017). The FB-dCas9 was cloned to pLV backbone under the control of human Ubc promoter and BirA was cloned to pRRL backbone under the control of human PGK promoter.

## 2.5 | Transfection of cells and luciferase reporter assay

Rat cortical astrocytes were transfected at 13 DIV using 190 ng of luciferase reporter plasmid (or 380 ng of DNA when cotransfected with effector plasmids) and 10 ng of pGL4.83-SR $\alpha$ -hRLuc normalizer plasmid (or 20 ng when cotransfected with effector plasmids) in unsupplemented DMEM with a DNA to Lipofectamine 2000 (Invitrogen) ratio of 1:3 (or 1:2 for cotransfection of reporter plasmid and effector plasmid). The transfection was terminated by changing the media to fresh supplemented DMEM.

After treatments at 15 DIV the cortical astrocytes were lysed in 1 $\times$  Passive Lysis Buffer (Promega) and luciferase assay was performed using Dual-Glo<sup>®</sup> Luciferase Assay (Promega) system. Luminescence signal was measured using GENios Pro Multifunction Microplate Reader (Tecan). For data analysis, background corrected Firefly luciferase signals were normalized with background corrected Renilla luciferase signals and the averages of duplicates were calculated.

## 2.6 | Lentiviral transduction

The production of lentiviral particles was performed as described previously (Koppel et al., 2018). qPCR was used to determine relative titers of lentiviral particles based on provirus incorporation (primers are listed in Supplementary file 1). For functional experiments, equal amounts of lentiviral particles with >95% transduction efficiency were used to transduce astrocytes. Rat cortical astrocytes were transduced after splitting the astrocytes to 6-well cell culture plates at 7 DIV.

## 2.7 | Drug treatments

Rat cortical astrocytes were treated at 15 DIV and mouse embryonic stem cell-derived astrocytes were treated on the 21st day of differentiation with 0.15% DMSO as a vehicle control, 150  $\mu$ M dopamine (Tocris Bioscience) or 25  $\mu$ M norepinephrine (Tocris Bioscience) in unsupplemented DMEM.

## 2.8 | RNA isolation, cDNA synthesis and qPCR

Total RNA from rat cortical astrocytes or mESC-derived cells was isolated using RNeasy Mini Kit (Qiagen) with on-column DNase digestion using RNase-free DNase set (Qiagen) according to the manufacturer's instructions. RNA concentration was measured with BioSpec-nano spectrophotometer (Shimadzu) or with Nanodrop 2000c spectrophotometer (Thermo Scientific). cDNA was synthesized from equal amounts of total RNA using Superscript III or IV Reverse Transcriptase (Thermo Fisher Scientific) with 1:1 mixture of oligo(dT)<sub>20</sub> (Microsynth) and random hexamer primers (Microsynth). qPCR was performed in triplicates using 1 $\times$  HOT FIREpol EvaGreen qPCR Mix Plus (Solis Bio-dyne) or 1 $\times$  LightCycler 480 SYBR Green I Master (Roche) on Light-Cycler 480 II Real Time PCR instrument (Roche). All used qPCR primers are shown in Supplementary file 1. *Ppib* mRNA levels in rat cortical astrocytes and *Cnot4* mRNA levels in mESC-derived NPCs and astrocytes were used to normalize mRNA and enhancer RNA expression.

## 2.9 | Chromatin immunoprecipitation (ChIP)

Rat cortical astrocytes were grown on 10 cm or 15 cm cell culture dishes. After treatments, chromatin was cross-linked with 1% formaldehyde (methanol-free, Cell Signaling Technology) for 10 min and quenched with 0.125 M glycine for 10 min at room temperature with gentle agitation. After washing two times with ice-cold PBS, cells were lysed in 1 mL L1 lysis buffer (50 mM HEPES NaOH [pH 7.5], 140 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.25% Triton X-100, 0.5% NP-40, 10% glycerol, 1 $\times$  cComplete protease inhibitor cocktail [Roche]). Cells were collected using cell scraper and centrifuged at 4°C for 5 min at 1400 g and the pellets were snap-frozen and stored



at  $-80^{\circ}\text{C}$  until further processing. After thawing, the nuclei were washed for 10 min at  $4^{\circ}\text{C}$  in 1 mL L2 buffer (10 mM Tris-HCl [pH 8.0], 200 mM NaCl,  $1\times$  cComplete protease inhibitor cocktail [Roche]), followed by lysis in 500  $\mu\text{L}$  L3 lysis buffer (10 mM Tris-HCl [pH 8.0], 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% sodium deoxycholate, 0.5% N-Lauroylsarcosine,  $1\times$  cComplete protease inhibitor cocktail [Roche]) for 5 min. For CREB, pCREB, and FOS ChIP the L1, L2 and L3 lysis buffer also contained phosphatase inhibitors (5 mM NaF, 1 mM beta-glycerophosphate, 1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM  $\text{Na}_4\text{P}_7\text{O}_2$ ). For histone ChIP, the solutions also contained 10 mM sodium butyrate to inhibit histone deacetylases. Chromatin was sonicated with sonication beads (Diagenode) using 20 cycles (30 s on, 30 s off) on a Bioruptor Pico sonicator (Diagenode) at  $4^{\circ}\text{C}$ . After sonication, lysates were cleared with centrifugation at  $4^{\circ}\text{C}$  for 5 min 10,500 g. Protein concentration was measured using BCA Protein Assay Kit (Pierce). Equal amount of protein was taken for ChIP and 10% of the ChIP volume was taken as input samples. The input samples were kept on ice until decrosslinking. 1% of final Triton X-100 was added to the ChIP samples, and then the samples were diluted 2-fold in dilution buffer (1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl [pH 8.0],  $1\times$  cComplete protease inhibitor cocktail [Roche]). The ChIP samples were incubated overnight at  $4^{\circ}\text{C}$  with 1  $\mu\text{L}$  (0.031  $\mu\text{g}$ ) of H3K27Ac (Cat #8173, Lot #8, Cell Signaling Technology), 1.5  $\mu\text{g}$  of H3K27me3 (Cat #07-449, Lot 31979, Upstate Biotechnology), 7.5  $\mu\text{L}$  (1.965  $\mu\text{g}$ ) of CREB (Cat #07-449, Lot 4820S, Cell signaling), 7.5  $\mu\text{L}$  (0.435  $\mu\text{g}$ ) of pCREB (Cat #87G3, Lot 9198S, Cell signaling) or 1  $\mu\text{L}$  (2  $\mu\text{g}$ ) of pan-FOS (Cat SC-253x, Lot K0110, Santa Cruz Biotechnology) antibody. At the same time, Dynabeads™ Protein G magnetic beads (Invitrogen) were washed twice with 1 mL PBS-0.05% Tween-20 and blocked overnight at  $4^{\circ}\text{C}$  with BSA (200  $\mu\text{g}/\text{mL}$ , Thermo Scientific). The following day, beads were washed twice with 1 mL PBS-0.05% Tween-20 and diluted in L3 lysis buffer. The beads were added to the ChIP samples and incubated for 5 h while rotating at  $4^{\circ}\text{C}$ . Then, antibody-bound beads were washed four times with 1 mL cold wash buffer (1% Triton X-100, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl [pH 8.0],  $1\times$  cComplete protease inhibitor cocktail [Roche]) and once with cold final wash buffer (1% Triton X-100, 0.1% SDS, 500 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl [pH 8.0],  $1\times$  cComplete protease inhibitor cocktail [Roche]). DNA-protein complexes were eluted three times with elution buffer (1% SDS, 100 mM  $\text{NaHCO}_3$ , 1 mM EDTA) and DNA cross-links were reversed with 250 mM NaCl at  $65^{\circ}\text{C}$  overnight. The next day, the samples were treated with RNase A (125  $\mu\text{g}/\text{mL}$ ) for at least 1 h, and then 6 mM EDTA was added and the samples were treated for at least 1 h with Proteinase K (240  $\mu\text{g}/\text{mL}$ ). The ChIP and input DNA was purified using QIAquick PCR Purification Kit (Qiagen) and DNA enrichment was analyzed with qPCR using  $1\times$  LightCycler 480 SYBR Green I Master (Roche). All used qPCR primers are shown in Supplementary file 1. The enrichment of binding was calculated relative to a non-conserved control region (chr3:99,937,231-99,937,310, Rn6 genome) located  $\sim 5$  kbp downstream of the  $-840$  kb enhancer region and selected specifically for this study.

## 2.10 | Immunocytochemistry

We used immunocytochemical analyses to characterize NPCs, cultured astrocytes and mESC-derived induced astrocytes. To analyze induced astrocytes, rat primary mixed culture was used as positive control for antibody staining. These primary cells were obtained from E20-21 Sprague Dawley rat cortex, plated in DMEM, and grown in supplemented NBA media as described previously (Esvald et al., 2022) without the addition of mitotic inhibitor. NPC-s, induced astrocytes at 21 days, primary mixed culture at 8 DIV, or cultured astrocytes at 15 DIV were fixed with 4% of paraformaldehyde for 15 min, neutralized and permeabilized with 50 mM  $\text{NH}_4\text{Cl}$  and 0.5% Triton X-100 in PBS for another 15 min and blocked with 2% of BSA for at least 30 min. Next, the cells were incubated overnight at  $4^{\circ}\text{C}$  with primary antibody in 0.2% BSA in PBS. The following primary antibodies were used: mouse anti-GFAP (Sigma-Aldrich, MAB360, 1:2000), rabbit anti-GS (Sigma, G2781, 1:2000), rabbit anti-TUJ1 (Sigma-Aldrich, T2200, 1:400), mouse anti-NEUN (Chemicon International, MAB377, 1:100), rabbit anti-MAP2 (Millipore, AB5622, 1:1000), mouse anti-NES antibody (Developmental Studies Hybridoma Bank, rat-401, 1:60), goat anti-DCX (Santa Cruz, C-18, 1:500), rabbit anti-IBA1 (Wako, 019-19741, 1:1000), and rabbit anti-CNP (Cell signaling, D83E10, 1:2000). Following the incubation with a primary antibody, cells were washed three times, each time for 5 min, using  $1\times$  PBS containing 0.1% Tween-20.

Goat anti-mouse Alexa 488 (Invitrogen, 1:2000), goat anti-rabbit Alexa 594 (Invitrogen, 1:1000), donkey anti-mouse Alexa 647 (Jackson ImmunoResearch, 715-605-150, 1:300), and donkey anti-goat CF488A (Sigma-Aldrich, 1:2000) were used as secondary antibodies and incubated at room temperature for at least 1 h. Hoechst 33342 (Thermo Scientific, 1:5000) was added to visualize nuclei. After incubation with the secondary antibodies, the cells were washed three times with  $1\times$  PBS with 0.1% Tween-20, and three times with  $1\times$  PBS. The cells were imaged using Axiovert 200M (Zeiss) fluorescent microscope (Axio Vision Rel 4.8 program) or LSM 900 (Zeiss) confocal microscope (Zen 3.3 program). The nuclei were counted using ImageJ software and Hoechst 33342 staining. Immunocytochemically labeled cells were counted manually. A total number of at least 300 cells were counted for each antibody staining.

## 2.11 | CAPTURE-3C-sequencing

At 7 DIV,  $\sim 5 \times 10^6$  rat cortical astrocytes were transduced with lentiviruses expressing biotin ligase BirA and *Bdnf* promoter VI-targeting gRNAs 1 and 2 (Supplementary file 1) according to titer measurements and 4-fold higher dosage of FB-dCas9-encoding lentiviruses. The whole media was changed every 2–3 days.

The previously published CAPTURE-3C-sequencing protocol (Botten et al., 2023; Liu et al., 2017, 2018, 2020) was adapted for cultured astrocytes. At 15 DIV, after 2 h of treatment, astrocytes grown on 145 mm dishes were washed two times with  $1\times$  PBS and chromatin was fixed with 2 mM ethylene glycol-bis(succinic acid

N-hydroxysuccinimide ester) (EGS, Santa Cruz Biotechnology, #sc-252807) in PBS with gentle agitation for 45 min. Then 1% formaldehyde (Cell Signaling Technology, #126065) was added and incubated for additional 10 min. The fixation was quenched with 250 mM glycine for 10 min at room temperature also on an orbital shaker. Next, the astrocytes were washed two times with ice-cold PBS and scraped in 1 mL ice-cold cell lysis buffer (25 mM Tris-HCl [pH 7.5], 85 mM KCl, 0.1% Triton X-100 [Triton™ X-100 Surfact-Amps™, Thermo Scientific, #85111], and freshly added 1× cComplete™ EDTA-free protease inhibitor cocktail [Roche] and 1 mM DTT [Invitrogen]). The cells were lysed for 30 min while rotating at 4°C. The nuclei were pelleted by centrifugation at 2300 g for 8 min at 4°C and the supernatant was removed with a pipette and the pellet was frozen in liquid nitrogen. Freezing the pellet at this stage is recommended as it appears to be an efficient way to help inactivate endonucleases that would otherwise interfere with the protocol.

Each pellet was gently resuspended in 500 µL 1× buffer O (Orange buffer, Thermo Fisher Scientific) and then centrifuged at 2300 g for 8 min at 4°C. The pellet was then resuspended in 0.5% SDS and heated at 62°C for 10 min to inactivate endonucleases. Finally, the lysate was cooled for 5 min on ice and SDS was sequestered by adding a final concentration of 1% Triton X-100 and incubated for 30 min at 37°C. Finally, buffer O (Orange buffer, Thermo Fisher Scientific) and 300 U of DpnII (NEB, #R0543M) were added (as a result, the SDS was also diluted down to 0.1%). Note that the use of buffer O instead of DpnII buffer is critical when performing CAPTURE-3C-sequencing from cultured astrocytes, to inhibit the endonucleases present in the cultured cells. DpnII restriction was performed for 4 h while rotating at 37°C. DpnII was inactivated at 65°C for 20 min. Next, the solution was diluted in a Falcon tube 5× up to 3 mL by adding ligase buffer (NEB, final 1×), Triton X-100 (final concentration 1%), 1× cComplete™ EDTA-free protease inhibitor cocktail (Roche), and Milli-Q. Finally, 2 million CELU units/mL of DNA ligase (NEB, #M0202M) was added per 1 mL. The solution was divided into three DNA LoBind tubes (Eppendorf) and rotated overnight (~16 h) at 16°C.

The next day the ligated chromatin was collected by centrifugation at 3000 g for 10 min at 4°C. The pellet was resuspended in 500 µL RIPA 0 buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA [pH 8.0], 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.1% sodium deoxycholate, freshly added protease inhibitors, 1 mM DTT), and final concentration of 0.25% N-lauroylsarcosine. The mixture was sonicated using sonication beads (0.2 g of beads per 0.5 mL solution and prewashed 3× with PBS) for 10 cycles (30 s on and 30 s off) at 4°C with Bioruptor Pico (Diagenode). Finally, the solution was transferred to a new DNA LoBind tube (Eppendorf), centrifuged at 10,500 g for 10 min, transferred to another DNA LoBind tube, and final concentration of 300 mM NaCl was added.

For streptavidin pulldown, 100 µL of Pierce™ Streptavidin Magnetic beads (Thermo Scientific, #88817) per one sample was used. The magnetic beads were prewashed two times with 0.1% Tween-20 in tris-buffered saline (TBS, pH 7.4). The samples were incubated with streptavidin beads for ~5 h while rotating at 4°C.

Finally, the beads were washed two times with 2% SDS, two times with RIPA buffer with high NaCl (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0], 500 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.1% sodium deoxycholate), two times with LiCl buffer (250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]), and two times with TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]). DNA was eluted from the beads in proteinase K and SDS elution buffer (1% SDS, 10 mM EDTA [pH 8.0], 50 mM Tris-HCl [pH 8.0], 0.2 mg/mL proteinase K [Thermo Scientific]) overnight at 65°C in a shaker. The next day, DNA was purified with QIAquick PCR Purification Kit (Qiagen). CAPTURE system enrichment to *Bdnf* promoter VI was verified with qPCR using 1× LightCycler 480 SYBR Green I Master (Roche) before library preparation and sequencing. Used qPCR primers are shown in Supplementary file 1.

Library preparation and 150 bp paired-end sequencing were conducted by Novogene (UK) Company Limited. Sequencing reads (392654811 for DMSO-treated and 337535553 for DA-treated cells) were processed and mapped to the Rn6 genome using MAXIM software [accessed 15. June 2023] (Chen et al., 2020). Reads with identical positions in both paired reads were discarded as PCR duplicates. The resultant read pairs were further processed using a model-based analysis as previously described in Chen et al. (2020) with modifications. For statistical analysis, only data of chromosome 3 was kept. Based on the sequencing read alignment, the region of *Bdnf* promoter VI, coordinates chr3:100,787,200-100,788,550 on the Rn6 genome, with the FB-dCas9 peak summit at 100,787,921, was selected as the anchor region. The region ±3 Mb of the anchor region was divided into 20 kb bins with a 5 kb step. For each bin, the number of reads with one end inside the anchor region and the other end in the respective bin was calculated. The distance of each bin from the anchor region was calculated as the distance between the bin midpoint and the FB-dCas9 peak summit ±10 kb. Read pairs with both ends outside the anchor region were used to define the background interaction distribution for each sample by subsampling  $n_{\text{anchor}}$  read pair distances from chromosome 3 for 10,000 times, where  $n_{\text{anchor}}$  is the number of read pairs with at least one end in the anchor region in the respective sample. Separate negative binomial distributions were fitted for each bin using maximum likelihood estimation, and  $p$ -values were calculated from the cumulative distribution function using the count of reads with one end mapped to the anchor region and the other end mapping to the respective bin region. For bins with zero reads interacting with the anchor region, the  $p$ -value was set as 1.  $p$ -values were corrected for multiple comparisons using Benjamini & Hochberg false discovery rate method. For graphical visualization, the number of anchor-interacting reads in each bin was divided by the number of  $n_{\text{anchor}}$  reads in the respective sample and multiplied by the mean of  $n_{\text{anchor}}$  reads in both DMSO- and DA-treated samples, to account for slight variations in sequencing depth. Consecutive and overlapping bins with adjusted  $p$ -value <.05 were flattened into one interacting region, and maximum read count was used for graphical depiction of the data. All regions analyzed in Capture-3C-sequencing are listed in the Supplementary file 1.

## 2.12 | Statistical analyses

Sample size estimation was not performed, and randomization and blinding were not used. All tested hypotheses were specified before conducting the experiments. For cultured cells, biological replicates were obtained from rat pups of different litters. For experiments with stem cell-derived neural cells, independent replicates were individual cell clones. For statistical analysis, normalized data was log transformed, mean centered and autoscaled. Statistical significance was calculated using two-tailed paired or unpaired *t*-test, as indicated in figure legends, and *p*-values were corrected for multiple comparisons with Holm-Šidak method using Prism 9.5.1 (GraphPad Software). For graphical representation, data was backtransformed and error bars indicate upper and lower limits of backtransformed means  $\pm$  SEM.

## 3 | RESULTS

### 3.1 | Selection of putative enhancer regions of *Bdnf* gene in astrocytes

The enhancer regions of *Bdnf* have not been studied in non-neuronal cells. In addition, previous research from our group suggests that catecholamines induce *Bdnf* expression through a yet undescribed enhancer region(s) in cortical astrocytes (Koppel et al., 2018). In search for the possible enhancers of the *BDNF* gene, we used the GeneHancer subsection of the Genecards database. GeneHancer gathers information about genomic regulatory elements from different human genome-wide analysis derived from numerous tissues (Fishilevich et al., 2017). Altogether 22 potential regions were predicted as *BDNF* enhancer regions based on the integrated data from several databases (Supplementary file 1). We selected four putative enhancer regions (Table 1, -840, -450, -40, +37 kb) based on the following criteria: (1) high *BDNF* gene-enhancer score, which indicates the likelihood of a region being an enhancer of the *BDNF* gene; (2) the region is predicted to act as an enhancer in astrocytes; (3) high conservation in mammals; (4) not previously studied as a potential enhancer candidate (Tuvikene et al., 2021); and (5) not located within a promoter region of another gene. All the selected putative enhancer regions were named after their approximate distance from the rat *Bdnf* exon I TSS. The metrics of the GeneHancer database are described in the legend of Table 1.

### 3.2 | Identification of the region 840 kb upstream of *Bdnf* as a putative dopamine-regulated *Bdnf* enhancer in cortical astrocytes

Kim et al. (2010) described bidirectional transcription of short enhancer-RNAs (eRNAs) from active enhancer regions and showed that eRNA expression levels are correlated with the expression of target genes, meaning that the transcription from an enhancer region can be used to determine the activity of an enhancer

(Kim et al., 2010). First, we used luciferase reporter assay to evaluate whether transcription can start from the putative enhancer regions upon stimuli. We cloned the selected potential rat enhancer regions (-840, -450, -40, +37 kb, Figure 1a,b) as promoters in front of the luciferase reporter gene (without an additional promoter) in either forward or reverse orientation. The cloned enhancer constructs were transfected into rat cortical astrocytes. To see whether these enhancers respond to dopaminergic stimulation, astrocytes were treated with dopamine or DMSO as a vehicle control for 8 h. The -840 kb region showed the highest orientation-independent induction in response to dopamine treatment (~6-fold) (Figure 1b). Although the -40 kb region showed the highest bidirectional transcription in basal conditions (Figure S2), the -450, -40 and +37 kb regions did not show dopamine-dependent induction (Figure 1b), implying that these regions are not dopamine-dependent enhancer regions in astrocytes.

Functionally important regulatory regions, including enhancers, are known to be evolutionarily conserved (Dickel et al., 2018; Pennacchio et al., 2013). As the -840 kb region showed the strongest transcriptional activation upon stimuli out of the screened candidates, we next inserted the highly conserved ~500 bp long core sequence of the -840 kb region from rat, mouse or human upstream of luciferase coding sequence in either forward or reverse orientation (Figure 1c). In unstimulated astrocytes, mouse and human core enhancer regions upregulated luciferase expression up to ~6-fold (Figure 1c). Robust upregulation was seen in cortical astrocytes (Figure 1c), where an 8 h dopamine treatment strongly (~14–56-fold) induced the expression of the reporter gene from all tested -840 kb enhancer core regions. These results demonstrate that the -840 kb region exhibits evolutionarily conserved dopamine-dependent activity in cortical astrocytes.

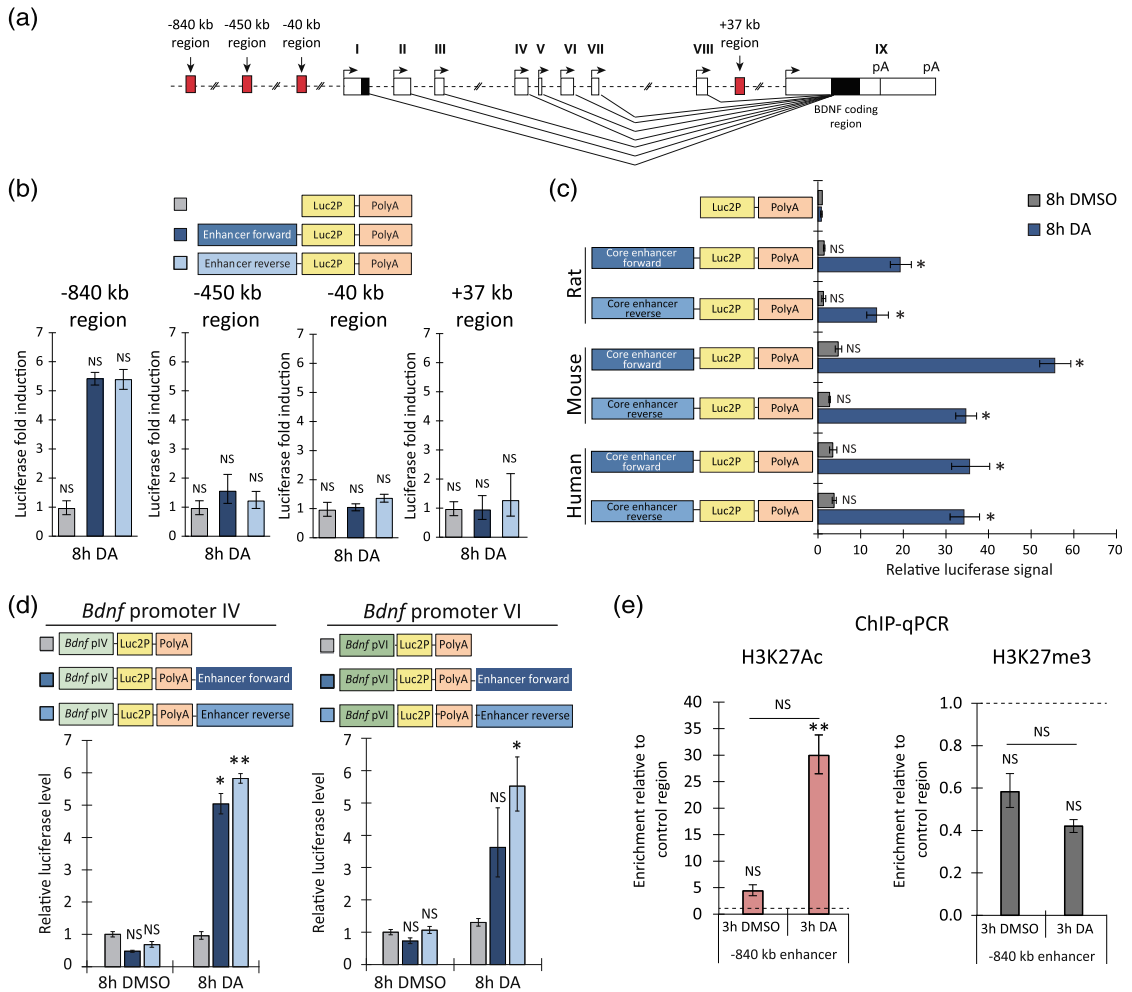
Next, we investigated whether the rat -840 kb region could potentiate the activity of *Bdnf* promoters in a heterologous context. For investigation, we chose *Bdnf* promoters IV and VI because *Bdnf* exon IV- and exon VI-containing mRNAs show the highest basal and catecholamine-induced expression among *Bdnf* transcripts in cortical astrocytes (Koppel et al., 2018). Therefore, we cloned rat *Bdnf* promoters IV or VI in front of the luciferase coding sequence and rat -840 kb enhancer region in forward or reverse orientation downstream of the polyadenylation signal (PolyA) in the same plasmid. Addition of the -840 kb region did not potentiate the activity of *Bdnf* promoter IV or VI (Figure 1d) in unstimulated cells, but strongly increased the stimulus-dependent activity ~5–6 and ~3–4-fold, respectively (Figure 1d). Collectively, our results show that the -840 kb region potentiates the dopamine-dependent activity of *Bdnf* promoters in rat cortical astrocytes.

Finally, to study whether the -840 kb region is an active enhancer region in endogenous context, we interrogated the chromatin state of the region in rat cultured astrocytes. For that, we performed ChIP-qPCR analysis to investigate the presence of H3K27Ac, a hallmark of active regulatory regions, and H3K27me3, a mark of repressed and condensed chromatin regions (Creyghton et al., 2010; Zhu et al., 2013). Analysis of the -840 kb region showed a ~4-fold

**TABLE 1** Potential human *BDNF* enhancer regions predicted by GeneHancer subsection in the GeneCards database and selected for investigation.

Our annotation	GeneHancer identifier	Enhancer score	Enhancer sources	<i>BDNF</i> gene-enhancer score	Total score	TSS distance (kb)	Size (kb)	Transcription factor binding sites within enhancer	Gene targets for enhancer	Coordinates
+37 kb	GH11G027696	1.2	Ensembl	11.1	13.32	+22.7	6.3	92 TFs	3 genes	chr3:100,804,694-100,805,402 (rn6) chr2:109,710,904-109,711,613 (mm10) chr11:27,693,530-27,694,267 (hg19)
			ENCODE							
-40 kb	GH11G027753	0.6	ENCODE	11	6.6	-32.3	1.3	6 TFs	3 genes	chr3:100,727,635-100,728,249 (rn6) chr2:109,626,756-109,627,366 (mm10) chr11:27,775,470-27,776,121 (hg19)
-840 kb	GH11G028565	1.3	FANTOM5 Ensembl	10.1	13.13	-844.6	2.2	19 TFs	3 genes	chr3:99,932,206-99,933,054 (rn6) chr2:108,827,249-108,828,106 (mm10) chr11:28,587,862-28,588,875 (hg19)
			ENCODE							
-450 kb	GH11G028145	0.9	Ensembl	5.9	5.31	-424.2	1.2	13 TFs	3 genes	chr3:100,321,518-100,322,555 (rn6) chr2:109,241,562-109,242,626 (mm10) chr11:28,167,349-28,168,475 (hg19)
			ENCODE							

Note: GeneHancer identifier is a unique tag for the enhancer region in the GeneHancer database. Enhancer score describes the likelihood of the region being an enhancer based on integrated data from different databases, *BDNF* gene-enhancer score reflects the association between *BDNF* gene expression and the activity of an enhancer, and total score is the multiplication of gene-enhancer and enhancer scores. Enhancer sources depict datasets that support the region as an enhancer. TSS distance (kb) shows the distance from the human *BDNF* exon I TSS to the midpoint of enhancer, positive values stand for regions downstream of the *BDNF* exon I TSS and negative values show upstream regions. Gene targets for enhancer shows the number of possible target genes. The table also provides the information on the size of the enhancer regions, numbers of transcription factors (TFs) that have been shown to bind the enhancer in ChIP-seq experiments, and the coordinates of the regions in mouse, rat and human genome are also listed. Our annotation takes into account the distance variations between rat and human genome and is based on the rat genome.



**FIGURE 1** The -840 kb region shows orientation-independent transcriptional activity and potentiates transcription from *Bdnf* promoters in a dopamine-dependent manner in cultured rat cortical astrocytes. (a) Schematic representation of rodent *Bdnf* gene structure together with putative enhancer regions screened in luciferase assay. (b-d) Rat cortical astrocytes were transfected at 13 DIV with luciferase reporter vectors indicated on the graphs: rat -840, -450, -40 or +37 kb putative enhancer region in either forward or reverse orientation (b), the conserved core region of either rat, mouse or human -840 kb enhancer in forward or reverse orientation in front of the luciferase coding sequence (Luc2P) followed by polyadenylation signal (PolyA) (c) or luciferase reporter constructs containing rat *Bdnf* promoter IV or VI upstream of the Luc2P coding sequence and the -840 kb region in either forward or reverse orientation downstream of the PolyA (d). Luciferase vector without an enhancer region was used as a negative control. At 15 DIV, transfected astrocytes were treated for 8 h with 0.15% DMSO as a control or 150  $\mu$ M dopamine (DA). The results are depicted relative to the luciferase signal measured from DMSO-treated astrocytes transfected with a no enhancer construct (b-d). (e) Histone modifications were analyzed with ChIP-qPCR assay using anti-H3K27Ac or anti-H3K27me3 antibodies. At 15 DIV, astrocytes were treated for 3 h with 0.15% DMSO as a control or 150  $\mu$ M DA. The region ~5 kb downstream of the -840 kb enhancer was used as a negative control region. Data is depicted as enrichment relative to the control region (dashed line equals to 1). (b-e) Error bars indicate SEM ( $n = 3$  independent experiments). Statistical significance was calculated with two-tailed paired *t*-test relative to the luciferase level measured from DMSO-treated cells transfected with luciferase vector without an enhancer (b, c), relative to the luciferase level measured from cells transfected with respective promoter-containing vector without an enhancer at respective treatment (d), relative to the enrichment of DNA at the negative control region at respective treatment or relative to the respective region in DMSO-treated astrocytes (e). NS, not significant, \* $p < .05$ ; \*\* $p < .01$ ; \*\*\* $p < .001$ , *p*-values were corrected for multiple comparisons with Holm-Šidák method.



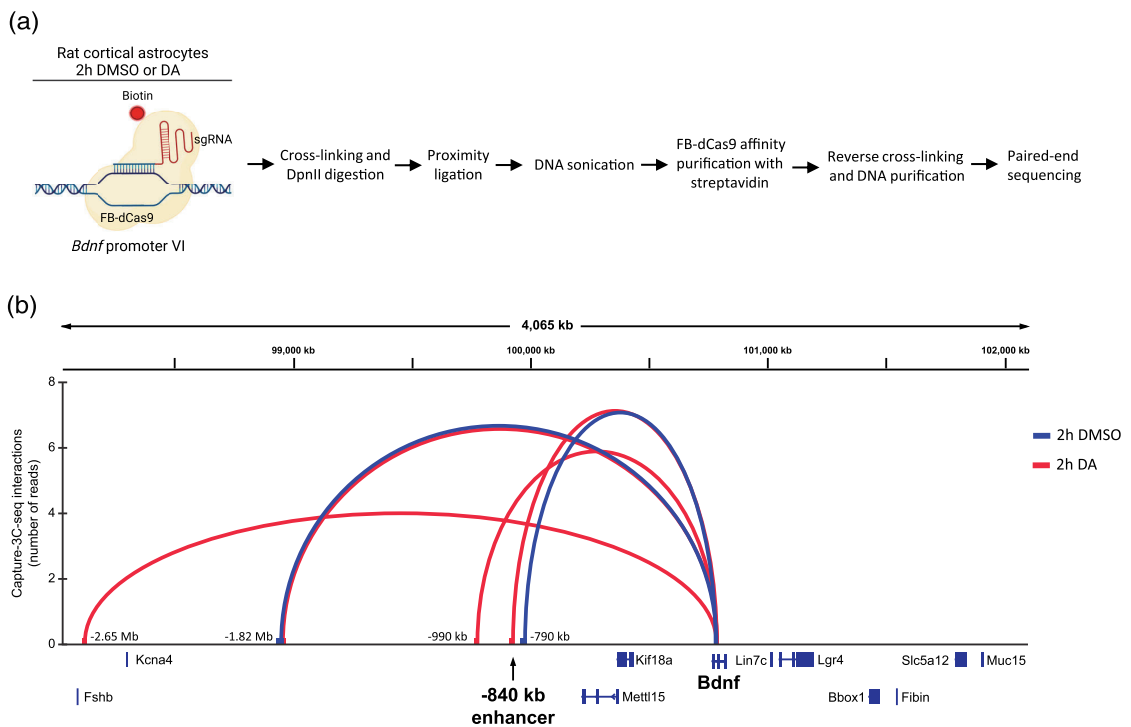
higher enrichment of H3K27Ac relative to control region in unstimulated astrocytes, and a robust  $\sim 7$ -fold increase in the H3K27Ac mark after 3 h dopamine treatment (Figure 1e). The levels of H3K27me3 were  $\sim 2$ -fold lower compared to negative control region in both DMSO- and dopamine-treated astrocytes, although this effect failed to reach statistical significance (Figure 1e). To conclude, our results show that the  $-840$  kb region is enriched with an active enhancer chromatin mark in cortical astrocytes especially upon dopamine treatment.

### 3.3 | The $-840$ kb enhancer region is in spatial proximity to *Bdnf* promoter VI in cortical astrocytes

The physical proximity between enhancer and its target promoter has been proposed to be necessary for the activation of transcription (Beagan et al., 2020; Chen et al., 2018). To identify the enhancer regions interacting with the *Bdnf* locus in astrocytes, we used the CAPTURE-3C-sequencing method (Botten et al., 2023; Liu

et al., 2017, 2018, 2020). Briefly, we used lentivirus-mediated expression of FLAG-tagged biotin acceptor site-containing nuclease deficient Cas9 (FB-dCas9), biotin ligase BirA, and gRNAs targeting *Bdnf* promoter VI region. We verified that gRNAs targeting *Bdnf* promoter VI did not have a major effect on the *Bdnf* mRNA expression (Figure S3A). The transduced astrocytes were treated with dopamine or DMSO as a vehicle control for 2 h and analyzed by CAPTURE-3C-sequencing (Figure 2a). The enrichment of *Bdnf* promoter VI was verified using qPCR before next-generation sequencing (Figure S3B). Our CAPTURE-3C-sequencing results also showed a strong enrichment of the *Bdnf* pVI region (Figure S3C). In total, we determined 10,559 and 9066 sequencing reads with at least one end in the *Bdnf* pVI anchor region, for DMSO- and DA-treated cells, respectively and of these, 220 and 249 reads, respectively showed long-range intrachromosomal interaction outside the pVI anchor region.

The CAPTURE-3C-sequencing experiment revealed that the *Bdnf* promoter VI region exhibits a few statistically significant interactions with distant upstream regions. Specifically, we identified a stimulus-independent interaction with a region located 1.82 Mb upstream of



**FIGURE 2** *Bdnf* promoter VI region interacts with various distal genomic regions in cultured cortical astrocytes in a stimulus-dependent manner. (a) Schematic depiction (created with BioRender.com) of CAPTURE-3C-sequencing protocol that was used to determine long-range chromatin interactions with the *Bdnf* promoter VI region. (b) The statistically significant long-range interactions of *Bdnf* promoter VI are shown with arcs, and the arc height is proportional to the number of sequencing reads for that interaction. x-axis shows the genomic coordinates in the rat Rn6 genome. Blue—DMSO-treated cells, red—DA-treated cells. The  $-840$  kb enhancer region is shown with an arrow. Only interactions with adjusted  $p$ -value  $< .05$  are shown. The number of reads and whole statistical analysis of  $\pm 3$  Mb region of *Bdnf* pVI is shown in Supplementary file 1.

*Bdnf* gene. Following dopamine treatment, we observed alterations in the three-dimensional structure of chromatin. Namely, an interaction with the –790 kb region was exclusively detected in DMSO-treated cells, while DA-induced interactions were noted with the –840 kb, –990 kb and –2.65 Mb regions. We did not detect any interactions near the *Bdnf* gene locus, even when looking with a smaller bin size of 5 kb (data not shown). Interestingly, we did not find any interactions downstream of the *Bdnf* gene in astrocytes. Collectively, our findings demonstrate that the –840 kb region physically associates with the *Bdnf* promoter VI in astrocytes in a catecholamine signaling-dependent manner, supporting the role of the –840 kb region as a stimulus-dependent enhancer of the *Bdnf* gene.

### 3.4 | The –840 kb enhancer regulates catecholamine-induced *Bdnf* expression in cortical astrocytes

Next, we investigated the functionality of the –840 kb enhancer region to potentiate *Bdnf* expression in the endogenous context. We hypothesized that if the region under investigation enhances the transcription of *Bdnf* gene, modulating the activity of the enhancer should affect the expression of endogenous *Bdnf*. For this purpose, we used lentivirus-encoded CRISPR interference and activator (CRISPRi/a) systems where a catalytically inactive Cas9 protein (dCas9) is fused with Krüppel associated box (KRAB) domain (dCas9-KRAB) or 8 copies of VP16 domain (VP64-dCas9-VP64), respectively. For the enhancer-specific repression or activation we used lentiviruses expressing five different gRNAs targeting the –840 kb region. As a control, we used lentiviruses expressing a gRNA that does not target any sequence in the rat genome. Cortical astrocytes were co-transduced at 7 DIV with lentiviruses expressing dCas9-KRAB or VP64-dCas9-VP64 and enhancer-specific or negative control gRNAs (Figure 3a,g). At 15 DIV, rat cortical astrocytes were treated for 3 h with dopamine or DMSO as a vehicle control (Figure 3a–f). We also investigated the effect of –840 kb enhancer after norepinephrine treatment (Figure 3g–i), another catecholamine known to regulate *Bdnf* expression in astrocytes (Koppel et al., 2018). The –840 kb region eRNAs, mRNA levels of total *Bdnf* and different *Bdnf* transcripts were measured using RT-qPCR.

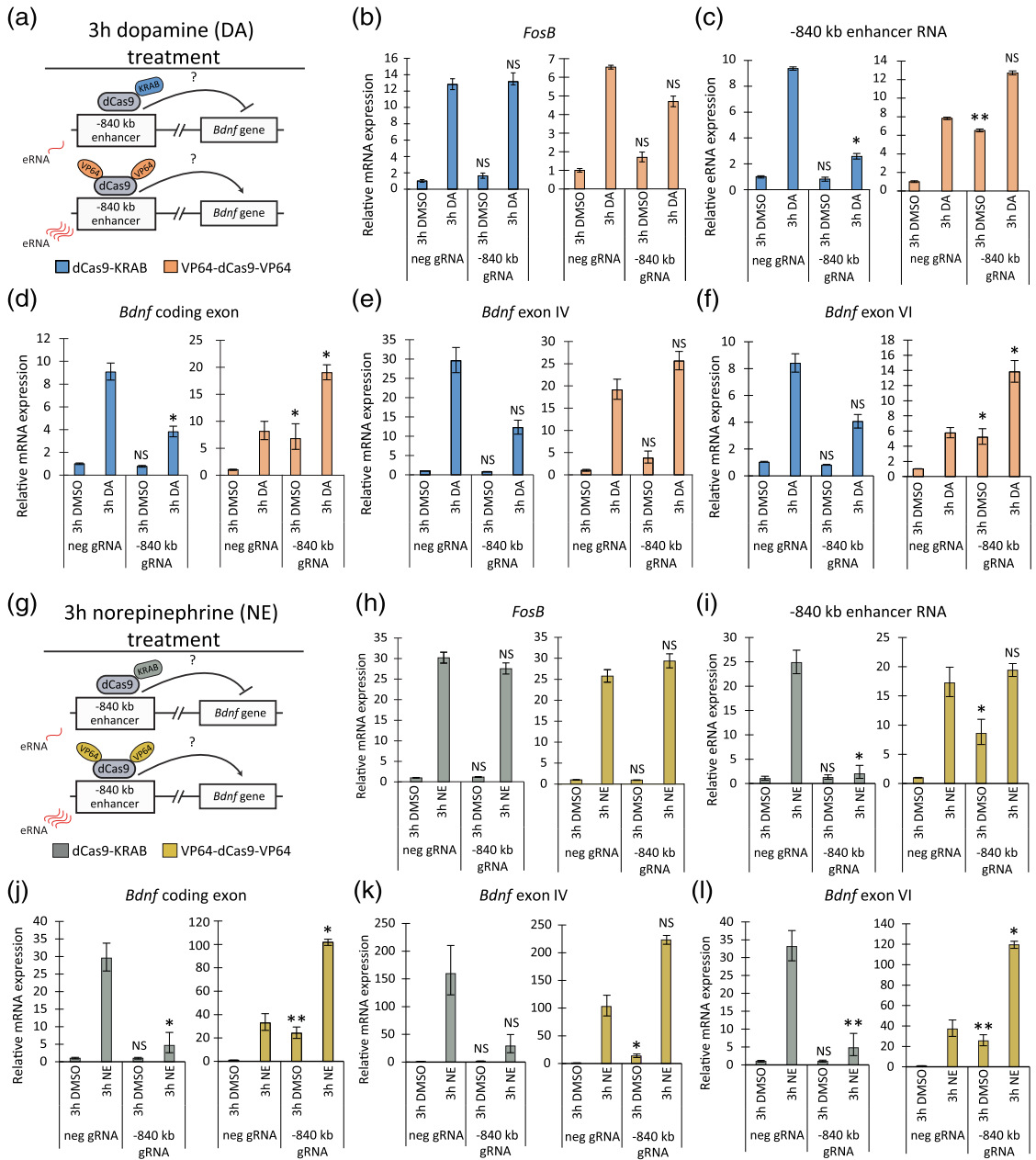
To test the specificity of our system, we first measured the expression of *FosB*, an unrelated stimulus-dependent gene. Neither repression nor activation of –840 kb enhancer region affected the expression of *FosB* (Figure 3b,h). The expression levels of –840 kb eRNAs were notably induced in cortical astrocytes—up to ~9-fold upon dopamine (Figure 3c) and ~25-fold after norepinephrine treatment (Figure 3i). Targeting dCas9-KRAB complex to the –840 kb region remarkably decreased the levels of –840 kb eRNAs ~4- and ~12-fold after dopamine and norepinephrine treatments, respectively, but did not affect the basal eRNA levels (Figure 3c,i). Activation of the –840 kb enhancer by VP64-dCas9-VP64 increased the –840 kb basal eRNA levels, but not stimulus-dependent levels, up to ~9-fold (Figure 3c,i). As the basal expression levels of the –840 kb

eRNAs are very low, our data suggests that the –840 kb enhancer is not active in unstimulated rat astrocytes, but is activated upon dopamine and norepinephrine treatments. However, nascent bidirectional transcription from –840 kb region is evident in previously conducted GRO-seq data obtained from human astrocytes (Figure S4) (Bouvy-Liivrand et al., 2017), indicating that this region is transcriptionally active in unstimulated human glial cells. Further mechanistic experiments in human astrocytes would be necessary to unequivocally show that the –840 kb enhancer is active at basal levels and determine whether its activity differs in a species-specific manner.

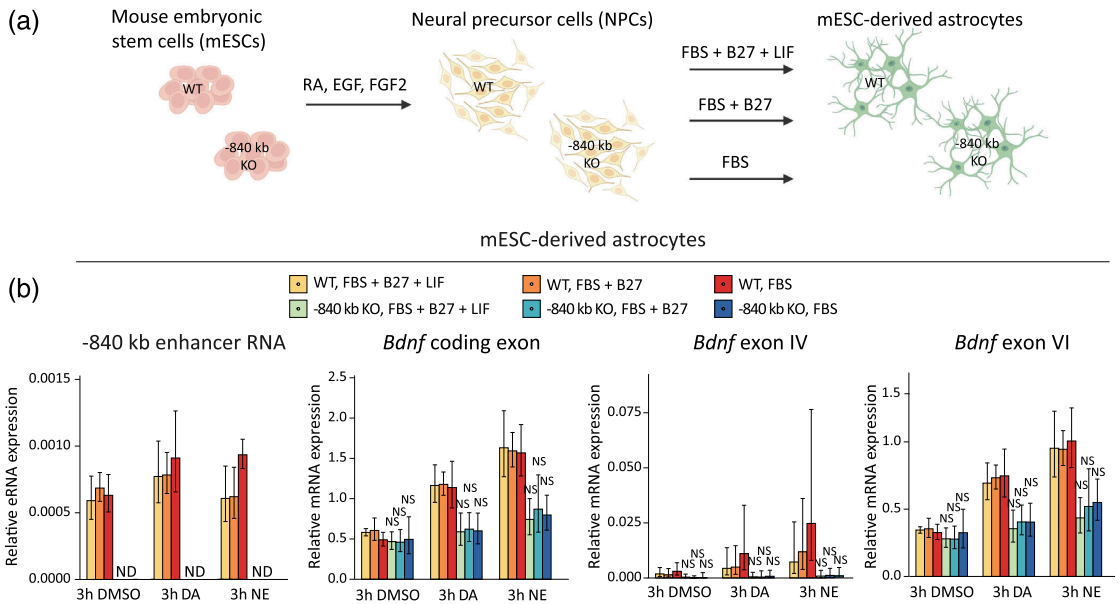
Repression of the –840 kb region with dCas9-KRAB had no effect on the basal *Bdnf* mRNA expression level (Figure 3d–fj–l), confirming the inactive/poised state of the enhancer at basal levels in rat cortical astrocytes. In contrast, dopamine- and norepinephrine-induced levels of *Bdnf* exon IV- (Figure 3e,k) and VI-containing transcripts (Figure 3f,l) were downregulated ~2- and ~6–7-fold, respectively, after targeting dCas9-KRAB to –840 kb enhancer region. Downregulation of different *Bdnf* transcripts was reflected in total *Bdnf* mRNA expression levels, which decreased ~2-fold in dopamine (Figure 3d) and ~6-fold in norepinephrine-treated cells (Figure 3j). Activation of the –840 kb region increased the basal levels of *Bdnf* ~4–25-fold and norepinephrine and dopamine-dependent expression levels ~2–5-fold (Figure 3d–fj–l). Collectively, these results confirm the –840 kb region as a functional *Bdnf* gene enhancer in the endogenous context regulating catecholamine-dependent but not basal expression of *Bdnf* in cortical astrocytes.

### 3.5 | Deletion of the –840 kb enhancer decreases the catecholamine-dependent expression of *Bdnf* in mouse embryonic stem cell-derived astrocytes

To confirm the endogenous function of the –840 kb enhancer, we deleted the enhancer region in mouse embryonic stem cells (mESCs). Based on genotyping results, wild-type mESCs and clones with homozygous –840 kb enhancer deletion (Figure S5) were differentiated into neuronal precursor cells (NPCs) (Figure S6A,B) and astrocytes (Figure S7, Figure 4a). Because differentiation of astrocytes from mouse stem cells lacks a standardized protocol, we tried three different approaches that were selected based on previously published research, and we aimed to obtain astrocytes that show induction of *Bdnf* expression in response to dopamine and norepinephrine treatments. The differentiation strategies used either serum, serum with B27, or serum with B27 and LIF. These conditions led to the maturation of induced astrocytes in time (Figure S7A) and resulted in varied astrocyte morphology and marker gene expression (Figure S7B,C), possibly representing different subtypes and/or maturation stages of astrocytes. The cells were differentiated for 21 days to obtain astrocytes responding to dopamine and norepinephrine treatment. On the 21st day of differentiation the induced astrocytes were treated for 3 h with dopamine or norepinephrine and the expression levels of different *Bdnf* transcripts and –840 kb eRNA levels were measured using RT-qPCR.



**FIGURE 3** Dopamine- and norepinephrine-induced *Bdnf* expression in rat cortical astrocytes is regulated by the –840 kb enhancer. (a, g) At 7 DIV, cultured rat cortical astrocytes were transfected with lentiviruses expressing dCas9-KRAB or VP64-dCas9-VP64 and 5 gRNAs specific for the –840 kb enhancer region (–840 kb gRNA) or negative control gRNA (neg gRNA) as shown in the schematic. (b–f, h–l) At 15 DIV, astrocytes were treated for 3 h with 0.15% DMSO as a vehicle control or 150  $\mu$ M dopamine (DA, b–f) or 25  $\mu$ M norepinephrine (NE, h–l). The expression of *FosB* (b, h), –840 kb enhancer RNA (c, i), *Bdnf* coding exon (d, j) and *Bdnf* exon IV- (e, k) and exon VI-containing (f, l) transcripts were measured using RT-qPCR. The expression level of respective transcript in cells transfected with dCas9-KRAB or VP64-dCas9-VP64 and negative gRNA-encoding lentiviruses and treated with DMSO was set as 1. Error bars indicate SEM ( $n = 4$  [b–f],  $n = 3$  [h–l] independent experiments). Statistical significance was calculated with two-tailed paired  $t$ -test relative to the levels of respective transcript in cells infected with lentiviruses encoding negative gRNA and either dCas9-KRAB or VP64-dCas9-VP64 upon respective treatment. NS, not significant, \* $p < .05$ ; \*\* $p < .01$ ; \*\*\* $p < .001$ ,  $p$ -values were corrected for multiple comparisons with Holm-Šidak method.

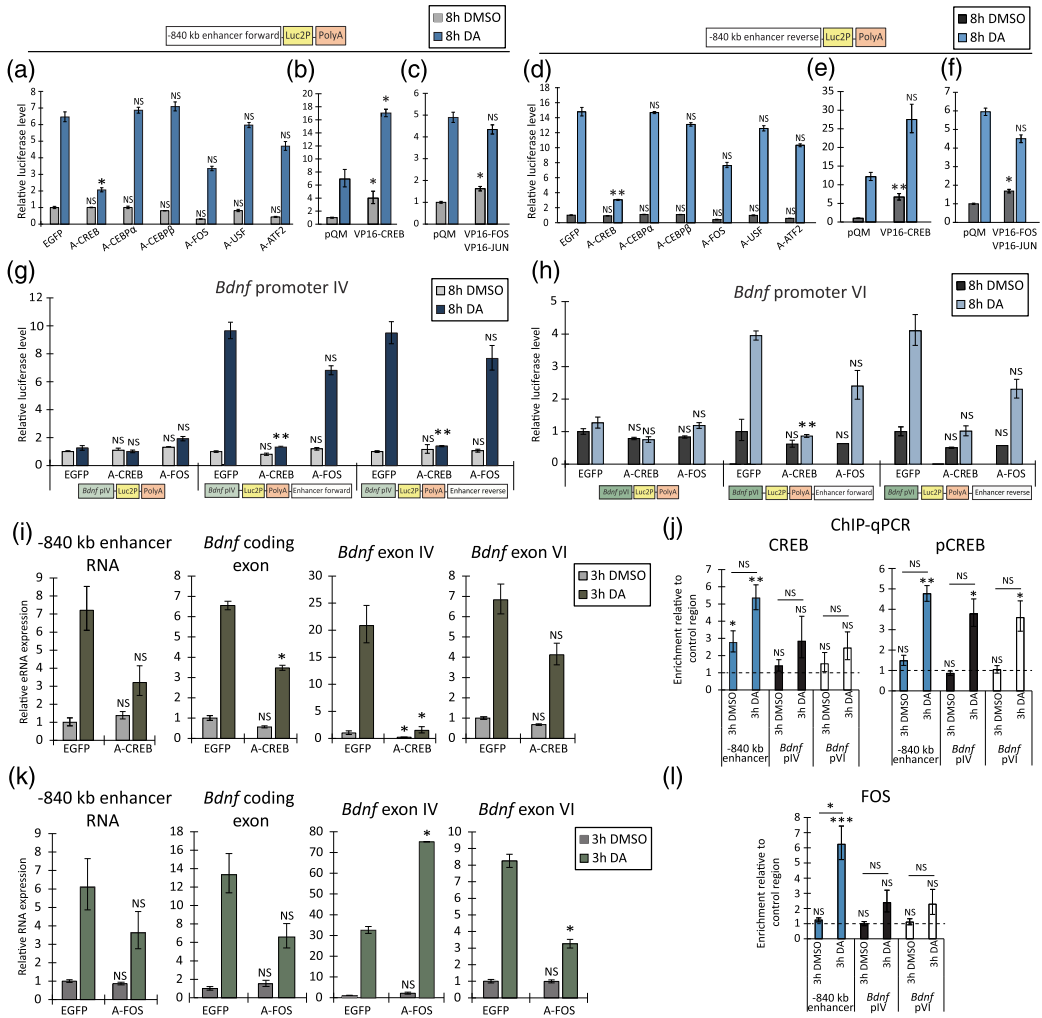


**FIGURE 4** Deletion of the  $-840$  kb enhancer region decreases the expression of *Bdnf* in mouse embryonic stem cell-derived astrocytes. (a) CRISPR/Cas9 system was used to delete the  $\sim 500$  bp conserved region of the  $-840$  kb enhancer. Mouse embryonic stem cells with an intact  $-840$  kb region (WT,  $n = 4$  clones) or with a homozygous deletion of the  $-840$  kb enhancer conserved core region ( $-840$  kb KO,  $n = 3$  clones) were differentiated to NPCs and then astrocytes using FBS alone or along with B27 (containing retinoic acid) or together with B27 and LIF as shown in the legend (illustration on panel a was created with [BioRender.com](https://BioRender.com)). On the 21st day the induced astrocytes were treated for 3 h with  $150 \mu\text{M}$  dopamine (DA) or  $25 \mu\text{M}$  norepinephrine (NE) or with DMSO as a control. (b) The expression levels of  $-840$  kb eRNAs, total *Bdnf* (*Bdnf* coding exon) or different *Bdnf* transcripts were measured using RT-qPCR. The average mRNA expression level relative to the housekeeper *Cnot4* expression level is shown with error bars indicating SEM. Statistical significance was calculated with two-tailed unpaired unequal variance *t*-test relative to the expression level of the respective transcript in wildtype cells at the respective treatment. NS, not significant, # $p < .1$ ; \* $p < .05$ ; \*\* $p < .01$ ; \*\*\* $p < .001$ , *p*-values were corrected for multiple comparisons with Holm-Sidak method. ND, not detected.

In NPCs, deletion of the  $-840$  kb region abolished the expression of the eRNAs and slightly decreased the levels of total *Bdnf* and *Bdnf* exon VI-containing transcripts (Figure S6C). In induced astrocytes, the deletion abolished the expression of  $-840$  kb eRNAs and decreased the induction of total *Bdnf*  $\sim 2$ -fold, *Bdnf* exon IV transcripts up to  $\sim 6$ -fold and *Bdnf* exon VI-containing transcripts  $\sim 2$ -fold in both dopamine and norepinephrine treated astrocytes independent of the differentiation protocol (Figure 4b). However, the effects of *Bdnf* mRNA decreases were not statistically significant due to high biological variability between cell clones (Figure 4b). Similar to the endogenous repression of the enhancer in rat cultured astrocytes (Figure 3), deleting the  $-840$  kb enhancer region did not decrease *Bdnf* expression in unstimulated mESC-derived astrocytes, further corroborating that the  $-840$  kb enhancer only participates in the regulation of *Bdnf* in stimulated cells. Collectively, homozygous deletion of the  $-840$  kb core enhancer region decreased the catecholamine-dependent expression of *Bdnf* in mESC-derived astrocytes, providing further evidence for the functional significance of this element in the regulation of *Bdnf* expression.

### 3.6 | CREB and AP1 family transcription factors regulate the $-840$ kb enhancer and *Bdnf* in rat cultured cortical astrocytes

To understand the molecular mechanism of the  $-840$  kb enhancer in cortical astrocytes, we first used luciferase reporter assays to screen transcription factors (TFs) important for the transcriptional activity of the enhancer region. We hypothesized that if we inhibit the binding of TFs that are important for the activity of  $-840$  kb region, then transcription from the enhancer region will decrease. To study this, rat cortical astrocytes were cotransfected with reporter vectors containing the  $-840$  kb enhancer region in either forward (Figure 5a) or reverse orientation (Figure 5d) in front of the luciferase coding sequence together with a panel of dominant-negative TFs, including A-CREB, A-CEBP $\alpha$ , A-CEBP $\beta$ , A-FOS, A-USF, and A-ATF2. Of note, all the selected and screened TFs have predicted binding elements in  $-840$  kb region based on JASPAR computational prediction (Castro-Mondragon et al., 2022). As a control, we cotransfected the EGFP-encoding vector together with the  $-840$  kb enhancer reporter construct. Transfected astrocytes were treated for 8 h with dopamine or DMSO as a vehicle control.



**FIGURE 5** CREB and AP1 family transcription factors regulate the expression of *Bdnf* and *-840 kb eRNA* in rat cultured cortical astrocytes. (a–h) At 13 DIV, rat cortical astrocytes were cotransfected with different dominant-negative transcription factors (termed as A in the name), constitutively active form of CREB (VP16-CREB), FOS (VP16-FOS), JUN (VP16-JUN) or EGFP and luciferase reporter vectors as indicated under the graphs: *-840 kb enhancer region* in forward (a–c) or reverse (d–f) orientation before luciferase coding sequence or rat *BDNF promoter IV* (g) or VI (h) before luciferase coding region in forward and reverse orientation after the polyadenylation signal (PolyA). Transfected astrocytes were treated for 8 h with 0.15% DMSO as control or with 150  $\mu$ M dopamine (DA). The results are shown relative to the luciferase activity measured from DMSO-treated astrocytes that were cotransfected with EGFP and respective reporter vector as shown below each panel. (i, k) At 7 DIV, rat cortical astrocytes were transduced with lentiviruses expressing either EGFP, dominant-negative for the CREB family (A-CREB, i), or dominant-negative for the AP1 family (A-FOS, k). At 15 DIV, astrocytes were treated with 0.15% DMSO as a control or 150  $\mu$ M dopamine (DA) for 3 h. The RNA expression of *-840 kb enhancer RNAs*, total *Bdnf* (*Bdnf* coding exon), *Bdnf* exon IV- and exon VI-containing transcripts was measured using RT-qPCR. The expression level of the respective transcript in cells treated with DMSO and transduced with EGFP-encoding lentiviruses was set as 1 (i, k). Error bars indicate SEM ( $n = 3$  independent experiments). (j, l) Endogenous binding of CREB, pCREB and FOS was analyzed with ChIP-qPCR. At 15 DIV, astrocytes were treated for 3 h with 0.15% DMSO as a control or 150  $\mu$ M DA. A region  $\sim 5$  kb downstream of the *-840 kb enhancer* was used as a negative control region. Data is depicted as enrichment relative to the control region (dashed line equals to 1). Error bars indicate SEM ( $n = 4$  independent experiments). (a–h) Statistical significance was calculated using two-tailed paired *t*-test relative to the luciferase signal measured from respectively treated cells cotransfected with the respective reporter vector and EGFP plasmid (a–h), relative to the mRNA levels of respective transcript in respectively treated cells infected with lentiviruses encoding EGFP (i, k), or relative to the enrichment of DNA at the negative control region at respective treatment or the respective region in DMSO-treated astrocytes (j, l). NS, not significant; \* $p < .05$ ; \*\* $p < .01$ ; \*\*\* $p < .001$ ; *p*-values were corrected for multiple comparisons with Holm-Sidak method.



Basal transcriptional activity of the –840 kb region was decreased ~2–3-fold in cells overexpressing A-FOS, a dominant-negative protein for AP1 family (Figure 5a,d). After dopamine treatment A-FOS inhibited the activity of the –840 kb enhancer region ~2-fold (Figure 5a,d). In addition to AP1 family, the dominant-negative form of the CREB family, A-CREB, almost abolished the induction of –840 kb transcription in cortical astrocytes upon dopamine treatment (Figure 5a,d). Other tested dominant negative proteins did not have a notable effect on the –840 kb region enhancer activity (Figure 5a,d). In addition, we overexpressed CREB or FOS and JUN fused to the VP16 activator domain together with –840 kb enhancer region to elucidate whether the CREB and AP1 family TFs can increase the transcription from the enhancer. The overexpression of constitutively active form of CREB (VP16-CREB) robustly increased the transcriptional activity of the –840 kb enhancer both in DMSO- and dopamine-treated cortical astrocytes (Figure 5b,e), while overexpression of VP16-FOS and VP16-JUN enhanced the transcriptional activity by ~2-fold only in DMSO-treated cortical astrocytes (Figure 5c,f), possibly due to saturation of –840 kb enhancer by endogenous AP1 family members after dopamine treatment.

Next, we studied whether the two dominant-negative TFs (A-CREB and A-FOS) regulate the –840 kb enhancer region-potentiated activation of *Bdnf* promoter IV and VI. For that, cells were cotransfected with A-CREB and A-FOS (Figure 5g,h) together with reporter constructs where *Bdnf* promoter IV (Figure 5g) or VI (Figure 5h) was in front of the luciferase coding sequence and the –840 kb enhancer region in forward or reverse orientation after the polyadenylation signal (PolyA). Overexpression of A-CREB markedly decreased the –840 kb region-enhanced activity of *Bdnf* promoter IV (Figure 5g) and VI (Figure 5h) in dopamine-treated cortical astrocytes, whereas A-FOS slightly decreased (~1.3–1.8-fold) the activity of *Bdnf* promoter IV and VI (Figure 5g,h). These results indicate that CREB and AP1 family members mediate the activation of *Bdnf* promoters by the –840 kb enhancer region in a heterologous context in cortical astrocytes.

To investigate whether the TFs controlling the activity of the –840 kb region in the reporter assay also participate in the regulation of endogenous *Bdnf* gene expression, rat cortical astrocytes were transduced at 7 DIV with lentiviruses encoding either EGFP, A-CREB (Figure 5i) or A-FOS (Figure 5k). The overexpression of dominant-negative CREB did not affect the basal expression levels of –840 kb eRNAs, but decreased the basal expression levels of exon IV-containing *Bdnf* levels ~3-fold (Figure 5i). Conversely, A-CREB inhibited the expression of –840 kb eRNAs, total *Bdnf* and exon IV- and VI-containing transcripts ~2–10-fold in dopamine-treated cells (Figure 5i). Using ChIP-qPCR, we confirmed direct binding of CREB to –840 kb enhancer region in DMSO- and DA-treated astrocytes (~3–5-fold higher enrichment relative to a control region) and binding of the active form of CREB, phospho-CREB (pCREB), which increased ~3-fold following a dopamine treatment (Figure 5j). Overexpression of A-FOS decreased the expression of –840 kb region eRNAs ~1.7-fold in dopamine-treated cells (Figure 5k). While

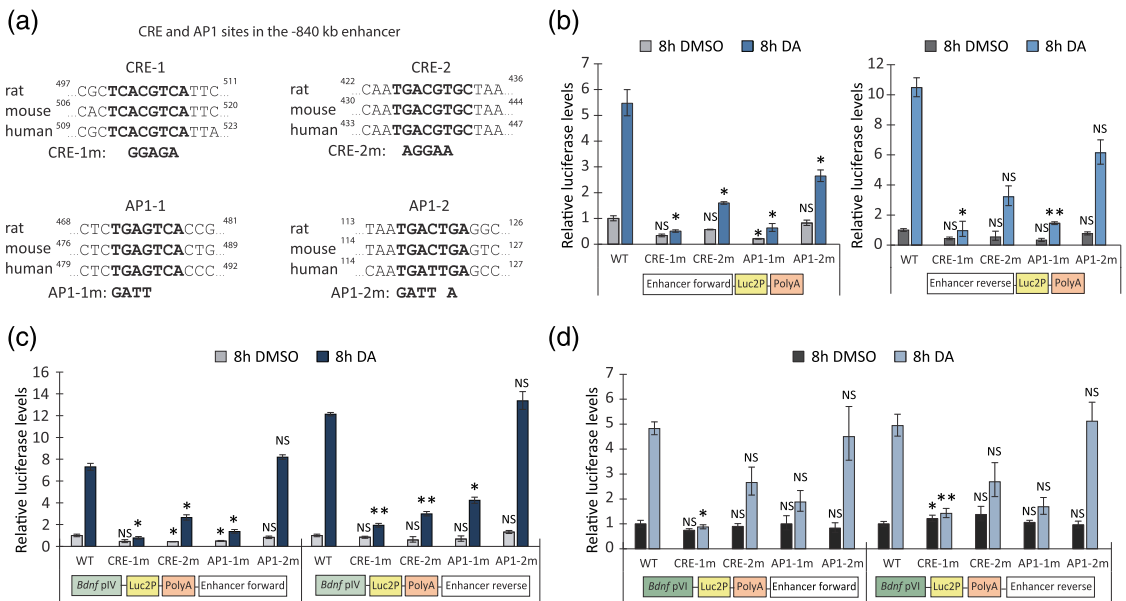
overexpression of A-FOS did not affect the basal levels of *Bdnf*, A-FOS decreased total *Bdnf* and exon VI-containing *Bdnf* mRNA (Figure 5k) levels ~2-fold in dopamine-treated astrocytes. Interestingly, *Bdnf* exon IV mRNA levels were upregulated ~2.3-fold in dopamine-treated A-FOS overexpressing cells (Figure 5k). We also detected endogenous binding of FOS-family TFs to the –840 kb enhancer region with ChIP-qPCR (Figure 5l), where FOS binding increased ~5-fold in dopamine-treated cells. Although CREB and AP1 family TFs also showed some interaction with *Bdnf* promoters IV and VI, our results collectively point to the importance of the recruitment of these TFs to the –840 kb enhancer region for stimulating *Bdnf* gene expression in cortical astrocytes.

### 3.7 | CREB and AP1 cis-elements within the –840 kb enhancer are required for the enhancer activity

Finally, we examined the role of CREB and AP1 transcription factor binding sites in the transcriptional regulation of –840 kb enhancer. First, we used JASPAR cis-element prediction of CREB and AP1 binding sites and found two CRE (cAMP responsive element, CRE-1 and CRE-2) and two AP1 sites (AP1-1 and AP1-2) in rat –840 kb enhancer (Figure 6a). Comparison of the sequences among rat, mouse and human revealed high evolutionary conservation of the identified binding sites, with the exception of AP1-2, which differs by a single nucleotide in human compared to rat and mouse (Figure 6a). Next, we mutated the CREB and AP1 binding sites in the rat –840 kb enhancer reporter constructs and used luciferase reporter assay to study the importance of these sites in the bidirectional transcription of the enhancer.

In DMSO-treated astrocytes, mutation only in the AP1-1 site showed ~3–4-fold downregulation of the –840 kb enhancer activity. The strongest stimulus-dependent effect on the –840 kb enhancer activity was seen when mutating the CRE-1 and AP1-1 sites (Figure 6b)—the activity decreased ~11-fold and ~7–9-fold upon dopamine treatment, respectively. Mutations in CRE-2 and AP1-2 site decreased the stimulus-dependent transcriptional activity ~2–3-fold (Figure 6b).

Next, we analyzed whether the identified cis-elements in the –840 kb enhancer affect the transcription from *Bdnf* promoters IV (Figure 6c) and VI (Figure 6d). Out of four tested cis-elements, mutations in the CRE-1, CRE-2 and AP1-1, but not in AP1-2, decreased the dopamine-dependent transcription from *Bdnf* promoter IV and VI. The strongest effect was seen with the mutations in the CRE-1 site which decreased the dopamine-dependent induction of *Bdnf* promoter IV ~6–9-fold (Figure 6c) and *Bdnf* promoter VI ~4–5-fold (Figure 6d). Taken together, these results show that all four identified transcription factor binding sites are responsible for the stimulus-dependent activity of the rat –840 kb enhancer region, and specifically CRE-1, CRE-2 and AP1-1 sites are necessary to increase the *Bdnf* promoter IV and VI activity after dopamine treatment in cortical astrocytes.



**FIGURE 6** Mutations in the CRE and AP1 sites in the -840 kb region decrease the activity of the enhancer and *Bdnf* promoters in cultured rat cortical astrocytes. (a) CRE and AP1 sites in the rat, mouse and human -840 kb enhancer. Nucleotides shown in bold indicate the CREB or AP1 family transcription factor core binding sites, numbers indicate the sequence location counted from the 5' of the cloned full-length -840 kb enhancer region. Mutated nucleotides are shown below the wild-type binding sites. (b-d) Rat cortical astrocytes were transfected at 13 DIV with luciferase reporter vectors indicated on the graphs: wild-type (WT) or mutated rat -840 kb enhancer region in either forward or reverse orientation (b) or luciferase reporter constructs containing rat *Bdnf* promoter IV or VI upstream of the Luc2P coding sequence and the WT or mutated -840 kb region in either forward or reverse orientation downstream of the PolyA (c, d). Luciferase vector with WT enhancer region was used as a control. At 15 DIV, transfected astrocytes were treated for 8 h with 0.15% DMSO as a control or 150 μM dopamine (DA). The results are depicted relative to the luciferase signal measured from DMSO-treated astrocytes transfected with WT enhancer construct (b-d). Error bars indicate SEM ( $n = 3$  independent experiments). Statistical significance was calculated with two-tailed paired  $t$ -test relative to the luciferase level measured from DMSO-treated cells transfected with luciferase vector with WT enhancer (b), or relative to the luciferase level measured from cells transfected with respective promoter-containing vector with WT enhancer (c, d). NS, not significant, \* $p < .05$ ; \*\* $p < .01$ ; \*\*\* $p < .001$ ,  $p$ -values were corrected for multiple comparisons with Holm-Šidak method.

## 4 | DISCUSSION

The multi-promoter composition of *Bdnf* gene allows generating alternative *Bdnf* transcripts, which result in identical BDNF protein. This kind of gene regulation ensures complex spatiotemporal expression of BDNF (Esvald et al., 2023; West et al., 2014). For instance, the first cluster of *Bdnf* transcripts is expressed in neurons, but not in non-neuronal cells, and, in contrast, the second cluster of *Bdnf* transcripts is expressed ubiquitously in both neuronal and non-neuronal tissues (Aid et al., 2007; Esvald et al., 2023; Pruunsild et al., 2007; Timmusk et al., 1993). Likely, this spatiotemporal regulation is maintained by both cell type-specific activity of proximal promoters (Palm et al., 1999; Zuccato et al., 2003) and recruitment of specific enhancer regions (Brookes et al., 2023; Flavell et al., 2008; Lyons et al., 2012; Tuvikene et al., 2021). In this study we aimed to identify distal regulatory regions that participate in the catecholamine-dependent expression of *Bdnf* in rodent astrocytes. Of the four candidate enhancer regions that we initially investigated, only one region mapping 840 kb

upstream of *Bdnf* gene showed properties of a stimulus-dependent enhancer in astrocytes, and interaction with *Bdnf* promoter VI. It is possible that the rest of the bioinformatically predicted enhancer regions (-450, -40 and +37 kb) would function upon different stimuli or in another cell type than studied here, highlighting the uniqueness of each enhancer.

The astrocytic BDNF seems to have similar roles as neuron-derived BDNF, for example, it modulates neuronal morphology, plasticity, and survival (De Pins et al., 2019; Giralt et al., 2010, 2011; Vignoli et al., 2016). However, in general the kinetics of exocytosis in astrocytes is slower compared to that in neurons (Kreft et al., 2004; Verkhatsky et al., 2016). Furthermore, due to different dynamics of BDNF secretion from neurons and astrocytes, the neuron- and astrocyte-derived BDNF distinctively modulates the hippocampal long-term potentiation and learning (Liu et al., 2022). The physiological function of dopamine- and norepinephrine-induced BDNF production in astrocytes remains to be investigated. In addition to neuron-derived BDNF, it is plausible that astrocytes within the dopaminergic and

noradrenergic tripartite synapses express BDNF to augment BDNF signaling, thereby contributing to several neuronal functions. What is the exact role of catecholamine signaling-derived astrocytic BDNF in the cerebral cortex and whether the –840 kb enhancer facilitates the expression of astrocytic BDNF similarly in different brain regions, are important questions to address in the future.

Koppel et al. (2018) showed for the first time that the catecholamine-dependent induction of *Bdnf* in astrocytes is not conferred only by the *Bdnf* proximal promoter regions. By using *BDNF*-bacterial artificial chromosome (BAC)-based reporters encompassing the whole rat or human *BDNF* gene locus, they found that at least some distal regulatory regions that facilitate the catecholamine-induced levels of *Bdnf* in cortical astrocytes are located within or near the *Bdnf* gene. Notably, the –840 kb region described in the present study is outside of the *BDNF*-BAC constructs used by Koppel et al. (2018). Together, these results show the existence of at least two enhancers, one 840 kb upstream of *Bdnf* described in the present study, and another within or nearby of *Bdnf* gene locus described by Koppel et al. (2018), that are responsible for catecholamine-induced regulation of *Bdnf*. It is also plausible that *Bdnf* gene regulation in astrocytes is even more complex, considering the numerous long-range chromatin interactions detected by our CAPTURE-3C-sequencing experiment. Namely, we determined that *Bdnf* pVI interacts with –840 kb region upon dopamine treatment, but we also showed dopamine-dependent interactions with –990 kb and –2.65 Mb regions. Moreover, we found interaction with –790 kb region only in DMSO-treated cells and a stimulus-independent interaction with a region located at –1.82 Mb. This indicates that the transcription of *Bdnf* gene is subject to specific combinatorial regulation by different enhancer regions, as it has been demonstrated for *c-Fos* (Joo et al., 2015). However, we acknowledge that our CAPTURE-3C-sequencing might lack the resolution and statistical power to detect the possible short-range interactions within the *Bdnf* locus. We have previously shown that *Bdnf* is regulated as two clusters of promoters (Aid et al., 2007; Esvald et al., 2023; Pruunsild et al., 2007; Timmusk et al., 1993), and that the intronic enhancer located downstream of exon III is responsible for neuron-specific expression of the first cluster of *Bdnf* exons (Tuvikene et al., 2021). It is plausible that the enhancers of *Bdnf* loop together with the anchor point located nearby the first, second, or both of the clusters depending on the cell type and stimulus. Further work is needed to determine the role and/or interplay of the potential enhancer regions to pinpoint the exact mechanism of the catecholamine-dependent *Bdnf* expression.

Although the transcriptional regulation of *Bdnf* in astrocytes is poorly investigated, catecholamines have been shown to induce *Bdnf* gene expression in astrocytes via CREB-dependent signaling (Jurič et al., 2008; Koppel et al., 2018). Our work provides further mechanistic insight by showing that AP1 family transcription factors act as regulators of astrocytic *Bdnf* expression. Interestingly, Koppel et al. (2018) showed that the well-studied CRE element in *Bdnf* promoter IV is not involved in catecholamine-dependent *Bdnf* regulation in astrocytes, whereas this site is critical for stimulus-dependent expression of BDNF in neurons (Esvald et al., 2020;

Hong et al., 2008; Pruunsild et al., 2011; Shieh et al., 1998; Tao et al., 1998). Similarly, AP1 family members are known to regulate the expression of the neuronal first cluster of *Bdnf* transcripts (Tuvikene et al., 2016) that are not expressed in astrocytes. These results argue for substantial differences in *Bdnf* gene regulation between different cell types even when the same transcription factor families are involved, implying involvement of other regulatory regions. Here, we demonstrate that the CREB and AP1 family-dependent regulation of *Bdnf* in astrocytes occurs via the –840 kb enhancer region. We show that mutations in any of the CRE sites and one AP1 site within the –840 kb enhancer decrease the activity of *Bdnf* promoters IV and VI. Collectively, our study is the first report showing an enhancer coordinating the transcription of *Bdnf* gene in non-neuronal cells and demonstrating the fine-tuning of gene expression based on cellular context and signaling.

In neurons, the AP1 family plays a crucial role in the regulation of activity-dependent enhancers. The AP1 motifs are one of the most prevalent cis-elements in enhancers, and the regulation of neuronal enhancers relies on the rapid induction and binding of AP1 family transcription factors (Malik et al., 2014). The AP1 family acts as an epigenetic regulator, modifying chromatin accessibility around their binding sites throughout the genome (Malik et al., 2014; Su et al., 2017). To achieve this, the AP1 factors recruit ATP-dependent BRG1/BRM associated factor (BAF) chromatin remodeling complexes, which, in turn, facilitate nucleosome remodeling and generate an accessible chromatin state (Vierbuchen et al., 2017). In our study, we identified two AP1 cis-elements within the –840 kb enhancer region, both of which are significant for enhancer activity. However, only one of these AP1 cis-elements (AP1-1), located in the center of the –840 kb enhancer, is involved in enhancing the activity of *Bdnf* promoters. Interestingly, the other AP1 cis-element (AP1-2) appears to be non-functional for *Bdnf* induction in astrocytes, but may play a crucial role in regulating *Bdnf* activity in other cell types. Currently, little is known about the general mechanism of enhancers in astrocytes, but our results studying this stimulus-specific enhancer in depth suggests a similar central role for AP1 factors in astrocytes as observed in neurons. A natural continuation of this line of work would be to investigate the role of AP1 family transcription factors in astrocyte-specific enhancers using genome-wide approaches.

Notably, the –840 kb enhancer is relevant in vivo. First, in 2006, a case-report of an 8-year-old girl suffering from hyperphagia, severe obesity, impaired memory and hyperactivity was published. Furthermore, it was determined that the BDNF protein levels were decreased in the patient's serum despite the fact that *BDNF* gene itself was not mutated (Gray et al., 2006). Genetic analysis revealed that the patient had a chromosomal inversion with a breakpoint located within a 9 kb region at the –840 kb enhancer locus. Second, a mouse line with transgene insertion in a conserved region located approximately 840 kb upstream of the *Bdnf* gene (Sha et al., 2007) was reported to have a similar phenotype as *Bdnf* knockout mice (Lyons et al., 1999; Rauskolb et al., 2010) and patient described in Gray et al. (2006). Furthermore, the transgene insertion strikingly reduced both *Bdnf* mRNA and protein levels in the hypothalamus and in the hippocampus



(Sha et al., 2007). However, neither of these studies unequivocally showed the exact enhancer region responsible for the decreased BDNF levels and the observed phenotype. Our current study provides one possible explanation for these findings by describing a functional enhancer in the –840 kb region regulating *Bdnf* transcription in astrocytes. Further dissecting the in vivo function and role of this enhancer in various cell types would improve the understanding of *Bdnf* gene regulation. Collectively, the phenotypic abnormalities in human and mice caused by alteration in the –840 kb region (Gray et al., 2006; Sha et al., 2007) should be considered as an example of enhanceropathy, a condition caused by a misregulation of an enhancer regulating a functionally important gene (Claringbould & Zaugg, 2021; Smith & Shilatifard, 2014).

In conclusion, our study provides the first description of an enhancer element regulating stimulus-specific transcription of *Bdnf* in an important type of non-neuronal cells. Whether the knowledge of the molecular mechanisms underlying BDNF production in astrocytes can be harnessed for therapeutic applications is an important question for the future.

#### AUTHOR CONTRIBUTIONS

AA designed research, performed experiments, wrote the first draft, and edited the manuscript. E-EE, IK and JT designed research, performed experiments, wrote and edited the manuscript. JT performed bioinformatical analysis. AP performed experiments and reviewed the manuscript. AZ and EVM provided funding, and edited the manuscript. TT and JT conceived the idea, supervised the study, TT provided funding, and edited the manuscript. All authors contributed to the article and approved the submitted version.

#### ACKNOWLEDGMENTS

This work has been supported by Estonian Research Council (institutional research funding IUT19-18 and grant PRG805), European Union through the European Regional Development Fund (Project No. 2014–2020.4.01.15-0012) and H2020-MSCA-RISE-2016 (Grant EU734791) and BBSRC (grants BB/R001049/1 and BB/V006258/1). This work has also been partially supported by TUT Institutional Development Program for 2016–2022 Graduate School in Clinical medicine receiving funding from the European Regional Development Fund under program ASTRA 2014–2020.4.01.16-0032 in Estonia. We thank Epp Väli for technical assistance; Kaie Uustalu for contribution in the early stage of the project; Florencia Cabrera-Cabrera and Olga Jasnovidova for critical reading of the manuscript.





#### CONFLICT OF INTEREST STATEMENT

Eli-Eelika Esvald, Jürgen Tuvikene, and Tõnis Timmusk were employees of Protobios LLC. The authors declare no other competing financial interests.

#### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

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**How to cite this article:** Avarlaid, A., Esvald, E.-E., Koppel, I., Parkman, A., Zhuravskaya, A., Makeyev, E. V., Tuvikene, J., & Timmusk, T. (2024). An 840 kb distant upstream enhancer is a crucial regulator of catecholamine-dependent expression of the *Bdnf* gene in astrocytes. *Glia*, 72(1), 90–110. <https://doi.org/10.1002/glia.24463>



## Appendix 3

### Publication III

**Avarlaid A**, Falkenberg K\*, Lehe K\*, Mudò G, Belluardo N†, Di Liberto V, Frinchi M, Tuvikene J#, Timmusk T#

An upstream enhancer and MEF2 transcription factors fine-tune the regulation of *Bdnf* gene in cortical and hippocampal neurons

J Biol Chem. 2024;300(6): 107411. doi: 10.1016/j.jbc.2024.107411





# An upstream enhancer and MEF2 transcription factors fine-tune the regulation of the *Bdnf* gene in cortical and hippocampal neurons

Received for publication, January 15, 2024, and in revised form, April 30, 2024. Published, Papers in Press, May 23, 2024.  
<https://doi.org/10.1016/j.jbc.2024.107411>

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Reviewed by members of the JBC Editorial Board. Edited by Elizabeth J. Coulson

The myocyte enhancer factor (MEF2) family of transcription factors, originally discovered for its pivotal role in muscle development and function, has emerged as an essential regulator in various aspects of brain development and neuronal plasticity. The MEF2 transcription factors are known to regulate numerous important genes in the nervous system, including brain-derived neurotrophic factor (BDNF), a small secreted neurotrophin responsible for promoting the survival, growth, and differentiation of neurons. The expression of the *Bdnf* gene is spatiotemporally controlled by various transcription factors binding to both its proximal and distal regulatory regions. While previous studies have investigated the connection between MEF2 transcription factors and *Bdnf*, the endogenous function of MEF2 factors in the transcriptional regulation of *Bdnf* remains largely unknown. Here, we aimed to deepen the knowledge of MEF2 transcription factors and their role in the regulation of *Bdnf* comparatively in rat cortical and hippocampal neurons. As a result, we demonstrate that the MEF2 transcription factor-dependent enhancer located at -4.8 kb from the *Bdnf* gene regulates the endogenous expression of *Bdnf* in hippocampal neurons. In addition, we confirm neuronal activity-dependent activation of the -4.8 kb enhancer *in vivo*. Finally, we show that specific MEF2 family transcription factors have unique roles in the regulation of *Bdnf*, with the specific function varying based on the particular brain region and stimuli. Altogether, we present MEF2 family transcription factors as crucial regulators of *Bdnf* expression, fine-tuning *Bdnf* expression through both distal and proximal regulatory regions.

Myocyte enhancer factor 2 (MEF2) transcription factors belong to the MADS box transcription factor superfamily, which in vertebrates consists of four paralogous members (MEF2A-D) (1–3). While the initial identification of MEF2

factors was predominantly associated with myogenesis (4, 5), subsequent research has demonstrated their pleiotropic roles within the central nervous system, where MEF2A, MEF2C, and MEF2D are mainly expressed (3, 6). It has become increasingly evident that MEF2 transcription factors have a significant role in synaptogenesis, neuronal survival, differentiation, and plasticity (7). All MEF2 proteins have a highly conserved N-terminus, containing the MADS-box and MEF2 domain, and a divergent C-terminus important for transactivation. The MADS-box and MEF2 domains mediate dimerization and binding of the MEF2 homo- or heterodimers to DNA consensus site (C/T)TA(A/T)<sub>4</sub>TA(G/A), known as MEF2 response element (MRE) (8–10). After binding, MEF2 transcription factors can activate or repress transcription of their target genes by recruiting coregulators like histone acetyltransferases or deacetylases, respectively (11–14).

One of the target genes of MEF2 transcription factors is *Bdnf* (6, 10, 15, 16), a neurotrophin that has an important role in the developing and mature organism by modulating neuronal survival, development, and plasticity (17). Rodent *Bdnf* gene contains eight 5' non-coding exons (I–VIII) and one 3' protein-coding exon (IX), with each exon regulated by its distinct promoter (18). The *Bdnf* transcripts are generated by splicing one of the non-coding exons (I–VIII) together with the coding exon (IX) (18, 19). In general, *Bdnf* transcripts of the first cluster (exon I, II, III) have a nervous system-specific expression pattern, whereas *Bdnf* exon IV- and VI-containing transcripts are expressed in both neural and non-neural tissues (18–20). The spatiotemporal expression of *Bdnf* gene is ensured by various transcription factors binding to its distinct promoters (16, 21–23) and through the use of different enhancer regions (24–26).

The role of MEF2 family transcription factors in the regulation and signaling of brain-derived neurotrophic factor (BDNF) has been previously demonstrated in numerous studies (6, 10, 15, 21, 27). For example, the BDNF-activated ERK5-MEF2 signaling pathway has been shown to induce the survival of newly generated cerebellar granule neurons (27) and embryonic cerebral neurons (28). Also, silencing of *Mef2a* and *Mef2d* using RNA interference reduced activity-dependent induction of *Bdnf* in rat hippocampal neurons (10). In rat

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cortical neurons, knockdown of *Mef2d* strongly increased activity-dependent expression of the *Bdnf* exon I-containing transcripts, while knockdown of *Mef2a* or *Mef2c* reduced neuronal activity-dependent expression of *Bdnf* exon I- or *Bdnf* exon IV-containing transcripts, respectively (6). Moreover, it has been shown that MEF2D needs the presence of CREB to bind *Bdnf* promoter IV in mouse cortical neurons (15), and we have previously shown the binding of MEF2A and MEF2C to *Bdnf* promoter IV by *in vitro* DNA pulldown assay in rat cortical tissue nuclear lysates (16). Finally, a region ~4.8 kb upstream of the *Bdnf* promoter I encompasses MRE, binds MEF2D transcription factor, and has been shown to regulate the neuronal activity-dependent transcription of *Bdnf* promoter I-driven reporter constructs in hippocampal neurons (10) but not in cortical neurons (6).

Although the role of MEF2 in the regulation of *Bdnf* has been described in several previous studies, the endogenous activity of the -4.8 kb MEF2-dependent enhancer and the contribution of MEF2 family transcription factors to the major *Bdnf* transcripts has not been addressed. Here, we aimed to comparatively study the role of MEF2 transcription factors in the regulation of *Bdnf* expression in cortical and hippocampal neurons. To achieve this, we used CRISPR/dCas9 system to show for the first time that the -4.8 kb MEF2 transcription factor-dependent enhancer regulates the endogenous expression of the first cluster of *Bdnf* transcripts in cultured hippocampal neurons. To elucidate the activity of the -4.8 kb enhancer *in vivo*, we used kainic acid-injected animals and detected robust activity-dependent transcription of -4.8 kb eRNAs in the hippocampus. Finally, RNA interference-mediated knockdown of MEF2 family members in both cultured cortical and hippocampal neurons revealed an interplay between MEF2 transcription factors in the regulation of *Bdnf* expression through promoter regions and the -4.8 kb enhancer. Together, these findings demonstrate how various members of the MEF2 family of transcription factors exert distinct effects on the expression of *Bdnf* depending on stimuli and brain regions.

## Results

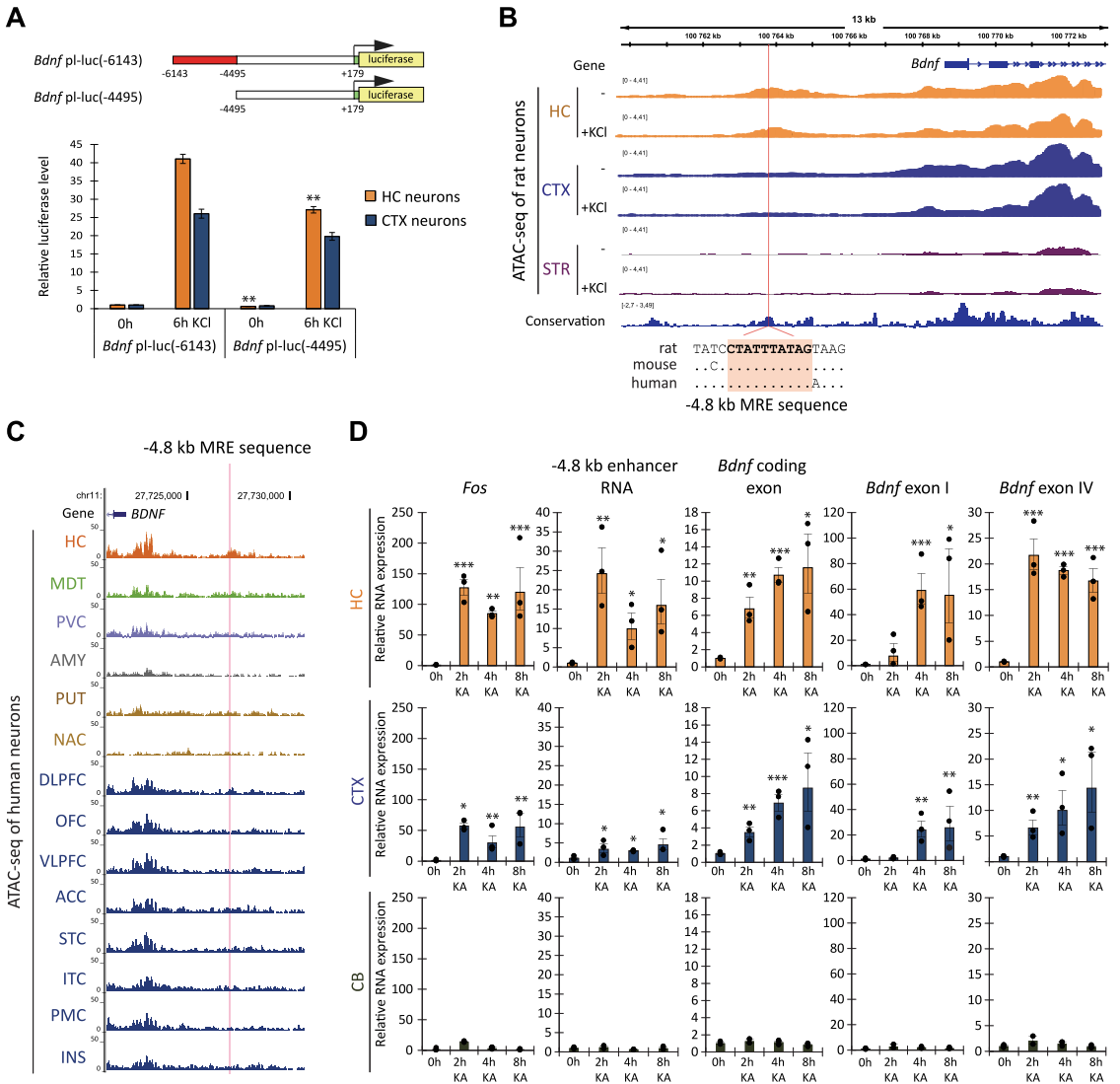
### The enhancer located 4.8 kb upstream of *Bdnf* exhibits neuronal activity-dependent transcription

Previous studies have demonstrated that a putative enhancer located ~4.8 kb upstream of the *Bdnf* exon I transcription start site promotes neuronal activity-dependent transcription from *Bdnf* promoter I in transient expression assays in hippocampal (10) but not in cortical neurons (6). Since different laboratories conducted these experiments using distinct protocols, we decided to comparatively investigate the impact of -4.8 kb enhancer on *Bdnf* promoter I activity in both cultured rat hippocampal and cortical neurons. For that, we conducted luciferase reporter assays using reporter constructs that were used previously (6, 10) and treated the neurons for 6 h with KCl to mimic neuronal activity. Our results showed that luciferase activity controlled by *Bdnf* promoter I without the -4.8 kb enhancer region decreased ~40%

( $p = 0.00153$ ) in untreated and ~34% ( $p = 0.00712$ ) in KCl-treated hippocampal neurons compared to luciferase activity driven by *Bdnf* promoter I with the -4.8 kb enhancer region (Fig. 1A). Notably, this effect was less evident and statistically not significant in untreated and KCl-treated cortical neurons, where the luciferase activity driven by *Bdnf* promoter I decreased ~20% ( $p = 0.09951$ ) and ~24% ( $p = 0.07329$ ) without the -4.8 kb enhancer, respectively (Fig. 1A). These results are in good agreement with previous findings (6, 10), and indicate that in heterologous context the -4.8 kb enhancer region potentiates the basal and KCl-induced activity of *Bdnf* promoter I specifically in hippocampal neurons.

Until now, research focusing on the -4.8 kb enhancer has been limited to luciferase assays. To study the -4.8 kb region as an enhancer in endogenous context, we initially analyzed previously obtained data of assay for transposase-accessible chromatin using sequencing (ATAC-seq) in rat hippocampal, cortical and striatal neurons (29) to study the chromatin state within the -4.8 kb region (Fig. 1B). The position of the -4.8 kb enhancer was determined by the MRE sequence previously described in Flavell *et al.* 2008 (10) and located at the center of the enhancer. The -4.8 kb enhancer exhibited stronger ATAC-seq signals in both untreated and KCl-treated rat hippocampal neurons than in cortical neurons. In striatal neurons the ATAC-seq signals within the *Bdnf* and -4.8 kb region were undetectable, which aligns with previous findings demonstrating that the mRNA levels of *Bdnf* are not detectable in rat striatum (Fig. 1B) (20, 30). As described previously (10), the -4.8 kb region is highly conserved in vertebrates and the -4.8 kb MRE element has identical sequence among rat, mouse and human, suggesting functionality of the -4.8 kb region also in other vertebrates (Fig. 1B). Next, to determine whether the -4.8 kb enhancer could be a regulatory region in humans, we used published ATAC-seq conducted in neurons from different human brain regions (31). Consistent with the ATAC-seq findings from rat neurons, the -4.8 kb region revealed open chromatin, particularly in human hippocampal neurons (Fig. 1C).

While open chromatin is widely associated with regulatory elements, it does not indicate a functional enhancer by default. In addition to chromatin accessibility, most of the active enhancers require the binding of RNA polymerase and transcription factors, which leads to the transcription of non-coding enhancer RNAs (eRNAs) (32, 33). It has been previously shown that eRNA levels correlate with the target gene expression (29, 33) and that the expression of *Bdnf* is robustly induced *in vivo* upon kainic acid (KA) treatment in different brain regions (30). Therefore, we hypothesized that -4.8 kb enhancer RNAs are also induced *in vivo* upon KA-induced neuronal activation. To test this hypothesis, rats were injected intracerebroventrically (ICV) with KA, a glutamate receptor agonist used as a robust model of epileptic seizures (34), or saline solution as a control. Rats were sacrificed 2 h, 4 h or 8 h after the onset of epileptic seizures and -4.8 kb eRNA together with *Bdnf* mRNA levels were analyzed using RT-qPCR from hippocampus, cerebral cortex and cerebellum, a brain region where *Bdnf* levels are not induced after KA



**Figure 1. The -4.8 kb enhancer exhibits enhancer-related characteristics in hippocampal neurons and adult hippocampal tissue.** *A*, schematics of luciferase constructs used for transfection. Red box represents mouse -4.8 kb enhancer region, white box mouse genomic region upstream of *Bdnf* promoter I, and mouse *Bdnf* promoter I (arrow indicates TSS) and yellow box indicates luciferase coding region. At 5 DIV, rat primary cortical and hippocampal neurons were transfected with the indicated constructs. At 6 DIV, tetrodotoxin (TTX) was added to neurons to inhibit spontaneous neuronal activity. At 7 DIV, transfected neurons were treated with KCl (25 mM) and D-APV (5  $\mu$ M) for 6 h or left untreated (CTRL). *Bdnf* promoter I activity was measured using luciferase assay. Luciferase levels measured from rat cortical or hippocampal neurons transfected with *Bdnf* pl-luc(-6143) and left untreated were taken as 1. Error bars indicate SEM (n = 4). For statistical analysis, two-tailed paired *t* test was performed between the luciferase levels in cells transfected with *Bdnf* pl-luc(-6143) and *Bdnf* pl-luc(-4495) upon the respective treatment. *B*, The Integrative Genomics Viewer tool was used to characterize the -4.8 kb enhancer region based on previously conducted ATAC-seq data (29), where rat cultured hippocampal, cortical and striatal neurons were treated with 10 mM KCl (1 h KCl) or vehicle (Neurobasal medium). The first cluster of rat *Bdnf* exons is shown as blue boxes (exons) and horizontal lines connecting boxes (introns). Orange-colored tracks represent data from hippocampal neurons (HC), blue-colored tracks indicate data from cortical neurons (CTX) and purple-colored tracks show data from striatal (STR) neurons. Light red vertical line represents the MRE sequence within the -4.8 kb enhancer. The conservation track shows conservation across 100 vertebrates (phyloP) and the genomic alignment of the -4.8 kb MRE sequence (CTATTATAG) in rat, mouse and human is indicated by light red shading. *C*, UCSC Genome Browser tool (<https://genome.ucsc.edu/>) was used to describe the -4.8 kb enhancer region based on previously conducted (31) NeuN+ ATAC-seq data in different human brain regions: (HC) hippocampus; (MDT) mediodorsal thalamus; (PVC) primary visual cortex; (AMY) amygdala; (PUT) putamen; (NAC) nucleus accumbens; (DLPCF) dorsolateral prefrontal cortex; (OFC) orbitofrontal cortex; (VLPFC) ventrolateral prefrontal cortex; (ACC) anterior cingulate cortex; (STC) superior temporal cortex; (ITC) inferior temporal cortex; (PMC) primary motor cortex; (INS) insula. The first exon (exon I) of human *BDNF* is shown as blue box, light red vertical line represents the MRE-sequence 4.8 kb upstream of *BDNF* exon I. *D*, Adult (3 months old) male Wistar rats were used for intracerebroventricular injection of kainic acid (KA). The experimental animals were randomly divided into four groups: ICV-injected with saline solution (CTRL) and ICV-injected with KA and sacrificed at the indicated time after the onset of seizures (2 h, 4 h or 8 h KA). The mRNA levels of *Fos*, -4.8 kb enhancer RNA, total *Bdnf* and *Bdnf* exon I- and IV-containing transcripts were measured from the rat hippocampus

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treatment (30). As a positive control of a neuronal activity regulated gene expression (35, 36), we measured the levels of *Fos* mRNA, that were induced after KA treatment *in vivo* in all analyzed brain regions (Fig. 1D). In addition, a strong, up to ~24-fold upregulation for -4.8 kb eRNAs was seen in rat hippocampus and ~3-5-fold induction in cerebral cortex, whereas in cerebellum the -4.8 kb eRNA levels were not induced upon KA treatment. Similarly, the mRNA levels of total *Bdnf*, *Bdnf* exon I and *Bdnf* exon IV were induced in the hippocampus and cortex but not in the cerebellum (Fig. 1D). Altogether, these results demonstrate that the -4.8 kb region exhibits enhancer-related characteristics, such as open chromatin and stimulus-dependent transcription, particularly in the hippocampus.

### CRISPR/dCas9 mediated targeting of the -4.8 kb region unveils a hippocampus-specific enhancer of the *Bdnf* gene

Enhancers can interact with and regulate one or several promoters located at considerable distances from their genomic location (37, 38). The -4.8 kb enhancer has been associated with the nearest regulatory region, *Bdnf* promoter I, in heterologous context (6, 10). Since the regulation of *Bdnf* expression involves several distinct promoters acting as independent regulatory hubs (18, 30), it is crucial to clarify the function of enhancer with the respect to each *Bdnf* promoter. Additionally, it is important to establish the connection between the enhancer and its target gene in the endogenous context. To study whether the alteration of the -4.8 kb enhancer affects the levels of different transcripts of *Bdnf* in an endogenous context, we used lentivirus-mediated CRISPR/dCas9 system to either repress (Fig. 2A) or activate (Fig. 2F) the enhancer in rat primary hippocampal and cortical neurons. Cultured neurons were treated for 3 h with KCl to mimic neuronal activity, and the levels of the -4.8 kb enhancer RNA and the *Bdnf* mRNAs were measured using RT-qPCR.

In cultured neurons the expression of -4.8 kb eRNA increased ~4-6-fold in hippocampal (Fig. 2, B and G) and ~2-3-fold in cortical neurons (Fig. 2, C and H) upon KCl treatment, showing the -4.8 kb region as an activity-dependent enhancer *in vitro*. To repress the enhancer, we used catalytically inactive Cas9 (dCas9) fused with the Krüppel associated box (KRAB) domain (dCas9-KRAB) coupled with 3 distinct gRNAs covering the -4.8 kb enhancer region. Repression of the -4.8 kb enhancer diminished the KCl-dependent expression of -4.8 kb eRNAs in hippocampal (Fig. 2B) but not in cortical neurons (Fig. 2C). Similarly, the repression of the -4.8 kb enhancer decreased the levels of the first cluster of *Bdnf* transcripts (*Bdnf* exon I-, IIc- and III-containing transcripts) in hippocampal neurons, where the strongest ~4-fold decrease was observed for *Bdnf* exon I-containing transcripts after KCl treatment (Fig. 2D). Repression of the enhancer neither

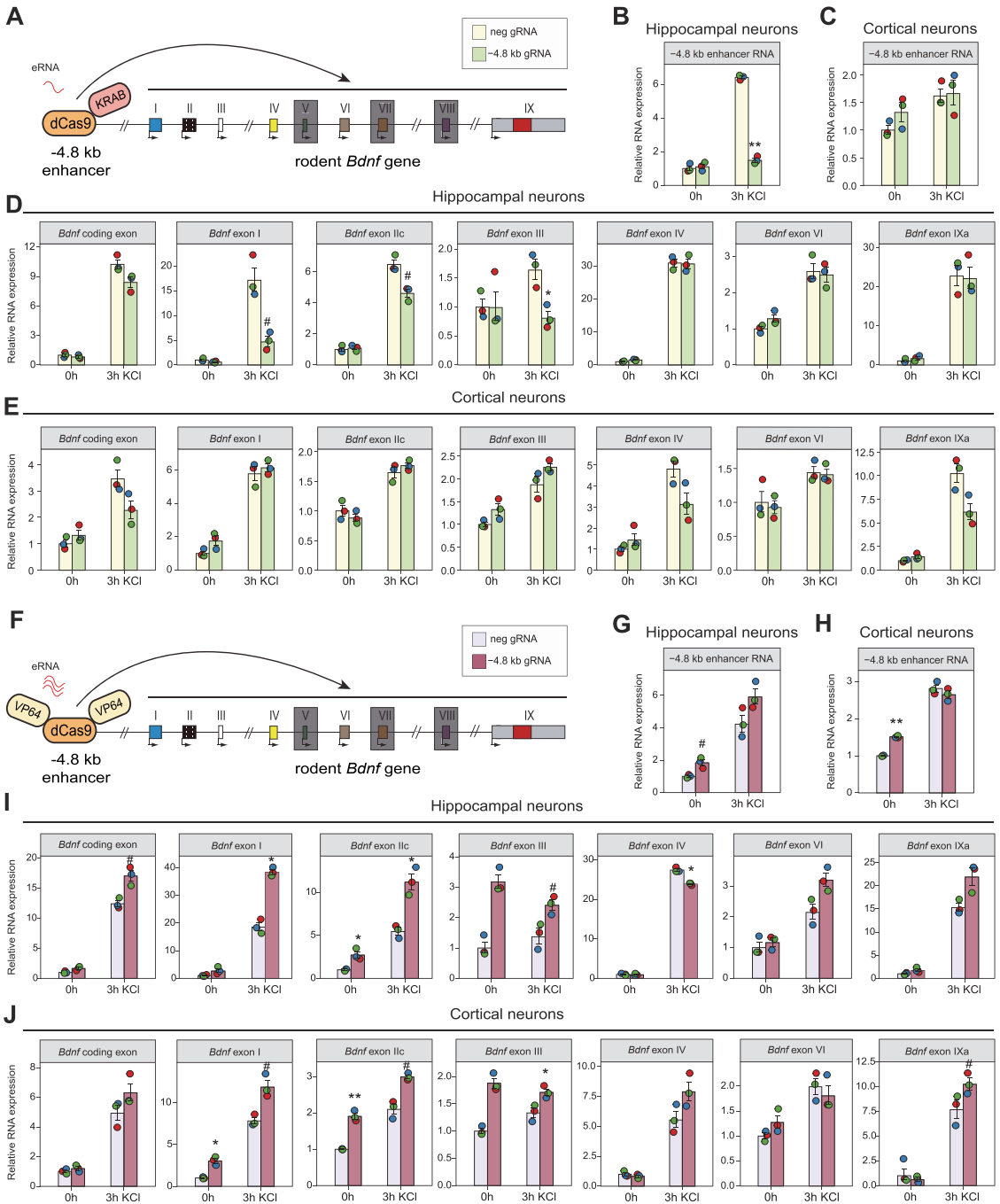
affected the other studied transcripts of *Bdnf* (*Bdnf* exon IV-, VI- and IX-containing transcripts) in hippocampal neurons (Fig. 2D) nor the expression levels of *Bdnf* mRNAs in cortical neurons (Fig. 2E).

Next, we used tandem repeats of transactivator VP16 domain fused with dCas9 (VP64-dCas9-VP64) together with 3 different gRNAs covering the -4.8 kb enhancer to induce the transcription from the enhancer. The activation of the -4.8 kb enhancer increased the -4.8 kb eRNA levels in untreated hippocampal (Fig. 2G) and cortical neurons (Fig. 2H). Activation of the -4.8 kb enhancer also increased *Bdnf* levels in untreated and KCl-treated hippocampal (Fig. 2I) and cortical neurons (Fig. 2J), where the first cluster of *Bdnf* transcripts was upregulated ~2-3-fold, indicating that the transcriptional activation by the CRISPR activator complex artificially induced the transcription from the -4.8 kb enhancer and upregulation of *Bdnf* expression. Although the -4.8 kb eRNA levels increased in both KCl-treated cultured cortical neurons (Fig. 2, C and H) and after KA treatment in adult cortical tissue (Fig. 1D), the repression of -4.8 kb enhancer had no effect on the -4.8 eRNA (Fig. 2C) and *Bdnf* levels in cultured cortical neurons (Fig. 2E). Therefore, the role of the -4.8 kb enhancer in the regulation of *Bdnf* in cortical neurons remains to be elucidated. We cannot rule out that the -4.8 kb enhancer regulates *Bdnf* expression in specific cortical cell type(s) or depending on developmental stage. Collectively, here we demonstrate for the first time that in the endogenous context the region 4.8 kb upstream of *Bdnf* is crucial for enhancing the neuronal activity dependent *Bdnf* expression in hippocampal neurons.

### The MEF2 family transcription factors regulate *Bdnf* via both proximal and distal regulatory regions

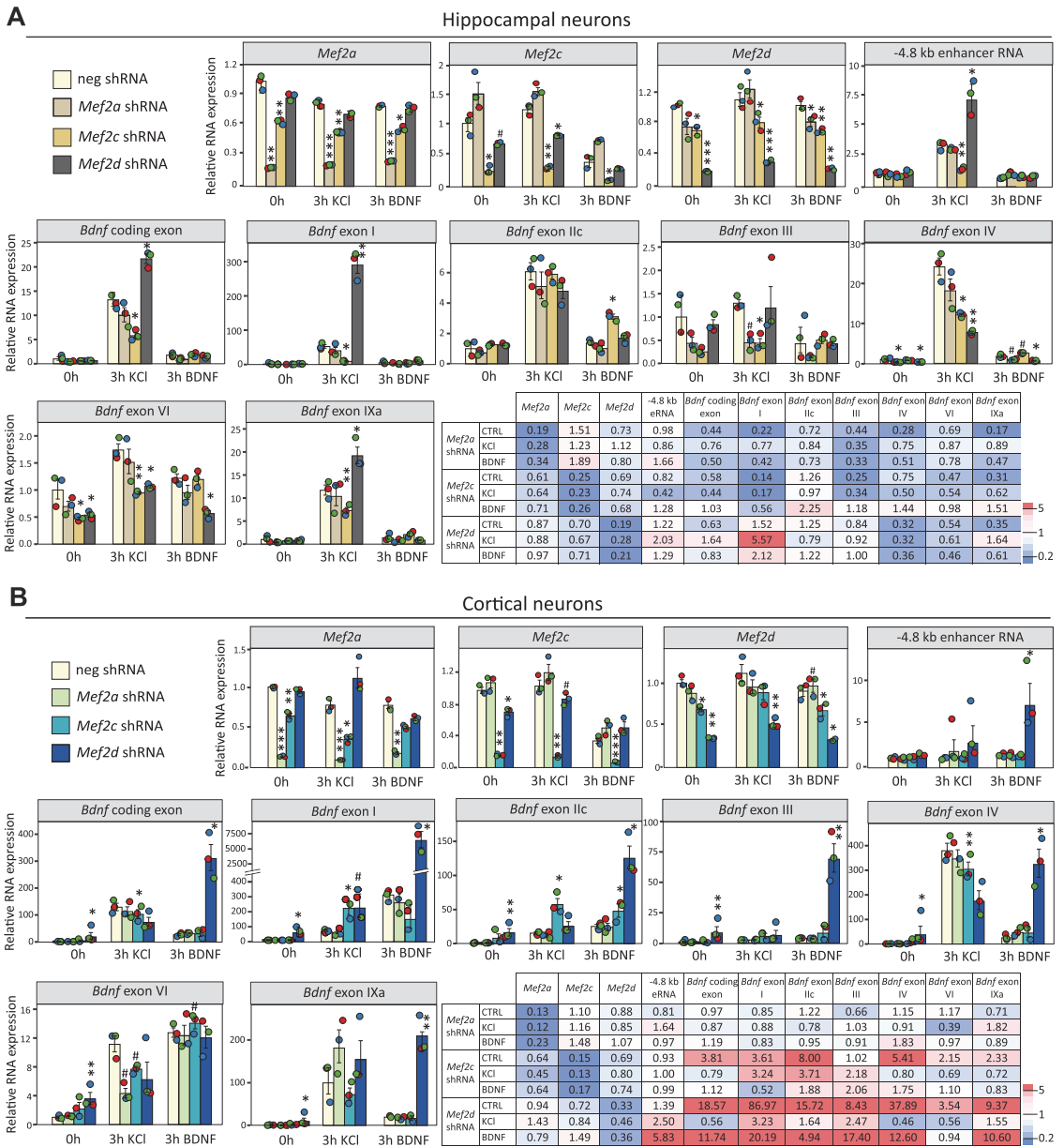
In addition to the -4.8 kb enhancer region, MEF2 transcription factors have been shown to regulate *Bdnf* promoter I and IV (10, 15), but the contribution to all the major promoters of *Bdnf* has not been addressed. Therefore, next we investigated the role of different endogenous MEF2 transcription factors in the regulation of *Bdnf* transcripts comparatively in cultured cortical and hippocampal neurons. For this, we used the RNAi-mediated knockdown of *Mef2a*, *Mef2c* or *Mef2d* transcription factors. *Mef2b* was excluded as it has been shown to have low expression in cultured cortical and hippocampal neurons (6). Rat hippocampal (Fig. 3A) or cortical (Fig. 3B) neurons were transduced with lentiviruses encoding *Mef2a*, *Mef2c*, *Mef2d* or negative control shRNA. As the MEF2 transcription factors are known to function upon stimuli, *for example*, neuronal activity and after BDNF-TrkB-signaling (39), neurons were left untreated or treated for 3 h with KCl or BDNF to induce membrane depolarisation or TrkB-signaling, respectively.

(HC), cerebral cortex (CTX) and cerebellum (CB). The expression levels were measured using RT-qPCR and normalized to *Hprt1* mRNA levels. The results from individual animals are depicted with dots and the average of respective enhancer RNA or mRNA levels in two to three animals is shown with the column. The average level of respective transcript measured from the CTRL group was set as 1. Error bars indicate SEM (n = 2-3 animals). Statistical significance was calculated with two-tailed unpaired unequal variance *t* test relative to the expression level of the respective transcript levels in the control animals. (A, D) \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001.



**Figure 2. Targeting the -4.8 kb enhancer with CRISPR/dCas9 system affects the expression of endogenous *Bdnf* in rat hippocampal and cortical neurons.** (A–J) At 0 DIV, rat hippocampal (B, D, G, I) and cortical neurons (C, E, H, J) were infected with negative control gRNA or with the pool of 3 gRNAs covering the -4.8 kb enhancer region (-4.8 kb gRNA) and dCas9-KRAB (A–E) or VP64-dCas9-VP64 (F–J) coding lentiviruses. At 7 DIV, tetrodotoxin (TTX) was added to neurons to inhibit spontaneous neuronal activity. At 8 DIV, neurons were treated with KCl (25 mM) and D-APV (5  $\mu$ M) for 3 h or left untreated. Expression of the -4.8 kb eRNAs (B–C, G–H), total *Bdnf* (*Bdnf* coding exon) and different *Bdnf* transcripts (D–E, I–J) were measured using RT-qPCR. For every experiment, the expression level of the respective RNA in cells transduced with dCas9-KRAB or VP64-dCas9-VP64 and negative gRNA-coding lentiviruses and left untreated was taken as one. The dots indicate results from biological replicates (n = 3), with error bars showing SEM. For statistical analysis, two-tailed paired t test was performed between the RNA levels in cells transduced with lentiviruses encoding -4.8 kb enhancer-specific gRNAs and negative control gRNA and either dCas9-KRAB or VP64-dCas9-VP64 upon the respective treatment, #p < 0.1, \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

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**Figure 3. Silencing of Mef2 transcription factors alters the transcription from different Bdnf promoters and -4.8 kb enhancer in cortical and hippocampal neurons.** A and B, at 0 DIV rat hippocampal (A) or cortical (B) neurons were infected with lentiviruses encoding Mef2 shRNAs (*Mef2a*, *Mef2c* or *Mef2d*) or negative control shRNA (neg shRNA). At 7 DIV, tetrodotoxin (TTX) was added to neurons to inhibit spontaneous neuronal activity. At 8 DIV neurons were treated with 25 mM KCl together with 5  $\mu$ M D-APV or 50 ng/ml BDNF for 3 h or left untreated (0 h). The mRNA expression levels of different Mef2 family members, Bdnf transcripts and -4.8 kb eRNA were measured using RT-qPCR. The expression levels in cells transduced with negative shRNA-encoding lentiviruses and left untreated was set as 1. All biological replicates (n = 3) are shown as dots. Error bars indicate SEM. Asterisks indicate statistical significance calculated using two-tailed paired t test between the respective transcript levels measured from neurons transduced with neg shRNA and indicated Mef2 shRNA at respective treatment, #p < 0.1, \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. To simplify the understanding of the results, we arranged the heatmap coloring based on the relative fold-change, which was calculated against the respective negative control shRNA treatment type. On color scale, red demonstrates increase, and blue shows decrease in the expression level. Note that the color scale has been capped for both hippocampal and cortical results to ensure comparability (i.e., fold-changes >5 in both heatmaps are shaded in the darkest red or blue).

First, we analyzed the efficiency of the knockdown tools by measuring the mRNA levels of *Mef2* family transcription factors. Silencing of *Mef2a*, *Mef2c* or *Mef2d* decreased the mRNA levels of all the respective *Mef2* family members in untreated as well as in KCl- or BDNF-treated hippocampal and cortical neurons. *Mef2a* and *Mef2c* shRNAs silenced the target genes with 80 to 90% efficiency (Fig. 3, A and B), silencing of *Mef2d* shRNA in untreated and treated cortical neurons was slightly weaker, resulting in 50 to 70% reduction of *Mef2d* mRNA (Fig. 3B).

Next, we studied the effect of MEF2-silencing on -4.8 kb eRNA expression (Fig. 3, A and B). In KCl-treated hippocampal neurons, silencing of *Mef2c* decreased the level of -4.8 kb eRNA ~2.5-fold, while silencing of *Mef2d* increased -4.8 kb eRNA levels ~2-fold (Fig. 3A). Surprisingly, silencing of *Mef2d* increased -4.8 kb eRNA levels ~6-fold in BDNF-treated cortical neurons, while silencing of other *Mef2* family members had no effect on the level of -4.8 kb eRNAs in treated cortical neurons (Fig. 3B). These results indicate that MEF2D represses the -4.8 kb enhancer in cortical and hippocampal neurons, and that MEF2C acts as a transcriptional activator of this enhancer in KCl-treated hippocampal neurons.

Silencing of *Mef2a* had a tendency to decrease the levels of total *Bdnf* and almost all *Bdnf* transcripts in untreated and BDNF-treated hippocampal neurons (Fig. 3A), while silencing of *Mef2a* did not have any effect on the levels of *Bdnf* mRNA in cortical neurons (Fig. 3B). Silencing of *Mef2c* decreased the expression of *Bdnf* in untreated, KCl- and BDNF-treated hippocampal neurons, with the highest decrease, ~6-7-fold, seen for *Bdnf* exon I-containing mRNA in untreated and KCl-treated neurons (Fig. 3A). Although silencing of *Mef2c* increased the levels of first cluster of *Bdnf* transcripts in KCl-treated cortical neurons, the expression levels of the second cluster of *Bdnf* transcripts and total *Bdnf* were decreased (Fig. 3B). Since *Bdnf* exon IV-containing transcripts are the major source of *Bdnf* mRNA in the cortical neurons (40), the small increase in the transcripts with exons from the upstream cluster did not influence significantly the total *Bdnf* mRNA levels. Silencing of *Mef2d* had the most drastic effects on the expression of *Bdnf*. In hippocampal neurons the *Bdnf* exon I-containing transcripts were upregulated ~6-fold after KCl-treatment and *Bdnf* exon IV-containing transcripts were downregulated regardless of treatment (Fig. 3A). In cortical neurons the silencing of *Mef2d* led to significant upregulation of *Bdnf* expression both in untreated and BDNF-treated, but not in KCl-treated neurons (Fig. 3B).

Collectively, we determined that among the MEF2 family of transcription factors, MEF2C and MEF2D primarily govern the expression of *Bdnf* in both hippocampal and cortical neurons. Although we cannot definitively ascertain whether the observed effects of silencing are direct or indirect, our findings strongly suggest that the MEF2 family transcription factors have a pleiotropic role in the complex regulation of *Bdnf* gene, acting through both proximal and distal regulatory regions in distinct ways in cortical and hippocampal neurons.

## Discussion

In this study, we show how the family of MEF2 transcription factors exerts multifaceted control over the expression of its target gene *Bdnf*. This regulation involves distinct mechanisms operating in response to different stimuli and brain regions. While previous research has shown the -4.8 kb region to enhance *Bdnf* promoter I in heterologous context (6, 10), our findings show for the first time that in endogenous context the -4.8 kb enhancer regulates *Bdnf* in hippocampal neurons. Notably, we provide evidence of the -4.8 kb enhancer activity in rodents *in vivo* and describe the -4.8 kb enhancer in an open chromatin conformation in human hippocampal neurons. Finally, we determine that after silencing the MEF2 family members, MEF2C and MEF2D have the most significant impact on the transcriptional regulation of *Bdnf* in both cultured hippocampal and cortical neurons. Altogether, our results highlight the intricate interplay between MEF2 family transcription factors in the regulation of BDNF expression.

We demonstrate that the -4.8 kb enhancer regulates the first cluster of *Bdnf* transcripts, containing *Bdnf* exons I, IIc, and III, after neuronal activity in hippocampal neurons. The first cluster of *Bdnf* exons is known to exhibit a highly neuron-specific expression pattern and is suggested to be coregulated as a single cluster of promoters (18–21). We have previously described an intronic enhancer region located downstream of the *Bdnf* exon III (+3 kb enhancer) which plays a crucial role in BDNF-TrkB signaling- and neuronal activity-induced expression of the first cluster of *Bdnf* transcripts in neurons (25). Although further studies are needed to determine the precise looping interactions, it is plausible that the -4.8 kb and +3 kb enhancer regions could synergistically facilitate the neuronal activity-dependent expression of the first cluster of *Bdnf* exons in hippocampal neurons. This aligns well with the hypothesis proposed in Tuvikene *et al.* 2021 (25), suggesting that the +3 kb enhancer functions as an anchor site for chromatin interactions between the *Bdnf* (promoters I, II, II) and the respective enhancers.

The hippocampus and cerebral cortex are the brain regions where BDNF is highly expressed (18–20). In addition to transcription factors involved in *Bdnf* regulation in both these brain regions (*e.g.*, CREB, NPAS4), numerous transcription factors regulate individual promoters of *Bdnf* depending on the brain region (16, 21). Although all *Bdnf* transcripts encode the same protein, it is known that transcript-specific modulation of *Bdnf* causes differential downstream cascades and phenotypes (41, 42). For example, in the hippocampus the upregulation of *Bdnf* exon I-containing transcripts increases the complexity of dendritic spine morphology, while upregulation of *Bdnf* exon IV-containing transcripts reduces fear expression (42). In contrast, mutations in *Bdnf* promoter IV impair the neuronal activity-dependent expression of BDNF important for the proper development of inhibitory synapses in the cerebral cortex (15). A differential engagement of enhancer regions could ensure this distinctive proportion of *Bdnf* transcripts in cortical and hippocampal neurons. Accordingly, the MEF2-dependent -4.8 kb enhancer is responsible for the

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increase in *Bdnf* exon I-containing transcripts upon neuronal activity and could thereby contribute to the complexity of dendritic spines in the hippocampus. Conversely, and as shown in the current study, the -4.8 kb enhancer is likely dispensable for the regulation of *Bdnf* exon IV-containing transcripts in cortical neurons, which comprise the vast majority of *Bdnf* transcripts needed there to refine synaptic connectivity. Moreover, the ability of MEF2 transcription factors to cooperate with cell type-specific transcription factors to activate tissue-specific enhancers (43) might explain the differential activation of regulatory regions crucial for BDNF expression in the cortex and hippocampus.

To date, the binding of MEF2 transcription factors has been linked to the -4.8 kb enhancer in hippocampus (10) and *Bdnf* promoter IV in cerebral cortex (6, 15, 16). The MEF2 transcription factor binding is not always restricted to the high affinity MRE site. For instance, MEF2 activates the transcription from *Bdnf* promoter IV through the CaRE1 element (6). In addition to proximal promoters, widespread binding of MEF2 transcription factors has been observed in distal regulatory regions (10, 43). Moreover, MEF2D binding to enhancers that lack the consensus MRE site has been shown to play a crucial role in photoreceptor cells, where MEF2D in cooperation with cell-specific transcription factors regulate the genes critical for photoreceptor functioning (43). Therefore, it is important to consider that our MEF2 knockdown results may arise from a combination of regulatory mechanisms, *i.e.*, binding of the MEF2 transcription factors both to proximal and distal regulatory regions of *Bdnf* gene and also to the regulatory regions of transcription factors regulating *Bdnf*.

While MEF2A, MEF2C, and MEF2D are widely expressed in the central nervous system, the proportion of each MEF2 family member varies significantly depending on the particular brain region (3, 6). For example, the expression of MEF2C in the hippocampus is highly restricted to the dentate gyrus (3, 44), where MEF2C is responsible for the development and morphology of dendritic spines (45, 46). Similarly, conditional deletion of MEF2C, but not MEF2A and MEF2D, is linked with altered memory formation and synaptic plasticity in the hippocampus (45, 47, 48), and MEF2C-related aberrations have been implicated in the development of autism-spectrum disorders (ASDs) (7). Recently it was shown that deletion of *Mef2c* specifically in adult-born dentate granule cells impaired contextual fear memory and caused deficits in social interactions, behavioral patterns associated with ASD (46). Although we have not addressed the exact region or subpopulation of neurons in the hippocampus in our studies, silencing of *Mef2c* had a profound effect, resulting in a significant reduction not only in the levels of the -4.8 kb eRNA but also in the overall expression of *Bdnf* mRNA in both basal levels and in KCl-treated hippocampal neurons. Further work on MEF2-regulated *Bdnf* expression in specific hippocampal subregions on single-cell level is needed. Nevertheless, our results describing *Bdnf* as a crucial downstream mediator of MEF2C could help to decipher the signaling cascade important for the proper synaptic development in the hippocampus.

Previous genome-wide studies have suggested that the redundancy and compensation among MEF2 family members can complicate the studies of MEF2 transcription factors (10, 48, 49). It is tempting to speculate that the observed effects on *Bdnf* regulation may also result from the intricate interplay between MEF2 family members. For example, when *Mef2d* is silenced, MEF2C might compensate for its absence, potentially enhancing the transcription of *Bdnf*. Studies in mouse cerebellar granule neurons have demonstrated that conditional MEF2D knockout induces compensatory genomic occupancy by MEF2A, but only at specific MEF2D sites (50). Although beyond the scope of this study, it would be interesting to study the endogenous binding of all the MEF2 transcription factors to *Bdnf* proximal and distal regulatory regions in wild-type and MEF2 knockout models in hippocampal and cortical neurons to confirm the direct participation in the transcriptional regulation.

From the MEF2 family members investigated in this study, silencing of *Mef2a* leads to the mildest effects when compared to the knockdown of *Mef2c* and *Mef2d*. It has been shown that brain-specific *Mef2a* knockout mice have no abnormalities, *Mef2a-Mef2d* double knockout mice have deficits in motor coordination but otherwise remain viable, and *Mef2a-Mef2c-Mef2d* triple knockout mice have decreased body weight together with neuronal apoptosis and postnatal lethality (48). Conversely, homozygous MEF2A knockout mice die during the first postnatal week due to the cardiac dysfunction (51), while MEF2D knockout animals appear to be vital and fertile, but exhibit problems in the circadian system as well as maturation and survival of photoreceptors (43, 52, 53). The described phenotypic peculiarities indicate that MEF2A might have a strong redundancy or it does not play a central role in the neural system, as seen for the regulation of *Bdnf* gene in cortical neurons.

In agreement with previous results (6), the silencing of *Mef2d* led to a significant increase in the mRNA levels of *Bdnf*. It is known that MEF2 transcription factors can, in addition to positive effects, repress their target genes (39). For example, in muscle cells, HDAC4 has been shown to form a repressor complex with MEF2D to control the regulation of genes (12, 13). Similarly, MEF2D in complex with HDAC4 could repress the expression of *Bdnf* gene in rat hippocampal and cortical neurons. We cannot rule out the possibility that MEF2D is crucial to refine the proper levels of BDNF, as abnormally increased levels of BDNF have been observed in the hippocampus and cortex of patients with temporal lobe epilepsy (54, 55). Whether the increase in *Bdnf* levels following the silencing of *Mef2d* indicates the effect of compensation or a way to refine the proper expression levels of BDNF, is a question that needs to be elucidated in the future.

In conclusion, the fine-tuning of *Bdnf* gene by MEF2 transcription factors illustrates distinct but robust regulatory mechanisms in the cortex and hippocampus. However, numerous exciting research questions are still awaiting exploration. For example, how do various MEF2 family members differentially control *Bdnf* transcription? How do the *Bdnf* promoters interact with the -4.8 kb enhancer and are the

interactions different across distinct cell types? How do the outcomes of the current study relate to the regulation of *Bdnf* at the single cell level? Future studies of this intricate relationship will hopefully unlock important insights, clarifying not only the regulation of *Bdnf* gene but also adding new knowledge on the functioning of the brain.

## Experimental procedures

### Rat primary cortical and hippocampal neuron culture

For cultured neurons, all animal procedures were conducted in accordance with European Directive 2010/63/EU, reviewed and approved by the Ministry of Agriculture of Estonia (Permit Number: 45). Animals were maintained under a 12 h light/dark cycle in a humidity ( $50 \pm 10\%$ ) and temperature ( $22 \pm 1^\circ\text{C}$ ) controlled room. Rats were group-housed (2–4 animals per cage), using either conventional polycarbonate or H-TEMP polysulfone cages with ad libitum access to water and food. The cultures of rat cortical and hippocampal neurons were generated from embryonic (E20/21) Sprague Dawley rats. Briefly, cortices and hippocampi were dissected and incubated in 1 ml of 0.25% trypsin and 1 mM EDTA solution (Gibco) for 10 min at  $37^\circ\text{C}$ . Next, DNaseI (Roche) and  $\text{MgSO}_4$  (Sigma) were added to the trypsinized tissue to a final concentration of 0.5 mg/ml and 12 mM, respectively, and incubated for 10 min at  $37^\circ\text{C}$ . Then, 275  $\mu\text{l}$  of trypsin inhibitor (1%, Gibco), 110  $\mu\text{l}$  of BSA (10%, Pan Biotech) and 50  $\mu\text{l}$  of DNaseI (5 mg/ml, Roche) were added. The tissue was triturated and the resulting suspension was diluted in  $1\times$  Hank's balanced salt solution. The solution was centrifuged at 200g for 30 s. The supernatant was centrifuged again at 200g for 6 min. The supernatant was removed, and the precipitation was resuspended in prewarmed DMEM (Corning) containing 10% FBS (Pan Biotech). Plates were pretreated in 0.1 M borate buffer with poly-L-lysine (0.2 mg/ml, Sigma Aldrich) for at least 1 h at room temperature. Cells were incubated for 2 h at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ . Then the medium was replaced with new cell medium (Neurobasal-A medium (NBA, Gibco) containing  $1 \times$  B27 supplement (Gibco), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Gibco) or 100  $\mu\text{g}/\text{ml}$  primocin (Invivogen) and 1 mM L-glutamine (Gibco)). At 2 days *in vitro* (DIV), all of the medium was changed and 10  $\mu\text{M}$  mitotic inhibitor 5'-fluoro-2'-deoxyuridine (Sigma-Aldrich) was added to inhibit proliferation of non-neuronal cells.

### Intracerebroventricular injection of kainic acid

Adult (3-month-old) male Wistar rats were used for intracerebroventricular injection (ICV) of kainic acid. The animals were housed in a specific pathogen-free environment, three per polypropylene cage in controlled temperature ( $23 \pm 2^\circ\text{C}$ ), humidity ( $50 \pm 5\%$ ) and light (12 h light/dark cycle), with ad libitum access to food and water. The experiments were carried out in accordance with the National Institute of Health Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (The National Academics Press), European Communities Council Directive 2010/63/EU revising Directive 86/609/EEC, and national Decree-Law No 26 on March 4, 2014, and were approved by the local Animal Care Committee (OPBA) of

University of Palermo, Italy and Ministry of Health, Italy. Up to 12 animals were randomly divided into four experimental groups: control (ICV-injected with saline solution), kainate 2 h, 4 h or 8 h (ICV-injected with kainate solution and sacrificed 2 h, 4 h or 8 h, respectively, after the onset of seizures). The surgical procedure was performed under aseptic conditions. Rats were initially anesthetized with intraperitoneal injection of chloral hydrate (200 mg/kg) and kept anesthetized for the entire duration of the surgical procedure with isoflurane. This procedure allows the early recovery of the animal from anesthesia immediately after the end of the surgery and the early and full manifestation of seizure behavior. Rats were placed in a David Kopf stereotaxic apparatus and received bilateral ICV injections of 0.35  $\mu\text{g}/\mu\text{l}$  of kainate (Merck, dissolved in 0.9% physiological saline), using the following stereotaxic coordinates from the Bregma (according to Ref. (56)): AP = 0, L = 1.4, and V = 4.3. The control group was ICV-injected with 1  $\mu\text{l}$  of 0.9% physiological saline. Injections were performed by 30-gauge injector cannula that was connected by a piece of polyethylene tube to the 10  $\mu\text{l}$  Hamilton syringe. Each injection was performed over 3 min, and following injection, the needle remained in the target location for 3 min to avoid kainate solution reflux along the needle tract and to achieve a proper diffusion of the drug. Animals were sacrificed by decapitation at different time points after the onset of epileptic seizures. Brain was quickly removed for dissection of hippocampus, frontal cortex, and cerebellum. Dissected brain areas were rapidly frozen and stored at  $-80^\circ\text{C}$  for later use.

### Lentivirus production and transduction

All used lentiviruses were produced in HEK293FT cell line (Thermo Fisher Scientific) on 145 mm cell culture dishes, cultured in DMEM (Pan Biotech) with 10% fetal bovine serum (FBS, Pan Biotech) and 1% penicillin-streptomycin (100 U/ml penicillin, 0.1 mg/ml streptomycin, Gibco) medium. The transfection solution contained 18.22  $\mu\text{g}$  effector, 13.67  $\mu\text{g}$  psPAX2 (Addgene) and 9.11  $\mu\text{g}$  pVSVG (Invitrogen) plasmids with 2:1 PEI to DNA ratio. HEK293FT cells were transfected for  $\sim 16$  h at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ . After that, the transfection solution was replaced with 24 ml lentivirus production medium (DMEM (Pan Biotech), 10% FBS (Sera Plus), 1 mM pyruvate,  $1\times$  non-essential amino acids (Gibco), and 20 mM HEPES, pH 7.4). Medium containing lentivirus particles were collected twice with 24 h interval. To purify lentiviruses, the collected medium was centrifuged at 4500g for 5 min at  $+4^\circ\text{C}$  and the supernatant was filtered through a 0.45  $\mu\text{m}$  polyvinylidene difluoride filter (Merck). 1/9 volume of Speedy Lentivirus Purification solution (Abm) was added to medium and lentiviral particles were precipitated with centrifugation at 7000g for 1 h at  $+4^\circ\text{C}$ . Lentiviral particles were resuspended in phosphate buffered saline (PBS) buffer.

To determine the relative lentivirus titres, neurons were infected with different dilutions of viruses at 0 DIV. At 7 DIV, 0.1 mg/ml DNaseI (Roche) with 10 mM  $\text{MgSO}_4$  (Sigma) was added to neurons and incubated for 20 min at  $37^\circ\text{C}$  to eliminate plasmid DNA contamination. Next, cells were lysed



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in proteinase K lysis buffer (0.08 mg/ml proteinase K, 30 mM Tris-HCl (pH 8.0), 1% Tween 20, 0.2% NP40, 1 mM EDTA). The lentiviral titers were analyzed based on provirus incorporation using provirus-specific Woodchuck Hepatitis virus post-transcriptional regulatory element or puromycin resistance (PURO) primers, and unrelated genomic region (for normalization (Supporting information)). For functional experiments, equal amounts of lentiviral particles were used to transduce neurons, achieving >95% transduction efficiency. Rat cortical neurons were transduced at 0 DIV. At 7 DIV, tetrodotoxin was added to neurons to inhibit spontaneous neuronal activity. At 8 DIV, cells were treated with 25 mM KCl (PanReac AppliChem) and 5  $\mu$ M D-(2R)-amino-5-phosphovaleric acid (D-APV, Cayman Chemical), with 50 ng/ml BDNF (Peprotech) or left untreated. After 3 h of treatment, neurons were lysed in 500  $\mu$ l RLT buffer (Qiagen) containing 1%  $\beta$ -mercaptoethanol (ROTH).

### RNA isolation, cDNA synthesis and qPCR

Total RNA from rat cortical neurons or ICV-injected rat brain tissue was isolated using RNeasy Mini Kit (Qiagen) or with RNeasy lipid tissue kit (Qiagen), respectively, with on-column DNase digestion using RNase-free DNase set (Qiagen) according to the manufacturer's instructions. RNA concentration was measured with a BioSpec-nano spectrophotometer (Shimadzu) or with a Nanodrop 2000c (Thermo Scientific) spectrophotometer. cDNA was synthesized from equal amounts of total RNA using Superscript III or IV Reverse Transcriptase (Thermo Fisher Scientific) with 1:1 mixture of oligo(dT)<sub>20</sub> (Microsynth) and random hexamer primers (Microsynth). qPCR was performed in triplicates using 1  $\times$  HOT FIREpol EvaGreen qPCR Mix Plus (Solis Biodyne) or 1  $\times$  LightCycler 480 SYBR Green I Master (Roche) on LightCycler 480 II Real Time PCR instrument (Roche). All used qPCR primers are shown in Supporting information. *Hprt1* mRNA levels were used to normalize gene and enhancer RNA expression.

### DNA constructs

The RNA interference (pLKO.1-neg-shRNA, pLKO.1-MEF2A-shRNA, pLKO.1-MEF2C-shRNA, pLKO.1-MEF2D-shRNA) and luciferase plasmids (pGL3-BDNFpI-luc(-6143) and pGL3-BDNFpI-luc(-4495)) were kind gift from A. E. West, Department of Neurobiology, Duke University, North Carolina, United States of America. The gRNA targeting sequences (Supporting information) of the -4.8 kb enhancer were designed using Benchling CRISPR tool (<http://www.benchling.com>) as described previously in Avarlaid *et al.* 2024 (24), and cloned into the pRRL-U6-gRNA-hPGK-EGFP plasmid. Briefly, a total of 3 gRNAs targeting the ~400 bp core region covering the -4.8 kb MRE sequence were used and the gRNA viruses were pooled for the CRISPR/dCas9 experiments. The plasmids (pLV-hUbC-dCas9-KRAB-T2A-GFP, pLV-hUbC-VP64-dCas9-VP64-T2A-GFP) used for CRISPR/dCas9-mediated modulation of the -4.8 kb enhancer were previously described in Tuvikene *et al.* 2021 (25).

### Transfection of cells and luciferase reporter assay

Rat cortical neurons were transfected at 5 DIV using Lipofectamine 2000 (Invitrogen) reagent. The transfection was carried out as duplicates in 48-well cell culture plates with each well containing 180 ng of luciferase reporter plasmid and 20 ng of pGL4.83-mPGK-hLuc normalizer plasmid in unsupplemented NBA with DNA to Lipofectamine ratio of 1:3. The transfection was carried out for 3 to 4 h and was terminated by changing the media back to the supplemented NBA that was previously collected from the neurons.

At 6 DIV, TTX (1  $\mu$ M, Tocris Bioscience) was added to prevent spontaneous neuronal activity. At 7 DIV, neurons were treated with KCl (25 mM, PanReac AppliChem) and D-APV (5  $\mu$ M, Cayman Chemical) for 6 h or left untreated. After treatments at 7 DIV the cortical and hippocampal neurons were lysed in 1  $\times$  Passive Lysis Buffer (Promega) and luciferase assay was performed using Dual-Glo Luciferase Assay (Promega) system. Luminescence signal was measured using GENios Pro Multifunction Microplate Reader (Tecan). For data analysis, background corrected Firefly luciferase signals were normalized with background corrected Renilla luciferase signals and the averages of duplicates were calculated.

### Statistical analysis

Sample size estimation was not performed, and randomization or blinding were not used. All tested hypotheses were specified before conducting the experiments. For cultured primary neurons, biological replicates were cultures obtained from rat pups of different litters. For *in vivo* experiments, biological replicates were individual animals. For statistical analysis, normalized data was log-transformed, mean centered, and autoscaled. The results were analyzed using Microsoft Excel 365 or RStudio version 2021.09.0 and R version R 4.1. Statistical significance was calculated using a two-tailed paired or unpaired *t* test, as indicated in figure legends, and *p*-values were not corrected for multiple comparisons. For graphical representation, data was backtransformed and error bars indicate upper and lower limits of backtransformed means  $\pm$  SEM.

### Data availability

All data are contained within the article.

**Supporting information**—This article contains supporting information.

**Acknowledgment**—We thank Epp Väli for technical assistance, Indrek Koppel and Florencia Cabrera-Cabrera for critical reading of the manuscript, and Anne E. West (Department of Neurobiology, Duke University) for providing the plasmids used in luciferase assay and RNA interference experiments.

**Author contributions**—T. T., M. F., J. T., A. A., N. B., V. D. L., and G. M. conceptualization; T. T. funding acquisition; T. T. project administration; T. T., J. T., and A. A. supervision; T. T., M. F., J. T., A. A., K. F., V. D. L., K. L., and G. M. writing—original draft; T. T., M.

F., J. T., A. A., V. D. L., and G. M. writing—review & editing; M. F., A. A., K. F., N. B., V. D. L., K. L., and G. M. investigation; M. F., N. B., V. D. L., and G. M. resources; A. A., K. F., and K. L. formal analysis; A. A., K. F., and K. L. visualization; J. T. and A. A. methodology

**Funding and additional information**—This work has been supported by Estonian Research Council (institutional research funding IUT19-18 and grant PRG805), European Union through the European Regional Development Fund (Project No. 2014–2020.4.01.15-0012) and H2020-MSCA-RISE-2016 (Grant EU734791), and European Commission and Estonian Research Council (ERA-NET NEURON Cofund2 programme grant GDNF UpReg).

**Conflict of interest**—Jürgen Tuvikene and Tõnis Timmusk were employees of Protobios LLC. The authors declare no competing financial interests.

**Abbreviations**—The abbreviations used are: ATAC-seq, transposase-accessible chromatin using sequencing; BDNF, brain-derived neurotrophic factor; ICV, intracerebroventricularly; KA, kainic acid; MEF2, myocyte enhancer factor; MRE, MEF2 response element.

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55. Martínez-Levy, G. A., Rocha, L., Rodríguez-Pineda, F., Alonso-Vanegas, M. A., Nani, A., Buentello-García, R. M., *et al.* (2018) Increased expression of brain-derived neurotrophic factor transcripts I and VI, cAMP response element binding, and glucocorticoid receptor in the cortex of patients with temporal lobe epilepsy. *Mol. Neurobiol.* **55**, 3698–3708
56. Paxinos, G., and Watson, C. (1998) *The Rat Brain in Stereotaxic Coordinates*, Academic Press, San Diego

# Curriculum vitae

## Personal data

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## Education

2018– Tallinn University of Technology, PhD  
2016–2018 Tallinn University of Technology, MSc in gene technology, diploma *cum laude*  
2013–2016 Tallinn University of Technology, BSc in applied chemistry and biotechnology  
2001–2013 Vinni-Pajusti High School

## Employment

2023– Estonian Accreditation Centre, technical assessor  
2018– Tallinn University of Technology, Early-Stage Researcher  
2016–2018 Protobios LLC, trainee  
2015–2016 Mektory, instructor of science course  
2015 AS Rakvere haigla, trainee

## Awards

2023 Kristjan Jaak scholarship  
2023 Best poster award in neuroscience, Physiology in Focus 2023, Tallinn  
2022 K. Falkenberg's master's thesis 2nd place in Estonian Students' Research Competition  
2021 Erkki Truve Doctoral study scholarship  
2018 President of the Republic of Estonia reception for the best graduates  
2016 The Bachelor Scholarship of Toomas Tamm

## Conferences and presentations

2024 EMBL conference Molecular Neurobiology, Fodele, **poster presentation**  
2023 Annual meeting of Estonian Society of Human Genetics, Rakvere, **oral presentation**  
2023 Physiology in Focus 2023, Tallinn, **poster presentation**  
2023 Glial Cells in Health and Disease, Berlin, **poster presentation**  
2023 EMBL conference Brain genome: regulation, evolution, and function, Heidelberg, **poster presentation**  
2022 TalTech School of Science conference, Tallinn, **oral presentation**,  
2022 Federation of European Neuroscience Societies (FENS) conference, Paris, **poster presentation**  
2021 Scandinavian Society for Laboratory Animal Science (Scand-LAS) meeting, Tallinn  
2019 Science Day for PhD students in medical field, **oral and poster presentation**

- 2019 Baltic summer school on Behavioural Characterization of Rodent Models of Major Brain Disorders, Pühajärve
- 2019 Nordic Neuroscience, Helsinki, **poster presentation**
- 2019 EMBL conference Chromatin and Epigenetics, Heidelberg, **poster presentation**
- 2016– Annual meeting of Estonian Society of Human Genetics

### Supervision

- 2020 **Kaisa Falkenberg**, BSc, "The role of a MEF2 transcription factor dependent enhancer in the regulation of BDNF gene in rat cortical and hippocampal neurons"
- 2020 **Ekke-Julius Hellat**, high school research, "Effect of MEF2-dependent enhancer on BDNF gene promoter I activity in rat cortical and hippocampal neuron"
- 2021 **Karin Lehe**, BSc, "The role of transcription factor MYT1L in the regulation of BDNF gene in rat cortical neurons"
- 2022 **Kaisa Falkenberg**, MSc, "The role of -4.8 kb enhancer and MEF2 transcription factors in the regulation of BDNF gene in rat cortical and hippocampal neurons", 2nd prize at the National Contest for University Students
- 2023 **Karin Lehe**, MSc, "The role of MEF2 transcription factor family in the regulation of Bdnf gene in rat cortical and hippocampal neurons"

### Teaching

- 2019–2023 Molecular and Cell Biology - laboratory
- 2019–2023 Introduction to Scientific Research
- 2019–2023 Anatomy and Physiology
- 2019 Basics of Gene Technology

### Membership in councils

- 2022 Member of the council of Doctoral Study programme in the School of Science
- 2019–2022 Member of the council of the Department of Chemistry and Biotechnology (Representative of doctoral students)
- 2017 Member of the council of MSc study programme in Applied Chemistry and Biotechnology
- 2016 Member of the council of BSc study programme in Chemistry, Gene and Food technology
- 2014–2018 Member of the Faculty of Science Student Council

## Publications

Esvald E.-E., Moistus A., Lehe, K., Tuvikene J., **Avarlaid A.**, Timmusk T., Stimulus-dependent expression of *Bdnf* is mediated by ATF2, MYT1L, and EGR1 transcription factors. *Under revision in Journal of Neuroscience*

**Avarlaid A.**, Falkenberg K\*, Lehe K\*, Mudò G, Belluardo N†, Di Liberto V, Frinchi M, Tuvikene #, Timmusk T#. An upstream enhancer and MEF2 transcription factors fine-tune the regulation of *Bdnf* gene in cortical and hippocampal neurons. *J Biol Chem.* 2024;300(6):107411. doi: 10.1016/j.jbc.2024.107411

**Avarlaid A.**, Esvald EE, Koppel I, Parkman A, Zhuravskaja A, Makeyev EV, Tuvikene J#, Timmusk T# (2024) An 840 kb distant upstream enhancer is a crucial regulator of catecholamine-dependent expression of the *Bdnf* gene in astrocytes. *Glia.* 2024;72(1):90-110. doi:10.1002/glia.24463

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Pupina N., **Avarlaid A.**, Sadam H., Pihlak. A., Jaago M., Tuvikene J., Rähni A., Planken A., Planken M., Kalso E., Tienari P. J., Nieminen J. K., Seppänen M., Vaheri A., Lindholm D., Sinisalo J., Pussinen P., Timmusk T., Palm K., (2022). Immune response to a conserved enteroviral epitope of the major capsid VP1 protein is associated with lower risk of cardiovascular disease. *EBiomedicine*, 76, 103835. doi: 10.1016/j.ebiom.2022.103835

Tuvikene J, Esvald EE\*, Rähni A\*, Uustalu K\*, Zhuravskaya A, **Avarlaid A.**, Makeyev EV, Timmusk T (2021) Intronic enhancer region governs transcript-specific *Bdnf* expression in rodent neurons. *Elife.* 2021;10: e65161. doi:10.7554/eLife.65161.

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### Hariduskäik

2018– Tallinna Tehnikaülikool, doktoriõpingud  
2016–2018 Tallinna Tehnikaülikool, magistrikraad geenitehnoloogias, diploma *cum laude*  
2013–2016 Tallinna Tehnikaülikool, bakalaureusekraad rakenduskeemias ja biotehnoloogias  
2001–2013 Vinni-Pajusti gümnaasium

### Teenistuskäik

2023– Eesti Standardmis- ja Akrediteerimiskeskus, erialaassessor  
2018– Tallinna Tehnikaülikool, nooremteadur  
2016–2018 Protobios OÜ, praktikant  
2015–2016 Mektory tehnoloogiakool, teaduskursuse juhendaja  
2015 AS Rakvere haigla, praktikant

### Tunnustused

2023 Kristjan Jaagu välislahetuse stipendium  
2023 Parima posterisitluse auhind neuroteaduste valdkonnas, Physiology in Focus 2023 konverents, Tallinn  
2022 K. Falkenbergi magistritöö 2. koht Eesti Üliõpilaste Teadustööde konkurssil  
2021 Erkki Truve nimeline doktoriõppe stipendium  
2018 Vabariigi Presidendi vastuvõtt parimatele koolilõpetajatele  
2016 Toomas Tamme bakalaureuseõppe stipendium

### Konverentsid ja esinemised

2024 EMBL konverents Molecular Neurobiology, Fodele, **posterettekanne**  
2023 Eesti Inimesegeneetika konverents, **suuline ettekanne**  
2023 Physiology in Focus 2023, Tallinn, **posterettekanne**  
2023 Glial Cells in Health and Disease, Berliin, **posterettekanne**  
2023 EMBL konverents Brain genome: regulation, evolution, and function, Heidelberg, **posterettekanne**  
2022 Taltech Loodusteaduskonna konverents, Tallinn, **suuline ettekanne**  
2022 Federation of European Neuroscience Societies (FENS) konverents, Pariis, **posterettekanne**  
2021 Society for Laboratory Animal Science (Scand-LAS) konverents, Tallinn  
2019 Meditsiiniteaduste doktorantide teaduspäev, **suuline ja posterettekanne**  
2019 Balti neuroteadlaste konverents teemal Behavioural Characterization of Rodent Models of Major Brain Disorders, Pühajärve

- 2019 Nordic Neuroscience konverents, Helsinki, **posterettekanne**
- 2019 EMBL konverents Chromatin and Epigenetics, Heidelberg, **posterettekanne**
- 2016 - Eesti Inimesegeneetika konverents

#### **Lõputööde juhendamine**

- 2020 **Kaisa Falkenberg**, BSc, "Transkriptsioonitegurist MEF2 sõltuva enhanseri roll BDNF geeni regulatsioonis roti kortikaalsetes ja hipokampaalsetes neuronites"
- 2020 **Ekke-Julius Hellat**, gümnaasiumi uurimustöö, "MEF2-sõltuva enhanseri mõju BDNF geeni promootor I aktiivsusele roti kortikaalsetes ja hipokampaalsetes neuronites"
- 2021 **Karin Lehe**, BSc, "MYT1L transkriptsiooniteguri roll BDNF geeni regulatsioonis roti kortikaalsetes neuronites"
- 2022 **Kaisa Falkenberg**, MSc, "The role of -4.8 kb enhancer and MEF2 transcription factors in the regulation of BDNF gene in rat cortical and hippocampal neurons", 2nd prize at the National Contest for University Students
- 2023 **Karin Lehe**, MSc, "The role of MEF2 transcription factor family in the regulation of Bdnf gene in rat cortical and hippocampal neurons"

#### **Õpetamine**

- 2019–2023 Molekulaar- ja rakubioloogia praktikum
- 2019–2023 Sissejuhatus teadustöösse
- 2019–2023 Anatoomia ja füsioloogia
- 2019 Geenitehnoloogia alused

#### **Organisatsioonidesse kuulumine**

- 2022– Loodusteaduste doktoriprogrammi nõukogu liige
- 2019–2022 Keemia ja biotehnoloogia instituudi nõukogu liige (doktorantide esindaja)
- 2017 Rakenduskeemia ja Biotehnoloogia magistriprogrammi nõukogu liige
- 2016 Rakenduskeemia, Toidu- ja Geenitehnoloogia bakalaureuseprogrammi nõukogu liige
- 2014–2018 Loodusteaduskonna üliõpilaskogu liige



## Publikatsioonid

Esvald E.-E., Moistus A., Lehe, K., Tuvikene J., **Avarlaid A.**, Timmusk T., Stimulus-dependent expression of Bdnf is mediated by ATF2, MYT1L, and EGR1 transcription factors. *Under review in Journal of Neuroscience*

**Avarlaid A.**, Falkenberg K\*, Lehe K\*, Mudò G, Belluardo N†, Di Liberto V, Frinchi M, Tuvikene #, Timmusk T#. An upstream enhancer and MEF2 transcription factors fine-tune the regulation of Bdnf gene in cortical and hippocampal neurons. *J Biol Chem.* 2024;300(6):107411. doi: 10.1016/j.jbc.2024.107411

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ISSN 2585-6901 (PDF)  
ISBN 978-9916-80-169-7 (PDF)